The fas Operon of Rhodococcus fascians Encodes New Genes Required for Efficient Fasciation of Host Plants

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Three virulence loci (*fas, att*, and *hyp*) of *Rhodococcus fascians* D188 have been identified on a 200-kb conjugative linear plasmid (pFiD188). The *fas* locus was delimited to a 6.5-kb DNA fragment by insertion mutagenesis, single homologous disruptive recombination, and in *trans* complementation of different avirulent insertion mutants. The locus is arranged as a large operon containing six open reading frames whose expression is specifically induced during the interaction with host plants. One predicted protein is homologous to P-450 cytochromes from actinomycetes. The putative ferredoxin component is of a novel type containing additional domains homologous to transketolases from chemoautotrophic, photosynthetic, and methylotrophic microorganisms. Genetic analysis revealed that *fas* encodes, in addition to the previously identified *ipt*, at least two new genes that are involved in fasciation development, one of which is only required on older tobacco plants.

The interaction between *Rhodococcus fascians* and host plants leads to the loss of apical dominance and the development of multiple malformed shoots at the site of infection (fasciation). This proliferation is governed by multiple bacterial loci located both on a linear Fi plasmid and on the chromosome (reference 8 and unpublished results). From the three identified loci on pFiD188, only inactivation of one (*fas*) leads to a complete loss of phytopathogenicity. In this locus, an open reading frame (ORF) coding for an isopentenyltransferase (*ipt*) has been identified by sequence homology to other cytokinin biosynthesis genes and by biochemical approaches (8). Expression of *ipt* in *R. fascians* is regulated by an inducing factor which can only be detected in significant amounts in extracts from tumor tissues (induced by *R. fascians*) (8).

In this paper, we present genetic data that allowed the definition of the *fas* locus on a 6.5-kb DNA fragment whose complete nucleotide sequence was determined. Six ORFs directed in the same orientation as the previously defined *ipt* were identified. ORF1 and the amino-terminal part of ORF2 have extensive homologies to P-450 cytochromes and ferredoxins from actinomycetes, whereas the carboxy-terminal portion of ORF2 has conserved regions homologous to the amino-terminal part of transketolases from chemoautotrophic, photosynthetic, or methylotrophic microorganisms.

The isolation of new *fas* mutants and complementation analysis revealed that *fas* encodes new genes involved in fasciation of the host plant.

MATERIALS AND METHODS

Bacterial strains and plasmids. Growth conditions and antibiotic concentrations for *R. fascians* strains have been described previously (11). The *Escherichia coli* strains used for

plasmid constructions were MC1061 (5) and DH5 α . *R. fascians* D188 is a highly virulent wild-type isolate. D188-2 is a virulent strain without the 138-kb circular pD188 plasmid not involved in virulence, whereas D188-5 is an avirulent derivative strain without any plasmid (10). *fas1* is an avirulent mutant strain derived from D188, carrying an insertion in the *ipt* gene (8). The construction of the partial *Sau3A1* library in a vector capable of replication in *R. fascians* has been reported previously (9). The plasmids used in this study and their relevant characteristics are listed in Table 1.

Insertion mutagenesis of *R. fascians* strains and complementation analysis. Insertion mutants in the linear plasmid were obtained by electrotransformation of D188 or D188-2 with the nonreplicating vector pTGV6b or pJDGV1 (9), followed by pool conjugation of the Cm^r transformants with D188-5 (Sm^r) (8). Appropriate insertion mutants were identified by hybridization with pFiD188-derived cosmids (e.g., pJGV30202 [8]). The isolated mutant strains were assayed for virulence by using either freshly germinated seedlings or axenically decapitated plants of different ages (8). *E. coli* clones of the right borders of the inserted sequences together with adjacent *R. fascians* were generated by *Bam*HI restriction of total DNAs of insertion mutant strains and self-ligation, followed by transformation of *E. coli* MC1061 with the ligation mixture and selection of Ap^r transformants (9).

trans-Complementation analysis was performed with replicating bifunctional vectors carrying bleomycin acetyltransferase as a selectable marker. The replicating cosmids pJGV-30202, pJGV26307, and pJGV26807 containing large pFiD188 fragments spanning *ipt* were isolated from a partial Sau3A1generated library cloned in pJGV9 by colony hybridization with the ³²P-labeled 5.4-kb StuI fragment (Fig. 1). The different constructs were introduced into the mutant strains (Cm^r), and Cm^r Bl^r transformants were selected. The presence of the plasmids in the transformants was verified by Southern blotting and plasmid analysis (11).

Disruptive single homologous recombination. Different overlapping fragments spanning the *fas* locus were cloned in pUC18, and a *cmr* encoding cassette (12) was inserted into the individual clones. The resulting plasmids pIN1 to pIN8 (Table 1) were introduced into *R. fascians* D188 by electroporation. Cm^r transformants were conjugated with strain D188-5, and

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TABLE 1. Plasmids used in this study

Plasmid	Size (kb)	Marker gene(s)	Relevant characteristics	Reference
pRF32	13.2	Cm ^r Ap ^r	Cointegrate of pRF28 and pUC18; integrates by illegitimate recombina- tion in <i>R. fascians</i>	11
pRF37	10.8	Bl ^r Ap ^r	Cloning vector for R. fascians	11
pRF41	6.1	Cm ^r Åp ^r	Clone of the cmr gene of R. fascians NCPPB 1675	11
pTGV6b	7.7	Cm ^r Ap ^r	pUC18-derived plasmid containing the <i>cmr</i> gene of pRF2 as a 2.5-kb XbaI-SstI fragment and the promoterless <i>uidA</i> gene of pGUS1	35 and unpublished data
pJDGV1	11	Cm ^r Ap ^r	Integrating vector for R. fascians	Unpublished data
pJGV9	12.1	Bl ^r Ap ^r	Cosmid vector containing an origin of replication for R. fascians	9
pRIPT1	4.4	Ap ^r	Clone of the <i>ipt</i> -coding region as a 1.9-kb <i>Eco</i> RI- <i>Bam</i> HI fragment in pUC18	8
pMGV1	13.7	Ap ^r	Clone of the 11-kb BamHI fragment 1 of pFiD188 in pUC18	This work
pMGV3	4.7	Ap ^r	Exchange of the <i>NcoI-Eco</i> RI fragment of pRIPT1 by the 1.05-kb <i>Eco</i> RI fragment (filled in by Klenow polymerase), containing the <i>cmr</i> promoter and first 66 amino acids of the coding region; translational fusion between <i>ipt</i> and <i>cmr</i>	This work
pJGV30202	42.1	Bl ^r Ap ^r	Cosmid clone from a partial Sau3A1 library in pJGV9, isolated by hybrid- ization with the 4.5-kb StuI fragment of pFiD188	This work
pJGV26807	43.3	Bl ^r Ap ^r	Cosmid clone from a partial Sau3A1 library in pJGV9, isolated by hybrid- ization with the 4.5-kb StuI fragment of pFiD188	This work
pJGV26307	46.9	Bl ^r Ap ^r	Cosmid clone from a partial Sau3A1 library in pJGV9, isolated by hybrid- ization with the 4.5-kb StuI fragment of pFiD188	This work
pMGV100	17.4	Bl ^r Ap ^r	Clone of the 6.6-kb <i>Hin</i> dIII fragment of pMGV1 (second <i>Hin</i> dIII in polylinker) in pRF37	This work
pMGV101	22.5	Bl ^r Ap ^r	Generated by deletion of a 21-kb HindIII fragment of pJGV26807	This work
pMGV102	21.3	Bl ^r Ap ^r	Generated by deletion of the internal 26-kb XhoI fragment of pJGV26807	This work
pMGV103	12.7	Bl ^r Ap ^r	Clone of the 1.95-kb XbaI fragment of pMGV3 in pRF37 linearized by XbaI	This work
pIN1	8.9	Cm ^r Ap ^r	Clone of the 1.2-kb NcoI-NcoI fragment of pFiD188 in pTGV6b	This work
pIN2	9.3	Cm ^r Ap ^r	Clone of the 1.6-kb NcoI-NcoI fragment of pFiD188 in pTGV6b	This work
pIN3	9.1	Cm ^r Ap ^r	Clone of the 1.4-kb <i>NcoI-NcoI</i> fragment of pFiD188 <i>Bam</i> HI fragment 1 in pTGV6b	This work
pIN4	7.1	Cm ^r Ap ^r	Clone of the 1.9-kb BamHI-EcoRI fragment of pFiD188 BamHI frag- ment 1 in pUC18 plus the 2.5-kb XbaI cassette containing the cmr gene of R. fascians NCPPB1675	This work
pIN5	8.2	Cm ^r Ap ^r	Clone of the 3-kb <i>StuI-XhoI</i> fragment of pFiD188 <i>Bam</i> HI fragment 1 in pUC18 plus the 2.5-kb <i>XbaI</i> cassette containing the <i>cmr</i> gene of <i>R. fascians</i> NCPPB1675	This work
pIN6	9.7	Cm ^r Ap ^r	Clone of the 4.5-kb StuI-StuI fragment of pFiD188 BamHI fragment 1 in pUC18 plus the 2.5-kb XbaI cassette containing the cmr gene of R. fascians NCPPB1675	This work
pIN7	6.0	Cm ^r Ap ^r	Clone of the 0.8-kb <i>Eco</i> RV- <i>Eco</i> RV fragment of pFiD188 <i>Bam</i> HI frag- ment 1 in pUC18 plus the 2.5-kb <i>Xba</i> I cassette containing the <i>cmr</i> gene of <i>R. fascians</i> NCPPB1675	This work
pIN8	5.8	Cm ^r Ap ^r	Clone of the 0.6-kb SstI-SstI fragment of pFiD188 BamHI fragment 1 in pUC18 plus the 2.5-kb XbaI cassette containing the cmr gene of R. fas- cians NCPPB1675	This work
pJDGV2	6.1	Ap ^r	Clone of the 1.5-kb <i>StuI-SnoI</i> fragment of pFiD188 <i>Bam</i> HI fragment 1 in pGUS1 resulting in a translational fusion between the 111 amino-terminal amino acids of ORF1 and <i>uidA</i>	This work
pJDGV3	16.2	Cm ^r Ap ^r	Clone of the 4.0-kb <i>HindIII-XbaI</i> fragment of pJDGV2 in pJGV131 plus the 2.5-kb <i>XbaI</i> cassette containing the <i>cmr</i> gene of <i>R. fascians</i> NCPPB1675	This work
pJDGV4	15.7	Cm ^r Ap ^r	Clone of the 2.9-kb AscI-XbaI fragment of pJDGV2 in pJGV131 plus the 2.5-kb XbaI cassette containing the cmr gene of R. fascians NCPPB1675	This work
pJGV131	7.8	Apr	Bifunctional cloning vector for R. fascians	This work
pGUS1	5.2	Apr	pUC19 containing a promoterless gus fusion	35

Cm^r Sm^r transconjugants were analyzed by Southern hybridizations against appropriate ³²P-labeled clones of pFiD188. Strains harboring linear plasmids with the desired single homologous recombination events were assayed for virulence on seedlings and decapitated plants.

GUS assays. For GUS assays, cells were harvested by centrifugation. Cell extracts were made by sonicating the cells

for four 20-second bursts at a 40-W output (sonicator cell disrupter B10; Branson Sonic Power Corp.) in buffer A (10 mM Tris-HCl, 10 mM Mg-acetate, 6 mM KCl, 6 mM β -mercaptoethanol [pH 7.5]) and centrifugation for 15 min at 12,000 \times g. Protein concentration was determined according to the method of Bradford (2). GUS activity was measured with *para*-nitrophenylglucuronide as the substrate (21).



FIG. 1. Genetic analysis of the *fas* locus of *R. fascians*. The central restriction map represents the region of the linear plasmid pFiD188 from *R. fascians* D188 spanning the *fas* locus. The dotted *StuI* fragment was used as a probe in colony hybridizations to detect the cosmids pJGV30202, pJGV26807, and pJGV26307. Above the restriction map, hatched bars indicate the DNA fragments cloned in the different intermediate vectors for the construction of single homologous recombinant strains. The dotted bar inside the restriction map is the *StuI* fragment used as probe for the isolation of complementing cosmids. The location of the *ipt* gene is indicated. Pcmr is the inducible promoter of the *cmr* gene of *R. fascians* (12). Under the restriction map, solid triangles represent the sites of insertion of the *R. fascians* vectors in the mutants pRF32 (*fas1*), pJDGV1 (M11, *fas5*), and pTGV6b (M9, M10, *fas6*). Stippled bars under the restriction map indicate the pFiD188 fragments cloned in vectors able to replicate in *R. fascians* used for the *trans*-complementation assays. At the bottom, the FAS bar represents the minimum (solid portion) or maximum (broken portion) size for the *fas* locus, as determined from the combination of the genetic experiments.

Other methods. The enzymatic isopentenyltransferase assays and the high-pressure liquid chromatography conditions used to identify reaction products, as well as the induction conditions for *ipt* expression, have been reported previously (8). Electrotransformation conditions for *R. fascians* were essentially as described previously (11). For general cloning purposes, pUC18 (45) was used, and recombinant DNA techniques were performed under standard conditions (36). The nucleotide sequence was determined with automated dideoxysequencing systems (A.L.F. DNA sequencer [Pharmacia] and the 370A DNA sequencer [Applied Biosystems]). Computerassisted interpretation of the sequence was performed by the IntelliGenetics Suite, whereas data base homology searches (with PIR release 35 and Swiss-Prot release 24 protein data bases) were carried out by the FASTDB program (4).

Nucleotide sequence accession number. The nucleotide sequence of the *fas* locus is available from EMBL under accession no. Z29635.

RESULTS

Identification of essential fasciation genes downstream of *ipt* by genetic analysis. Insertion mutants in the linear plasmid of *R. fascians* strain D188 (generated by integration of pTGV6b or pJDGV1 [see Materials and Methods]) were screened by Southern analysis (with a 30-kb cosmid covering *ipt* [pJGV-30202] as probe) to isolate new insertions in the vicinity of the previously described *fas1* (Fig. 1). Four such insertion mutants (*fas5, fas6, M9, and M10*) were assayed for their ability to induce fasciations on decapitated *Nicotiana tabacum* plants or to inhibit tobacco seedling growth (Table 2).

Mutant strains M9 and M10 were fully virulent in both

assays, delimiting the maximum left and right borders of *fas*, respectively. The mutant strain *fas6*, harboring a pTGV6b insertion located 3 kb to the right of the *fas1* insertion (same orientation as in Fig. 1), was not virulent in both assays. Strain *fas5*, with a pJDGV1 insertion located 1 kb to the left of the insertion in *fas1*, however, behaved differently in the two assay systems. Whereas seedling growth was inhibited by infection with these bacteria, older plants did not develop any symptoms upon infection, indicating that *fas5* inactivates at least one gene required for fasciation of older plants. The younger the plants were at the time of infection, the more severe the fasciation symptoms were which developed (data not shown).

In addition, plasmids containing several fragments spanning the ipt-coding region were tested for their ability to complement in trans the avirulent phenotype caused by the pRF32 insertion in fas1. Introduction of either pJGV30202, pJGV-26307, or pJGV26807 (Fig. 1) into fas1 yielded transformants that were fully virulent both on decapitated plants and on seedlings. The smallest DNA fragment that could complement fas1 in trans in both virulence assays (Fig. 1 and Table 2) extended from 5.8 kb upstream of the pRF32 insertion in fas1 to 3.2 kb downstream (pMGV101 [Fig. 1 and Table 1]). A plasmid containing only 3.5 kb of the upstream sequences (pMGV102 [Table 1 and Fig. 1]) could not complement fas1 in either of the assays (Table 2). Restriction of the complementing DNA to 200 bp downstream beyond the stop codon of ipt, as in pMGV100 (Table 1 and Fig. 1), and introduction into fas1 resulted in bacteria that could inhibit tobacco seedling growth but that could not incite fasciations on older plants (Table 2).

Finally, the size of the *fas* locus was accurately estimated by single homologous disruptive recombination experiments. These also allowed us to circumvent possible instability prob-

TABLE 2. Summary of virulence assay results^a

Strain	Seedling growth inhibition	Leafy gall formation on decapitated plants
D188	+	+
D188-5	-	-
Insertion mutants		
fas1	_	-
fas5	+	-
fas6		_
M9	+	+
M10	+	+
Merodiploid strains		
fas1(pJGV26807)	+	+
fas1(pJGV26307)	+	+
fas1(pJGV30202)	+	+
fas1(pMGV100)	+	-
fas1(pMGV101)	+	+
fas1(pMGV102)	-	-
fas1(pMGV103)	+*	ND
fas6(pMGV100)	+	-
fas6(pMGV103)	_b	ND
Single homologous recombinants		
D188-5(pFiD188::pIN1)	+	+
D188-5(pFiD188::pIN2)	+	-
D188-5(pFiD188::pIN3)	_	-
D188-5(pFiD188::pIN4)	+	-
D188-5(pFiD188::pIN5)	-	-
D188-5(pFiD188::pIN6)	+	+
D188-5(pFiD188::pIN7)	+	ND
D188-5(pFiD188::pIN8)	+	ND

^a +, effect present; -, effect absent. ND, not determined. Seedling growth inhibition and leafy gall formation assays were performed as described previously (8)

(8). ^b Chloramphenicol (5 μ g/ml) was added to the MST plant growth medium (Murashige and Skoog medium supplemented with 0.001% thiamine and 1% sucrose) (8).

lems with the *trans*-complementing plasmids on plants. Plasmids pIN1 to pIN8, containing different fragments of the presumed *fas* locus cloned in nonreplicative (Cm^r) vectors for *R. fascians* (Table 1 and Fig. 1), were used to generate recombinant *R. fascians* strains (Materials and Methods). Only plasmids that contained fragments with either of the ends of the *fas* locus could regenerate a functional copy of *fas* after integration by single homologous recombination with the linear plasmid pFiD188 and hence yield virulent bacteria.

When tested on decapitated *N. tabacum* plants, the *R. fascians* strains harboring the recombinant linear Fi plasmids pFiD188::pIN2, pFiD188::pIN3, pFiD188::pIN4, and pFiD188::pIN5 were avirulent. In contrast, strains D188-5 (pFiD188::pIN1) and D188-5 (pFiD188::pIN6) were virulent, so the 1.2-kb *NcoI* fragment (cloned in pIN1) and the 4.5-kb *StuI* fragment (cloned in pIN6) can be considered to contain the left and right ends of *fas*, respectively. The strains containing the plasmids pFiD188::pIN2 and pFiD188::pIN4 were virulent, however, on tobacco seedlings. The virulent phenotype of strains harboring the plasmids pFiD188::pIN7 and pFiD188::pIN8 (Table 2) allowed us to locate the right end of *fas* within the 0.5-kb *Eco*RV-*SstI* fragment (Fig. 3).

Combination of all of these genetic data allowed us to delimit the *fas* locus maximally to the 6.5-kb fragment between the left *NcoI* site of pIN1 and the right *SstI* site of the fragment

cloned in pIN8 (Fig. 1). The part of the *fas* locus located downstream of *ipt* is only required for fasciation of fully developed plants.

Nucleotide sequencing of the *fas* **locus.** The nucleotide sequence of a 7.14-kb pFiD188 fragment spanning the identified *fas* locus was determined (Fig. 1 and 2). Six ORFs oriented in the same direction as the previously identified *ipt* and separated by small intergeneric regions were detected within this region (Fig. 2 and 3). The fourth ORF corresponds to the previously identified *ipt*. A fifth ORF starts 1 base before the stop codon of ORF4, resulting in a 4-bp translational overlap. The final ORF6 also has a 1-bp overlap with the previous ORF and is not followed by any inverted repeat which could constitute a transcription terminator. It is separated from the next significant ORF (outside of the genetically determined *fas* locus) by a 200-bp intergenic region.

All of the ORFs, except the translationally coupled ORF5 and ORF6, are immediately preceded by a similar pattern of nucleotides (GAPuPuNGAPuTC), which could represent a ribosome-binding site for R. fascians, because this pattern can also be found in front of other coding regions determined (e.g., cmr gene [12]) (Fig. 2). The exact location of the insertion events in fas5 and fas6 was determined by nucleotide sequencing (Fig. 2). fas6 is located within the amino terminus of ORF1, whereas fas5 is located at the carboxy-terminal region of ORF5. The nucleotide sequence of fas indicates that fas is arranged as an operon. A fusion was constructed between uidA and a DNA fragment containing the N-terminal part of ORF1 and the upstream region until the StuI site (Fig. 2 and Table 1). Introduction of a plasmid containing this fusion (pJDGV3) in strain D188-5 led to transformants exhibiting β -glucuronidase activity (10.53 µmol of para-nitrophenol per h per mg of protein versus 0.81 µmol of para-nitrophenol per h per mg of protein for a D188-5 control). Restriction of the upstream region until the AscI site (500 bp; plasmid pJDGV4) still resulted in a plasmid that, when introduced into D188-5, expressed β -glucuronidase (14.83 µmol of para-nitrophenol per h per mg of protein), indicating the possible presence of a promoter.

The fas operon contains a P-450 cytochrome and a new type of ferredoxin. Protein data bases were searched for homologous proteins by using the six putative fas-encoded ORFs defined above as query sequences. ORF1 has a striking homology to P-450-type cytochromes that play a central role in biosynthetic and biodegradative reactions in actinomycetes (ranging from 33% to 37% overall identical amino acids [Fig. 4A]). In particular, the carboxy-terminal parts of the peptide, with the heme-binding loop and the absolutely conserved cysteine residue, are homologous. In most actinomycetes, an ancillary small iron-sulfur redox protein is encoded in the vicinity of the monooxygenase, and, indeed, the amino-terminal 60 amino acids of ORF2 are 70% identical to these actinomycete 4Fe-3Su-type ferredoxins, with conservation of the three critical cysteine residues (Fig. 4B). Nevertheless, ORF2 is significantly larger than the known ferredoxins, and, surprisingly, the carboxy terminus of ORF2 shares conserved amino acid stretches with the amino termini of transketolases from photosynthetic and chemoautotrophic bacteria, as well as with the dihydroxyacetone synthase from methylotrophic yeast species (Fig. 4C). In particular, the proposed nucleotide binding site is well conserved. ORF6 is 52% similar to the available N-terminal sequence of a hypothetical protein detected 5' on the opposite strand of the azurin protein of Pseudomonas aeruginosa (Fig. 4D). No homologous proteins were found with ORF3 or ORF5.

An essential fasciation gene is located upstream of *ipt*. To test whether the nonpathogenic phenotype caused by the

121 361 ACGATCACTCTGTACTCGCTGCATCTGCGGAGCTGCGGGAGTCACTGATCCTGCGCGAACTTCACCGGATGGAGTCGCTGGAAATCAACCTTATCGTCGATGCATTAGGGGAGGTCTCAC 481 CGAGCTCTCGTGTCATGGACGCGGGATCGGGGCGAGGCGGAACGACCTTCACAATCGCTGATCGATTCGGCTGCCGGGTAGACGGGGTGAACTACTGCGGCTCACCATGTCGAGTTCGCAG 601 AGAAGTTGGCCCGTGAACGCGGGAGCTCGGACCGTGTGCAATTCCACTTCGCAAACATGGTTCAGGCCCCCTTCGAGGACAACACCCTTCGATTACATCGTCTCCAACGAAACCACGATGT GTGTCGACCTAGGGGAGGCGTTCACCGAGTTCGCCCGGTGCGCCCCGGCGGCCGCTATGTCGCAGTCACCTGGTGCCGCAACGACGTAGTTGCTGAGCGTTCCGAGGCATCACGGT TGATCGACGAAGAATATCAGTGCGCTATGCACACCCGCAGTACTTATTTCCAAACTCTGGCAGCAAATGGTCTGGTGCCGTATCACGGTACACCGACGAAGCATCCCGACG GGGACCTGCGCAACCAGGCAGCGTTGCGCACAGGTGTCGAGGAACCATTCCTCCAGGGCTTCCGCGAACGCAGCATCGACTACTTGGTTATCGCGTGCGAGCGTCTGTAGCGGTGGCCTG 721 841 961 1081 GTADLP LEMR ORF1 <u>⊽fas6</u> ACGCAACGGCCTGAACCCGAACGAGGGCCCGGCCAGGGTCCGTGACCGCGGACGGGGTTATCCCGGTGGGGAGACTCTACGGGGGCCCCGGCTTTCTCGTCTGCCGTTACGAAGACGTCCG R N G L N P T E E L A Q V R D R D G V I P V G E L Y G A P A F L V C R Y E D V R 1201 1321 A D S N R F S N A H T P M F A I P S G G D V I E D E L A A M R A G N ACTCGATCCCCCCGACCACCCCCCTTGCGTCATATCCTTGCTGCGGGAGTTCTCAGTGCACCGGCTCAGTCGCCGCGCGCACAGGAGATAGTGGACAGTGCATTGGATGGTCT 1431 1561 A D L M D R Y A L P V S L L V L C E L L G V P Y A D GQP RDEL 1681 AGCACGCCTCCTGGATCTGTCGGCATCTGCAGAACAACGAGCGGTTGCCCAGCGAGAAGAACCGTCGATACATGGCCACCCTGGTTACCCGTGCGCAGGAACAACCCGGTGACGACTTGTT 1801 G I L A R K I G D N L S T D E L I S I I S L I M L G G H E T T A S M I G L S V L GGCCCTTTTGCATCACCCGGAACAAGCCGCCATGATGATGAGGAACCGCGCACTGCGGCCATTCGCGGAACTACTGCGGTGGTCGTCGGCCCATTCGCAGCCACCGCGCAT 1921 ннр E Q A A M M I E D P N C V N S G I E E L L R W L S V A HSQP 2041 G V T I P A G S F V I P S L L A A N R D S N L T D R P 2161 2281 ORF2 2401 GGAGGTGTTTCGGCTCCGGTCAGTGCGTGCTCGTCGCCCGAGGTCTTCGAGCAGAGTAACGACGGCACAGTCACATTGCTCGTGGACAAACCTTCCCCAGACAACCATTCGCTGGTAC 2521 R A A A R S C P A T A I R F E E N A M R Q E P T E F S Y D D L P A L I S R M R G ACGAAAGGCACTCGTTCTCATCCATCGACGATGGACGTGCTGTGGGTACTCTACGACGAAATCCCCCAACGTATCCCCCGAGAGGCCCCGACGACGACGGCGACGGCCGCTTCTTGCTGT 2641 D E R H S F S S S T M D V L W V L Y D E I P N V S P E S P D D D D R D R F L L CCAAAGGTCACGGCCCGATGGCCTACTACGCGGTACTTGCGGCCAAAGGGTTCCTCCGACCAGAGCTCCTCGGGCGGACAAAGAACTCACCACTCGGCTGGGCCGGATCGAA 2761 SKGHGPMAYYAVLAAKGFLRPELLDTWATKNSPLGFAPDR CCAAAATCTCGGGTGTAGAAATGTCGGGTGGCTCGCGCTGGGCCTTGCCGCTCGCAGTGGGCGTCGCGATGGGACTCCGTATACAAAATCGTCACGCACCGAGAGTCTTCGTGCTCA 2881 G V E M S G G S L G H G L P L A V G V A M G I R T O N R H A P 3001 G E F D E G S N H E A M A F A G R A R L N Q L T V I VLDNGT ASMGW CGCATGGAATCGATAAACGGTTCGACGGTGAGGGTTGGGACACAATCAACATCAACGTGACGTGCTGACCATGAGGAGATCGCTGCGGCACTGAATCGAGATCACAACGACCGCTCGCGG 3121 3241 VATVTRQSARSSIQQR. MNSADTQEPKSFNHTDMWTA ORF3 3361 E T D P R A V V L A D I G A H L F K A A A I A D P 3481 TAGGGATCAGGGAACAGCTGATGATGGGGGTAGCCGGCGGGCTCGCCATGTGTGGCATGCGACCCGTGGTACACACAGTCGCCGCCTTTCTCGTCGAGCGGCCCTCTTGAACAAATAAAGC V G I R E Q L M M G V A G G L A M C G M R P V V H T V A A F L V E R P L E Q I K TGAACTICGCCCAGCAAGATGTCGGCGCAGTCCTGGTCAGTTGGGGAGCCTCCTACGACTTGTCGGAGTTCGCCTTCTCCCATTTCACCCGGGCGATATCACCGTCATCGATTGGAGC 3601 AQQD V G A V L V S W G A S Y D L S E F A F SHF Ρ Т G D D 3721 P N W T V H V P G H P Q E A A D L L L E S L P G D G R V Y L R L S S Q V N R Y P ATGCGGTCCGAGGGACATCGTTCACACCTATCAAGTACGGCACCCGCGGGAGTCGTCCTCGCAGTCGGGCCCTGTTTAGATGCGGTGCTGTCGGCAACCTCCATGCTCGACGTAACCATCC 3841 3961 IRP F D A T G L C A A V Q A V N R P NVVLVEP YLAG Т S A н 4081 R L L S L G V R R E M E D R H Y G T P D D HDH 4201 S L S N S I N S F L G M K E S T M A Q T Q A R F D R ORF4 4321 GAACCCGGCGTATACGCAATCGTCGGTGCCACCGGAATTGGAAAGAGCGCCGAAGCGAGCAAGTTGGCATTGAGTCACTCGGCTCCGATTGTTGCTGGCGACCGTATCCAGTGTTACTCC Y A I V G A T G I G K S A E A S K L A L S H S A P I VVAD 4441 GATCTCCTGGTCACCAGTGGTCGAGCGTTCGACGCGAAAGTGGAAGGGCTCAACCGCGTTTGGCTCGACAACCGGACCATACATCAGGGCAACTTCGATCCGGACGAGGCCTTTGACCGG D L L V T S G R A F D A K V E G L N R V W L D N R T I H Q G N F D P D E A F D R CTGATAAAAGTACTGACCTCGTACGTTGATCGCGGCGAAGCGGTGGTAATGGAGGGGGGGTCGATATCGCTCATTCTTCGATTCGCGCAAACGATATCCAACCTACCGTTCCCAGCTGTC 4561 LIKVLTSYVDRGEAVVMEGGSISLILRFAQTISNLPFPAV GTGAATGTTATGCCTATTCCTGATAGGCAACTACTTTGCCCAGCAGTGCGCAGGAGGGCAGGACAGAGTGTACGGGGGAGATTCAACGGGCAGGAACTGCCGGAACGGCGGAAGGG 4681 V N V M P I P D R Q H Y F A Q Q C A R A R Q M L R G D S T G R N L L T E L A E A TGGGTTCTAGGCGATCAGCACAACTTCATAGCCTCGGTGCGGGTCTGGGACTGTGTACTCGACTGGTGCGCCACGCATTCCGTAACACCAGAGGAGTTGGCCAACCGAGACCTCACCACG 4801 V L G D Q H N F I A S V A G L D C V L D W C A T H S V T P E E L A N R D L T T

insertion in *fas6* was due to polar reduction of *ipt* expression, we assayed the isopentenyltransferase activity of wild-type strain D188 and the mutant strain *fas6* under inducing conditions. A slight reduction of the radioactivity peaks corresponding to 6-isopentenyladenine and 6-isopentenyladenosine was

observed in the reactions with *fas6* extracts compared with the reactions with wild-type extracts but was not sufficient to ascribe the avirulent phenotype of *fas6* to polar inactivation of *ipt* expression (data not shown). The introduction of pMGV103 (a plasmid that contained the *ipt*-coding region

4921 GAGGTGCTCGACGAACTGGCGGCATCAATGGGTGGCCGGTATGTCGAGCACGGCGTCCTTCAGCAGGAAATATTTTTAAGAACCTTCGGTGCCCCAGGTGTGACCGCCAGATGAGCGGGA EVLDELAASMGGRYVEHGVLQQEIFLRTFGAPGVTAR. MSG ORF5

5041	\$1 TCTGGCACACCGACGACGTGCACCTGACATCCGCCGGGGCTGATTTCGGAAACTGCATGCGAAACCGCCCGTCGTTGTCGTGCCACGGACCGT	TGGCCGACGTGCAGGAAGCCCTGC
	I W H T D D V H L T S A G A D F G N C I H A K P P V V V V P R T V	VADVQEAL
5161	51 GCTACACCGCGGGCGCGGAACCTATCGCTCGCGGTGCGCGGATCCGGACACTCGACTTACGGGCAATGCCAGGCAGATGGCGGAGTCGTGCTGGACAT	TGAAAAGGTTCAACACCGTCCACG
	_ RYTAARNLSLAVRGSGHSTYGQCQADGGVVLDM	M K R F N T V H
5281	31 ACGTTCGATCGGGGCAGGCCACGATCGATGCGGGTGTGCGATGGAGCGACGTGGTCGCTGCCACGCTCAGCCGCCAGCAAACCCCTCCGGTACTCAC	CCGACTATCTCGGGACCACCGTCG
	D V R S G Q A T I D A G V R W S D V V A A T L S G T L S V G G F G	GGSSHGFG
5401	J1 GCGGAACGCTTTCGGTCGGCGGCTTCGGGGGATCGAGTCACGGCTTTGGCTTGCAAACCGACAACGTGGACTCTCTTTGCTGTAGTTACCGGATCGGG	GAGATTTTCGGGAATGCTCCGCCG
	L Q R Q Q T P P V L T D Y L G T T V G T D N V D S L A V V T G S G	GDFRECSA
5521	21 TATCGAACAGTGAACTTTTCGACGCCGTGCGCGGCGGCGGCCTCGGTCAATTCGGCGTAATCGTCAACGCGACAATCCGTCTGACCGCTGCTCACGAGTC	CGGTTCGACAGTACAAATTGCAGT
	VSNSELFDAVRGGLGQFGVIVNATIRLTAAHES	SVRQYKLQ
5641	TTCCAACCTCGGCGTATTCCTTGGCGACCAACTCCGCGCCATGTCCAACAGACTATTCGACCATGTACAAGGACGAATTCGTGTCGATGCCGACGG	GCCACTTACGTTATCGACTGGACC
	Y S N L G V F L G D Q L R A M S N R L F D H V Q G R I R V D A D G	GHLRYRLD
5/61	51 TAGCCAAGTACTTCACCCCACCACCACGAAGGCCAGACGACGATGCGCTGTTGTCATCGCTCCAATACGATTCGTGCGCCCGAATACAACTCGGACGTAGA	ATTATGGTGACTTTATCAACCGTA
	LAKYFTPPRRPDDDALLSSLQYDSCAEYNSDVD	DYGDFINR
5881	31 TGGCGGATCAGGAGCTTGATCTTCGGCACACAGGTGAGTGGTTCTATCCACATCCATGGGCCAGCCTGCTGATCCCGGCAGACAAGATCGAGCAGTT	TCATCGAAACTACCAGCTCCTCAT
	MADQELDLRHTGEWFYPHPWASLLIPADKIEQF	FIETTSSS
6001	JI TGACGGATGACCTAGGAAACTCCGGGCTGATCATGGTTTACCCGATTCCAACAACACCGATCACCGCACCGTTCATTCCGATTCCTCACTGCGACAC	CATTCTTCATGTTGGCCGTACTTC
	LTDDLGNSGLIMVYPIPTTPITAPFIPHCDT	TFFMLAVL
		V <u>tas5</u>
6121	21 GAACAGCATCCCCAGGGGCCGAAGCCCGAATGATTGCCAGCAACCGCCTGCTCTATGAACAGGCTCGGGATGTAGGCGGGGTGGCCTACGCAGTCAA	ATGCTGTCCCCATGTCACCGGGAG
	R T A S P G A E A R M I A S N R L L Y E Q A R D V G G V A Y A V N	NAVPMSPG
6241	41 ATTGGTGTACGCACTTCGGTTCGCGATGGCAGGCCATCGCACGGGCTAAGCGTCGCTTCGACCCCATACAGGATCCTCGCCCCTGGATACAGAATGTC	CATTEGACTGATGAACCTTEGACE
	D W C T H F G S R W Q A I A R A K R R F D P Y R I L A P G Y R M S	SFD.
		MNLKP
4744		
0301		ORF6
	61 AATGCCCGCGACTACGGTCTCGGCACAGGCCCGCCCGACCCCCAAGAGCGTCACGGTCTTCTGCGGAGCTATGCCAGGGCGCGGGACCAAATATGGA	ORF6 ACAGCTCGCAGAGGGGATGGGGCG
4/91	61 AATGCCCGCGACTACGGTCTCGGCACAGGCCCGACCCCCAAGAGCGTCACGGTCTTCTGCGGAGCTATGCCAGGGCGCGGGACCAAATATGGA M P A T T V S A Q A R P T P K S V T V F C G A M P G R G T K Y G	ORF6 Acagctcgcagagggatggggcg Q L A E G M G R
6481	61 AATGCCCGCGACTACGGTCTCGGGCACGCGGGCCCGACCCGACCCCAAAAGAGCGTCACGGTCTTCTGCGGAGCTATGCCAGGGCGCGGGGCCCAAATATGGA M P A T V S A P T P K V T C G M P G T K Y G 81 TGCGATTGCTAGATCGAAGCTGCGCCTCGTTTATGGTGGCGCCCCGAGTCGGCCTCATGGGCACCCTCGCGCAACGCGGGCCTTGGACTCCGCGCGAACC G M P G R G T K Y G 81 TGCGATTGCTAGATCGAAGCTGCGCCTCGTTTATGGTGGCGCCCCGAGTCGGCCTCATGGGCACCCTCGCGAACGCGGGCCTTGGACTCCGCGCGAACC G G C G C G A M P G R G T K Y G G C G C G C G C G C G C G C G C G C G C G C G C G G C G C G C G C G G G C G C G G G G G	ORF6 ACAGCTCGCAGAGGGGATGGGGCG Q L A E G M G R CGTCGTTGGCGTGATCCCTGAGAG
6481 6601	61 AATGCCCGCGACTACGGTCTCGGCACAGGGCCCGACCCGACCCCAAGAGGCGTCACGGTCTTCTGCGGAGGCTATGCCAGGGCGCGGGACCAAATATGGA M P A T T V S A Q A R P T P K S V T V F C G A M P G R G T K Y G B1 TGCGATTGCTAGATCGAAGCTGCGCCTCGTTTATGGTGGCGCCCGAGTCGGCCTCATGGGCACCCTCGCGAACCGCGGCCTTGGACTCCGGCGGAACC A I A R S K L R L V Y G G A R V G L M G T L A N A A L D S G G T CTCTATGCTGACTCGCCATCGCCTCGCCGAACCACCACCTCGCGAACCGCGCAACCGCGCGAACCGCGAACCGCGCGAACCGCGCGAACCGCGAACCGCGCGAACCGCGCGAACCGCGCGAACCGCGCGCCGAACCGCGAACCGCGCGAACCGCGCGAACCGCGCGAACCGCGCGCGAACCGCGCGAACCGCGCGCCGC	ORF6 ACAGCTCGCAGAGGGATGGGGGG Q L A E G M G R Cgtcgttggcgtgatccctgagag V V G V I P E S Carcottrattcatccreatscrea
6481 6601	61 AATGCCCGCGACTACGGTCTCGGCACAGGGCCCGACCCGACCCCCAAGAGCGTCACGGTCTTCTGCGGAGCTATGCCAGGGCGCGGGACCAAATATGGA M P A T T V S A Q A R P T P K S V T V F C G A M P G R G T K Y G B1 TGCGATTGCTAGATCGAAGCTGCGCCTCGTTTATGGTGGCGCCCCGAGTCGGCCTCATGGGCACCCCTCGCGAACGCGGCCTTGGACTCCGGGCGAACC A I A R S K L R L V Y G G A R V G L M G T L A N A A L D S G G T D1 CTTCACTGCGATACCCGAGGCTGCGCTCATGGACGAACTACACGTCGTCCATGACACGACAGCACTCCAGGCCGAACTCCGGT A I A R S K L R L V Y G G A R V G L M G T L A N A A L D S G G T D1 CTTCACTGCGATACCCGAGGCTGCCGCATCATGGACGAACTACACGTCCATGACATGACCACGAACGCCAAAGCTCTCATGGCCGAACTCGGT A I A R S K L R L V Y G G A R V G L M G T L A N A A L D S G G T D1 CTTCACTGCGATACCCGAGGCTGCCGCATCATGGACGAACTACACGTCGTCCATGACATGCACCAACGCAAAGCTCTCATGGCCGAACTCGGT A A A A A A A A A A A A A A A A A A A	ORF6 ACAGCTCGCAGAGGGATGGGGGCG Q L A E G M G R CGTCGTTGGCGTGATCCCTGAGAG V V G V I P E S TGACGCATTCATTGCCCTCCCCGG
6481 6601 6731	61 AATGCCCGCGACTACGGTCTCGGCACAGGCCCCGACCCCCAAGAGCGTCACGGTCTTCTGCGGAGCTATGCCAGGGCGCGGGGACCAAATATGG/ M P A T T V S A Q A R P T P K S V T V F C G A M P G R G T K Y G B1 TGCGATTGCTAGATCGAAGCTGCCCCTCGTTTATGGTGGCGCCCCGAGCCGCCCTCATGGCACCCCGCGCGAACC A I A R S K L R L V Y G G A R V G L M G T L A N A A L D S G T 01 CTCACTGCGATACCCGAGGCTGCGCCTCATGACATGCACCTACGCAAGCCCTCCTCGGCGAAACCCGCG F T A I P E A A H H G L T E L H V V H D M H Q R K A L M A E L G 21 COTCTCGCGATAACCCGCATTGCTCATGCACCTAAGCATCTCAAGCACTAACCGCTAAGCACATACCGCAAACCACCGCAAACCTAACCGCAAACCTAACCGCAAACCTAACCGCAAACCTAACCGCAAACCTAACCGCAAACCTAACCGCAAACCTAACCGCAAACCTAACCGCAAACCAACC	ORF6 ACAGCTCGCAGAGGGATGGGGGCG Q L A E G M G R CGTCGTTGGCGTGATCCCTGAGAG V V G V I P E S TGACGCATTCATTGCCCTCCCCGG D A F I A L P G
6481 6601 6721	ATGCCCGCGCACTACGGTCTCGGCACAGGCCCGCCCGACCCCCAAGAGCGTCACGGTCTTCTGCGGAGCTATGCCAGGGCGCGGGGACCAAATATGG/ M P A T T V S A Q A R P T P K S V T V F C G A M P G R G T K Y G B1 TGCGATGCTAGATCGAAGCTGCGCCTCGTTTATGGTGGCGCCCGAGTCGGCCTCATGGGCACCCCGGCAGCCGGGCCTTGGACTCCGGCGGAACC A I A R S K L R L V Y G G A R V G L M G T L A N A A L D S G G T 01 CTTCACTGCGATACCCGAGGCTGCGCATCATGGACTGACAGACTACACGCGTCGTCATGGCCGAACCCGAACCGCAAACGCAAAGCTCTCATGGCCGAACCGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCAACGCGAACCCGCAGAAGGTTCTTCGAGGTCCTTACGTGGTCACACCGTGGGCCTTCACATAAACCCCTGTGTAACTGCCTGAACGCAAACGACAACGGCAACGCAAACGGCAACGCGAACGCGGAACCGCGAAGGGTTCTTCGAGGTCCTTACGTGGTCACACCGCGGGCTTCACATGACCCCTGTGTAACTGCCTGAACGCAAACGCAAACGGCAACGCGAAACGGCAAACGGCAAACGGCAACGGGTTCACACCGCTGGTCGAACCCGCTGAAGGGTTCTCGCGAAACGCGCAAACGGCAAACGGCAAACGGCAACGCGAAACGGCAAACGGCAAACGGCTTCACCTGGTGGAACCCCTGTGTACTGCTGAACGACAACGGCAAACGGCAAACGGCAACGCGAAACGGCAAACGGACAACGGCAACGCGCAACGCGAAAGGTTCTTCGAGGTCCTTACGTGGTCACACCCTGGGGCTTCACAATAAACCCCTGTGTAACTGCCTGAACGACAACGGCAAACGGCAACGGACAACGGCAACGCGACGCGCAACGGACGAC	ORF6 ACAGCTCGCAGAGGGATGGGGGG Q L A E G M G R CGTCGTTGGCGTGATCCCTGAGAG V V G V I P E S TGACGCATTCATTGCCCTCCCGG D A F I A L P G TTACCGCCCCTGCTCTCCTACAT
6481 6601 6721	 AATGCCCGCGACTACGGTCTCGGCACAGGCCCGACCCGA	ORF6 ACAGCTCGCAGAGGGGATGGGGGCG Q L A E G M G R CGTCGTTGGCGTGATCCCTGAGAG V V G V I P E S TGACGCATTCATTGCCCTCCCGG D A F I A L P G TTACCGCCCCTTGCTCTCCTACAT Y R P L L S Y I ACCCTATLATCCCACTTGCTCT
6481 6601 6721 6841	 AATGCCCGCGACTACGGTCTCGGCACAGGGCCCGACCCGACCCCCAAGAGCGTCACGGTCTTCTGCGGAGCTATGCCAGGGCGCGGGACCAAATATGG/ M P A T T V S A Q A R P T P K S V T V F C G A M P G R G T K Y G B1 TGCGATTGCTAGATCGAAGCTGCGCCTCGTTTATGGTGGCGCCCCGAGTCGGCCTCATGGGCACCCCTCGCGAACGCGGCCTTGGACTCCGGGCGGAACCA A R S K L R L V Y G G A R V G L M G T L A N A A L D S G G T C11 CTTCACTGCGATACCCGAGGCTGCGCATCATGGACTGACAGAACTACACGTCGTCCATGACATGCACAAGCCCCAAGCCCAAGCCCAAGCCCAAGCCCCGAACCCGGT F T A I P E A A H G L T E L H V V H D M H Q R K A L M A E L G C11 CGGTGTCGGAACCGCGAGAGCTTCTCGAGGTCTTACGTGGTCACACCTGGGGCTTCACAATAAACCCTGTGTACTGGTGAACGACAACGACAAGCACTACGAGATTGGTGCCGAACCGCGGAACCACCGGTGCCGTCGCGTAACGACATCGAGGGGGCTATCGCGGCACTCGCCGAACCACCGGTCTCGCGTAATGCTGCGAAGACTACGAGGGGCTATCGCGGCAATCGCCGGCCATCGCCGAACCGCGGTCTCGCGTAATGCTGCGAAGACTACGAGGGGGCTATCGCGGCCATCGCCCGAACCCGGTCTCGCGTAATGCTGCGAAGACTCGAGGGGGCTATCGCGGCCATCGCCCA 	ORF6 ACAGCTCGCAGAGGGGATGGGGGCG Q L A E G M G R CGTCGTTGGCGTGATCCCTGAGAG V V G V I P E S TGACGCATTCATTGCCCTCCCCGG D A F I A L P G TTACCGCCCTTGCTCTCCTACAT Y R P L L S Y I ACCCTAATTAGTGGGGACTTCTGCC
6481 6601 6721 6841	 AATGCCCGCGACTACGGTCTCGGCACAGGCCCCGACCCCCAAGAGCGTCACGGTCTTCTGCGGAGCTATGCCAGGGCGCGGGGACCAAATATGG/ M P A T T V S A Q A R P T P K S V T V F C G A M P G R G T K Y G B1 TGCGATTGCTAGATCGAAGCTGCGCCTGTTTATGGTGGCGCCCCGAGTCGGCCTCATGGGCACCCGCGCGCAGCCGGAACC A I A R S K L R L V Y G G A R V G L M G T L A N A A L D S G G T CTTCACTGCGATACCCGAGGCTGCGCCATCATGGACTGACAGAACTACACGTCGTCCATGACATGCACCAAAGCTCTCATGGCCGAACCGCGAACCGGAACCGCAGACCGGAACCGCGAACCGCGAACCGCGAACCGCGAACCGCGAACCGCGAACCGCGAACGCGAAGCATCTTCGAGGTCCTTACGTGGTCACACCGGGGCTTACGCACAATAAACCCTGTGTACTGCTGCAACGAAACAACGACAACGAAGAACGACGAACAACGAAGTTCTCGAGGACTCCGGGCTTCGCGGGCTTCGCGGGCTTCGCGGCATCGCGGCCATCGCGGCCATCGCGGCCATCGCCGCGAACCCGGGCTATCGCCGCGAACCCGGTCTGGCGCAAGCACGAGGACCAACGAAGAACGACGACGACGAC	ORF6 ACAGCTCGCAGAGGGATGGGGGCG Q L A E G M G R CGTCGTTGGCGTGGCATCCCTGAGAG V V G V I P E S TGACGCATTCATTGCCCTCCCCGG D A F I A L P G TTACCGCCCCTGCTGCTCCTACAT Y R P L L S Y I ACCCTAATTAGTGGGACTTCTGCC P .
6481 6601 6721 6841 6961	ATGCCCGCGACTACGGTCTCGGCACAGGCCCGACCCGAC	ORF6 ACAGCTCGCAGAGGGGATGGGGGCG Q L A E G M G R CGTCGTTGGCGTGATCCCTGAGAG V V G V I P E S TGACGCATTCATTGCCTCCCCCGG D A F I A L P G TTACCGCCCCTTGCTTCCTACAT Y R P L L S Y I ACCCTAATTAGTGGGACTTCTGCC P TACGAAAATTTCACCTAGGCTGGT
6481 6601 6721 6841 6961 7081	 AATGCCCGCGACTACGGTCTCGGCACAGGCCCGACCCGA	ORF6 ACAGCTCGCAGAGGGGATGGGGGCG Q L A E G M G R CGTCGTTGGCGTGATCCCTGAGAG V V G V I P E S TGACGCATTCATTGCCCTCCCCGG D A F I A L P G TTACCGCCCCTTGCTCTCCTACAT Y R P L L S Y I ACCCTAATTAGTGGGGACTTCTGCC P TACGAAAATTTCACCTAGGCTGGT

FIG. 2. Nucleotide sequence of the *fas* locus. The nucleotide sequence of the 7.14-kb fragment between the *StuI* (far right) and *NcoI* (far left) sites (see Fig. 3) spanning the *fas* locus was determined. Six ORFs were identified and are indicated in the one-letter code under the sequence. ORF1 (position 1166 to 2365) has coding capacity for 399 amino acids and is followed by two ORFs (ORF2, position 2379 to 3293, corresponding to 304 amino acids; ORF3, position 3300 to 4238, corresponding to 312 amino acids). ORF4 corresponds to the previously identified isopentenyltransferase. A fifth ORF (position 5031 to 6347, with coding capacity for 438 amino acids) starts 1 base before the stop codon of ORF4, resulting in a 4-bp translational overlap. The final ORF, ORF6 (position 6347 to 6943, putatively encoding 198 amino acids), also has a 1-bp overlap with the previous ORF. The putative ribosome-binding sites upstream from ORF1 to ORF4 are indicated by shaded boxes. The insertion points of pJDGV1 and pTGV6b in *fas5* and *fas6*, respectively, were determined by the nucleotide sequence of the right border fragments (see Materials and Methods) and are indicated by triangles. The box corresponds to the amino-terminal amino acids of ORF3 indicating the predicted signal peptide.

under control of the *cmr* promoter of *R. fascians* [12]) in *fas1* restored the ability to inhibit the growth of tobacco seedlings when small amounts of chloramphenicol ($5 \mu g/ml$) were added to the plant growth medium, consistent with the inducible character of the promoter used (Table 2). However, introduction of the same construct could not restore the avirulent



FIG. 3. Schematic representation of the restriction map of the *fas* locus with indication of the different ORFs (solid arrows). The open arrow represents a hypothetical ORF belonging to the locus immediately upstream of *fas*. Open bars above the restriction map represent the different DNA fragments used in the promoter fusion constructs to *uidA*.

phenotype of fas6 (Table 2), even in the presence of inducing amounts of chloramphenicol. Such a complementation was observed, however, when pMGV100 was introduced in fas6(Table 2), indicating that at least one of the three ORFs, or a combination thereof, located upstream of *ipt* is essential for pathogenesis.

DISCUSSION

Infection of plants by the nocardioform bacterial species R. fascians (42) has for a long time been considered as the prototype of a plant-bacterium interaction requiring only cytokinin production to cause neoplastic growth (15). Several cytokinins have been detected in small amounts in the supernatants of some virulent R. fascians cultures (25, 30, 31) but not in other virulent cultures (1, 8), indicating that the phytopathogenicity of R. fascians involves more than mere cytokinin secretion. Indeed, we have previously demonstrated that fasciation is a multigenic trait with determinants located both on extrachromosomal linear plasmids (Fi plasmids) and on the chromosome (8). Of the three identified pFiD188-located virulence-determining loci (fas, att, and hyp [8]), one (fas) is involved directly in cytokinin production. Further genetic

A M A G T A D L P L E M A A P V T F S T V R E N Y F G P P A E M G A L R H - K A P V M T Q A P A F P S N R S C P Y Q L P D G Y A Q L R D T P G P L M T D T A T T D P A T Q N L D P T S P A P A T S F P Q D R G C P Y H P P A G Y A Q L R D T P G P L T T A E R T A P P D A L T V P A S R A P G C P F D P A P D V T E A A R T - E P V M T T V P D L E S D S F H V D W Y R T Y A E L R E T - A P V M L T T I D E V P G M A D E T A L L D W L L G T M R E - K Q P V	PV 1 TR 2 HR 3 SR 4 TR 5 TP 6 WQ 7
GELY-GAPAFLVCRYEDVRRIFADSNRFSNAHTPMFAIPSGGDV TAFADGRPGWLVTGYSAARAVLSDSRFTARGEREH	IE 1 2 3 4 5 FP 6 7
DELLAAMRAGNLIGLDPPDHTRLRHILAAEFSVHRLSRLQPRIAEIVDS PAVPRAATLEDERCRRLIAGQFTARRMRQLTGRTERIVRE -EAVRESPQAFIGLDPPEHGTRRRMTISEFTVKRIKGMRPEVEEVVHG -AGAQRRRVALLGVDDPEHNTQRRMLIPTFSVKRIGALRPRIQETVDR -REIIGTNPTFLRMDDPEHARLRRNLTADFIVKKVEAMRPPEVQRTAD EDVRNYFATNMGTSDPPTHTRLRKLVSQEFTVRRVEAMRPRVEQITAE -EGASPTPGMIHEIDPPEHRALRKVVSSAFTPRTISDLEPRIRDVTRS	A L 1 H L 2 F L 3 L L 4 L V 5 L L 6 L L 7
D G L E QA G Q P A D L M D R Y A L P V SL L V L C E L L G V P Y A D R D E L R D R T A R L L D D A M E H M G S P A D L V E H F A L P V P SL V I A E L L G V P P A D R E Q F Q H D T L R W G G D E M L A A G P T A D L V S Q F A L P V P S M V I C R L L G V P Y A D H E F F Q D A S K R L V Q D A M E R Q G P P A E L V S A F A L P V P S M V I C A L L G V P Y A D H A F F E E R S Q R L L R D R M E R Q G P P A E L V S A F A L P V P S M V I C A L L G V P Y A D H A F F E E R S Q R L L R D R M T T G R T S A D L V T E F A L P L P S L V I C L L G V P Y E D H A F F Q E R S R V L L T D E V G D S G - V V D I V D R F A H P L P T K V I C E L L G V D E K Y R G E F G R W S S E I L V A D A G E S F D L V D V L A F P L P V T I V A E L L G L P P M D H E Q F G D W S G A L V D	L - 1 F - 2 - 3 - 4 L - 5 M - 6 I Q 7
SAS-AEQRAVAQREDRRYMATLVTRAQEQPGDDLLGILAR-K- GRS-TEEVTEAFVSLGGQLQRLVRLKRTEPGDDLLSGLIA STD-AQSALTARNDLAGYLDGLITQFQTEPGAGLVGALVA-DQ GPG-ADDVNRARDELEEYLGALJORKRAEPGDGLLDELIH-RD RST-PEEVRAAQDELEYLARLARTKRERPEDAJISRLVA-R- DPERAEQRGQAAREVVNFILDLVERRRTEPGDDLLSALIRVQD MDDPTDPALAERTADVLNPLTAYLKARCAERRADPGDDLISRLV-LAE	- I 1 - A 2 L A 3 H P 4 5 D D 6 V D 7
GD N L ST D E L I S I I S L I M L G G H E T T A S M I G L S V L A L L H H P E Q A A M M I D P A L T D E E L A S I A F L L L V A G H G T T A H Q I A L G A F L L L E H P D Q L A A L R N G E I D R E E L I S T A M L L L I A G H E T T A S M T S L S V I T L L D H P E Q Y A A L R D G P V D R E Q L V A F A V I L L I A G H E T T A NM I S L G T F T L L S H P E Q L A A L R - G E L D D T Q I A T M G L L L V A G H E T T A NM T A L S T L V L L R N P D Q L A A L R D G R L S A D E L T S T A L V L L L A G F E A S V S L I G I G T Y L L L T H P D Q D Q L A L V R G R A L D D E E A A N F S T A L L L A G H I T T T V L L G N I V R T L D E H P A H W D A A A	ED1 AD3 AG4 AE5 RD6 ED7
PN CVNSGIEELLRWLSVAHSQPPRMAVITEVQIAGVTIPAGSFVIPSLL PALTESAVEELLRWLSVVHHGPTRAALQDADIEGTPVKAGEVVVVSLG RSLVPGAVEELLRYLAIADIAGGRVATADIEVEGHLIRAGEGVIVVNS GTSTAVVVEELLRFLSIAE-GLQRLATEDMEVDGATIRKGEGVVFSTS PALVKGAVEELLRFLSIAE-GLQRLATEDVLIGGRTIAAGEGVLCCMIS PSALPNAVEEILRYIAPPETT-TRFAAEEVEIRGVAIPQYSTVLVANG PARIPAIVEEVLRY-RPPFQMQRTTTKATEVAGVPIPADVMVNTWVL	A A 1 A A 2 I A 3 L I 4 S A 5 A A 6 S A 7
N R D SNLT D R P D D L D I T R G V A G H L A F G H G V H F C L G H S L A R M T L R T A N R D P A R F E R P D A V D V T R E D T G H L A F G H G M H Q C L G R Q L A R I E L R V A N R D G T V Y E D P D A L D I H R S A R H H L A F G F G V H Q C L G Q N L A R L E L E V T N R D A D V F P R A E T L D W D R P A R H H L A F G F G V H Q C L G Q N L A R A E L D I A N R D A D V F P R A E T L D W D R P A R H H L A F G F G V H Q C L G Q N L A R A E L D I A N R D A E V F P G D L D V A R D A R R H V A F G F G V H Q C L G Q N L A R A E L D I A N R D A E V F P G D L D V A R D A R R H V A F G F G V H Q C L G Q P L A R V E L Q I A N R D A E V F P G D D D V A R D T R G H L S F G Q G I H F C M G R P L A K L E G E V A N R D P K Q F P D P H R F D V T R D T R G H L S F G Q G I H F C M G R P L A K L E G E V A N R D S D A H D D P D R F D P S A Q V R P A P R T S S F G H G V H F C L A A P L A R L E N R VA	VP 1 LT 2 LN 3 MR 4 IE 5 LR 6 LE 7
A V L R R F P D L A L S - P S H D V R L R S - A S I V L G L E E L Q L T W A L L E R F P H L R L A C P A A E I P L R H - D M Q V Y G A D R L P V A W A L M D R V P T L R L A V P V E Q L V L R P - G T T I Q G V N E L P V T W T L F E R L P G L R L A V P A H E I R H K P - G T T I Q G L D L P V A W T L R R L P D L R L A V P A H E I R H K P - G D M A I Y G V N S L P I A W A L R R L P D L R L A V P A H E I P F R G - D M A I Y G V N S L P I A W A L F G R F P A L S L G I D A D D V V W R R - S L L L R G I D H L P V R L D G E I I A R F G R L T V D R D D E R L R H F E Q I V L G T R H L P V L A G S S P R Q S A	1 2 3 4 5 6 7
B * * * * MKVVVN E RRCFGSGQCVLVAPEVFEQS - NDGTVTLL- VDKPSPDNHSL MRIHVDQDKCCGAAGSCVLAAPDVFDQREEDGIVVLLDTAPPAALHDA- MRVSADRTVCVGAGLCALTAPGVFDQ- DDDGIVTVL- TAEPAADDDRR	- 1 - 2 T 3
V RAAAR SC PATAIR FEENAM V REAATIC PAAAIT V TD AREAGH LC P S G A V R V VED T E	1 2 3



FIG. 4. Sequence comparison between different *fas*-encoded gene products of *R. fascians* and homologous proteins found in data bases. Chemically conserved residues are boxed. (A) Amino acid alignment of ORF1 *R. fascians* (no. 1 [right]) and P-450 cytochromes from actinomycetes. The heme-binding cysteine residue is indicated by an asterisk. The following P-450 cytochromes (numbers on the right) were used in the alignment: 2, CHOP, involved in cholesterol metabolism by *Streptomyces* sp. (19); 3, the herbicide-inducible cytochrome P-450 (SUAC) from *Streptomyces griseolus* (34); 4, a soybean flower-induced P-450 cytochrome (SOYC) from *Streptomyces griseus* (43); 5, a second herbicide-inducible P-450 cytochrome (SUBC) from *S. griseolus* (34); 6, a hydroxylase (ERYF) from *Saccharopolyspora erythraea* involved in erythromycin biosynthesis (16); and 7, the P-450 cytochrome (ERYK) from *S. erythraea* involved in the final C-12 hydroxylation of erythromycin B and D (40). (B) Amino acid alignment between the N-terminal residues of ORF2 of *R. fascians* (no. 1 [right]) and herbicide-inducible ferredoxins Fd₂ (no. 2 [right]) and Fd₁ (no. 3 [right]) from *S. griseolus* (32). The conserved cysteine residues are indicated by asterisks. (C) Amino acid alignment between the carboxy-terminal residues of the ORF2 of *R. fascians* (no. 1 [right]) and the amino-terminal residues of the incomplete CFXs from the chromosome (no. 2 [right]) and the megaplasmid pHG1 (no. 3 [right]) involved in the reductive pentose phosphate cycle for CO₂ fixation in the chemoautotrophic bacterial species *A. eutrophus* (26), the transketolase (TKLB) from *R. sphaeroides* involved in photosynthetic CO₂ fixation in the chemoautotrophic bacterial species of the methylotrophic yeast *H. polymorpha* (transfer of glycoaldelhyde group of xylulose 5'-phosphate to formaldehyde) (no. 5 [right]) (20). (D) Alignment between the N-terminal amino acids of the ORF6-encoded product from *R. fascians* (no. 1 [right]) and the hypothetical protein found u

analysis of this locus, described here, revealed that *fas* consists of at least three genes involved in fasciation development.

The earlier identified isopentenyltransferase-coding region (ipt [8]) is essential for the seedling growth inhibition phenotype, and a mutation in it (fas1) can be complemented by the wild-type *ipt* region in *trans*, on the condition that it has been provided with an *R. fascians* promoter (Fig. 1 and Table 2).

At least one gene downstream from *ipt* is required for fasciation on older *N. tabacum* plants (older than 3 weeks) but not on younger plants (characterized by *fas5*) (Fig. 1 and Table 2). This transition is not abrupt, because mild leafy gall symptoms can be observed on 2-week-old plants. Germinating (growing) seedlings go through a series of changes in hormonal balance (29, 41), and a possible function for these genes could be to counteract these changes. Equally, these genes could allow an efficient colonization of the older plants which are more resistant to pathogen attack. *R. fascians* isolates, which cannot efficiently induce fasciations on 5- to 8-inch (12.70- to 20.32-cm)-high sweet pea plants but do so when inoculated on

young seedlings, have been described in early reports on the causative agent of sweet pea fasciation. These strains were unable to fasciate tobacco and geranium (Pelargonium zonale) plants (3). Less-pathogenic strains were also described in later reports on R. fascians (27, 31) and do not fasciate tobacco (unpublished data). Therefore, it is tempting to speculate that these isolates lack specifically the downstream genes present in highly virulent strains such as D188. Two ORFs were identified in this region. In mutant fas5, ORF5 is carboxyl-terminally truncated by the pJDGV1 insertion, but a polar effect on the expression of ORF6 cannot be excluded. The translational coupling of ORF4, ORF5, and ORF6 suggests a tight stoichiometrical requirement of the three gene products. The ORF6encoded protein shares extensive homology with a hypothetical peptide from P. aeruginosa (18). This latter bacterium is an opportunistic pathogen of humans (14) and, occasionally, of plants (7).

Finally, at least one of the ORFs upstream of *ipt* is essential for fasciation. The mutation in *fas6* did not polarly inactivate

ipt expression and was not complemented by introduction of the plasmid with ipt under control of the cmr promoter (Table 2 and Fig. 1). Two of three ORFs in this region have extensive homologies to P-450 cytochromes and the ancillary iron-sulfur redox proteins (ferredoxins) found in biodegradative and biosynthesis pathways of actinomycetes (for reviews, see references 33 and 37). No ferredoxin reductase, the third component of these oxido-reduction systems, could be detected. Frequently, these reductases are not encoded within the P-450-ferredoxin operon (33). The coexpression of a P-450 monooxygenase, ferredoxin, and isopentenyltransferase in R. fascians could indicate a functional relationship between these enzymes by which isopentenyladenine-adenosine (generated by the isopentenyltransferase reaction) is oxidized to trans- or cis-zeatin-riboside. These latter cytokinins have been detected in R. fascians supernatants (1, 25, 38). The oxidation reaction from 6-isopentenyladenine to zeatin has been demonstrated not to be a property of Agrobacterium ipt or tsz genes and to be catalyzed by E. coli extracts in well-aerated tubes (17). R. fascians might have evolved this specialized system to fulfill the specific oxygen demands in the bacteria during the infection. P-450 monooxygenases were found in other microorganisms interacting with plants (e.g., pisatin demethylase in Nectria haematococca and pinF in octopine-type Agrobacterium tumefaciens strains [23, 28]). The role of Nectria pisatin demethylase in degrading phytoalexins of the plant host, allowing infection by the fungus, has been clearly demonstrated (24). A similar putative role has been attributed to the P-450 cytochromes of A. tumefaciens, since pinF mutant strains require larger inocula to successfully infect plants (23). R. fascians mutants fas6 and fas6(pMGV103), however, are completely avirulent, rendering an analogous role for the gene products of ORF1 and ORF2 in phytoalexin degradation less likely.

The ORF2 product has an additional carboxy-terminal domain which resembles the amino-terminal part of transketolases from *Rhodobacter sphaeroides* and *Alcaligenes eutrophus* and the amino-terminal part of dihydroxyacetone synthase of the methylotrophic yeast *Hansenula polymorpha*. These enzymes catalyze the transfer of two carbon units from a sugar to another sugar or to formaldehyde in the case of dihydroxyacetone synthase (6, 20, 26). The functional relevance of the coupling between ferredoxinlike and transketolaselike domains in one protein remains obscure. Equally, no function can be ascribed to the peptide encoded by ORF3, although protein sequence analysis predicted the presence of a signal peptide (Fig. 2).

Expression of the *fas* operon is regulated by the inducing substance or substances released during R. fascians plant interaction (8). Large operons involved in plant-microbe interactions, whose expression is induced during the interaction, have been described in virtually all of the well-studied gramnegative phytopathogenic and symbiotic bacteria (nod [13], vir [22, 39], and hrp [44]), and the data presented here seem to indicate a similar arrangement in gram-positive phytopathogens. These operons are involved in the synthesis of the molecular mediator of the interaction (be it a protein-coated T-DNA, elicitor molecule, or Nod factor) and in the export of this molecule towards the site of action. In Agrobacterium spp., expression of several of the vir loci is coordinately regulated by acetosyringone. Also, in R. fascians, other loci with plantinducible expression were detected in the vicinity of the fas locus (unpublished data). The different loci of this regulon might be involved in the biosynthesis by R. fascians of a specific cytokininlike molecule and/or transport towards the responsive sites in the plants, explaining the massive shoot production after infection. Biochemical characterization of the biomolecule or biomolecules produced by *R. fascians* will facilitate elucidation of the functions of the different identified gene products and might also add to understanding of cytokinin action and shoot development in plants.

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