pFiD188, the Linear Virulence Plasmid of *Rhodococcus fascians* D188

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Rhodococcus fascians is currently the only phytopathogen of which the virulence genes occur on a linear plasmid. To get insight into the origin of this replicon and into the virulence strategy of this broad-spectrum phytopathogen, the sequence of the linear plasmid of strain D188, pFiD188, was determined. Analysis of the 198,917 bp revealed four syntenic regions with linear plasmids of *R. erythropolis*, *R. jostii*, and *R. opacus*, suggesting a common origin of these replicons. Mutational analysis of pFi_086 and pFi_102, similar to cutinases and type IV peptidases, respectively, showed that conserved region R2 was involved in plasmid dispersal and pointed toward a novel function for actinobacterial cutinases in conjugation. Additionally, pFiD188 had three regions that were unique for *R. fascians*. Functional analysis of the *stk* and *nrp* loci of regions U2 and U3, respectively, indicated that their role in symptom development was limited compared with that of the previously identified *fas*, *att*, and *hyp* virulence loci situated in region U1. Thus, pFiD188 is a typical rhodococcal linear plasmid with a composite structure that encodes core functions involved in plasmid maintenance and accessory functions, some possibly acquired through horizontal gene transfer, implicated in virulence and the interaction with the host.

Among the gram-positive Actinomycetales, members of the genus *Rhodococcus* are renowned for their biotechnological applications and for their ability to degrade a wide variety of organic compounds and xenobiotics (Larkin et al. 2005; van der Geize and Dijkhuizen 2004). Several large, mostly linear, plasmids, harboring the genes encoding characteristic meta-

bolic functions, have been identified in different *Rhodococcus* spp. (Meinhardt and Klassen 2007). For instance, *Rhodococcus jostii* RHA1, one of the most effective polychlorinated biphenyl degraders, has three linear plasmids (McLeod et al. 2006), whereas the genes involved in alkane degradation of *R. erythropolis* PR4 (Sekine et al. 2006) and those required for isopropylbenzene and chlorocatechol catabolism of *R. erythropolis* BD2 (Stecker et al. 2003) and *R. opacus* B4 (GenBank accession number AP011116), respectively, are all located on linear plasmids.

The only plant pathogen within the genus, *R. fascians*, also contains large plasmids that encode special traits, such as heavy metal and antibiotic resistance (Desomer et al. 1988; Lawson et al. 1982). A conjugative circular plasmid (pD188) of 138 kb had been isolated from the highly virulent strains D188 and NCPPB 1488 and, although cadmium resistance was associated with its presence, virulence was not (Desomer et al. 1988). In their search to isolate the pathogenicity determinants of *R. fascians* D188, Crespi and associates (1992) discovered a conjugative linear plasmid, designated pFiD188, as the carrier of the virulence genes. In other *R. fascians* isolates as well, the occurrence of a linear plasmid was strictly correlated with pathogenicity (Crespi et al. 1992; Stange et al. 1996). In both gram-negative and gram-positive phytopathogens, virulence genes are often located on genomic pathogenicity islands or on circular plasmids (Barash and Manulis-Sasson 2009; Bentley et al. 2008; Bignell et al. 2010; Gal-Mor and Finlay 2006; Vivian et al. 2001) but *R. fascians* is the only phytopathogen known to harbor a linear plasmid that carries the virulence genes (Francis et al. 2007).

R. fascians takes a unique position among the hyperplasia-inducing plant pathogens because it provokes the formation of differentiated tumors, called leafy galls. The pathology is triggered by the secretion of plant growth regulators, such as cytokinins and auxins, that perturb the hormone balances in the host and turn on a signal transduction cascade in the plant, resulting in the activation of existing meristems and de novo meristem formation (Depuydt et al. 2008; Pertry et al. 2009; Simón-Mateo et al. 2006; Stes et al. 2011; Vandeputte et al. 2005).

By pulsed-field electrophoresis and macro-restriction analysis, the size of pFiD188 was estimated to be between 200 and 210 kb (Crespi et al. 1992; Pisabarro et al. 1998). Since its discovery, three virulence loci have been identified (Crespi et al. 1992). Two of them have been analyzed in detail and proved to be insufficient to render strain D188-5, the plasmid-free derivative of strain D188, pathogenic (Crespi et al. 1992; 1994; Maes

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et al. 2001; Pertry et al. 2010; Temmerman et al. 2000). Therefore, and because these loci represented less than 18% of the coding potential of the replicon, we set out to uncover the remaining information encoded by pFiD188. The termini were cloned, the sequence was determined, the structural features were analyzed and compared with those of other linear plasmids, and the genes were annotated. Functional information on three previously uncharacterized loci was obtained through mutation, expression, or biochemical analyses, singly or in combination.

RESULTS AND DISCUSSION

Sequence characteristics of pFiD188, an invertron-type linear plasmid.

Linear plasmids of actinomycetes are predominantly of the invertron type, with terminal inverted repeats (TIR) and terminal proteins (TP) covalently linked to their 5' ends (Meinhardt and Klassen 2007). Pulsed-field gel electrophoresis of bacterial agarose plugs prepared with and without proteolytical treatment and digested with *KpnI* identified 11.0- and 1.7-kb fragments as the protein-bound termini of pFiD188 (data not shown).

Both fragments were cloned and, although hybridization experiments confirmed their linear plasmid origin, no cross hybridization was observed between the fragments, hinting at a lack of extensive homology between the termini and, hence, of extended TIR (data not shown). Indeed, when the nucleotide sequences of the termini were compared, only one short 7-bp perfect TIR and several other short inverted repeats were identified (Fig. 1A), similar to the termini of the linear replicons of other *Rhodococcus* spp. but in contrast to the linear plasmids of *Streptomyces* spp. that have long TIR (Kalkus et al. 1998; Sekine et al. 2006; Stecker et al. 2003).

The sequence of the termini also hinted at a probable linkage of the terminal proteins of pFiD188 to the 5' end via dCMP, as observed for all other linear plasmids from actinomycetes studied so far (Parschat et al. 2007). Calculations based on the energy minimization algorithm mfold (SantaLucia 1998) predicted that, in a single-stranded 3' overhang, nucleotides might fold back to form a complex secondary structure

containing stable hairpins (Fig. 1B). Two central GCTxCGC motifs, conserved in the termini of linear replicons of *Rhodococcus* and *Streptomyces* spp. and part of the TP recognition sites (Bao and Cohen 2003; Kalkus et al. 1998), were detected in the telomeric sequences of pFiD188 (Fig. 1A).

To determine the complete sequence of pFiD188, template DNA was obtained by combining several approaches (discussed below) and primer walking resulted in a sequence of 198,917 bp (GenBank accession number JN093097). The calculated average G+C content of pFiD188 was 61.8%, which is within the range described for genomic DNA of the genus *Rhodococcus* (61 to 68%) (LeChevalier 1986); still, a strong variation was observed in a 100-bp window plot with maxima as high as 77% and minima as low as 41% (Supplementary Fig. S1).

pFiD188 shares a common origin with other rhodococcal linear plasmids.

By combining computer prediction, manual curation, and similarity searches, we identified and annotated 184 open reading frames: 91 gene products had a functional homolog, 56 were conserved hypothetical proteins, and 37 were unique for *R. fascians* (designated "hypothetical proteins") (Supplementary Table S1). The G+C content of the majority of the predicted genes ranged between 60 and 65% but two continuous regions, consisting of *pFi_074* to *pFi_084* and *pFi_111* to *pFi_123*, had a G+C content that was markedly lower than the pFiD188 average (58 and 58.5%, respectively). Analysis with the Alien Hunter application (Vernikos and Parkhill 2006) predicted that these loci were part of these regions, which, together with three other ones (i.e., *pFi_011* to *pFi_019*, *pFi_028* to *pFi_036*, *pFi_072* to *pFi_087*, *pFi_107* to *pFi_125*, and *pFi_141* to *pFi_146*), might have been acquired through horizontal gene transfer (HGT) events.

Most pFiD188-encoded proteins were homologous to proteins of other actinomycetes. This finding is in contrast to the proteins encoded by pVAP, the circular virulence plasmid of the only animal pathogen within the *Rhodococcus* genus, *R. equi*, that have little or no homology with proteins of any other species (Letek et al. 2010). Even more, pFiD188 was strikingly similar to linear plasmids of several environmental *Rhodococ-*

Fig. 1. Structural features of the pFiD188 termini. **A**, Alignment of the left- and right-end telomeres. Identical bases are boxed in gray; inverted repeats and the GCTxCGC motifs central in the terminal protein recognition site are indicated by converging arrows and in black, respectively. **B**, Predicted secondary structures formed by the 3' single-stranded DNA sequence of the left- and right-end telomeres (mfold) (SantaLucia 1998). Structures with the lowest energy ($\Delta G = -30.49$ kcal/mol and $\Delta G = -37.52$ kcal/mol for the left and right end, respectively) are shown.

cus spp. Analysis with the Artemis Comparison Tool (Carver et al. 2005) (threshold similarity 90%) revealed that pFiD188, pBD2, and pREL1 of *R. erythropolis* strains BD2 and PR4 (Sekine et al. 2006; Stecker et al. 2003); pRHL2 of *R. jostii* RHA1 (McLeod et al. 2006); and pROB01 of *R. opacus* B4 (GenBank accession number AP011116) shared four regions of extensive homology and colinearity (designated R1, R2, R3, and R6 according to Sekine and associates [2006]), implying a common origin for these five linear replicons. Whereas these conserved regions carry functions that are essential for plasmid maintenance, the unique regions of each plasmid (designated U regions) code for typical features of the particular bacterial strain (Fig. 2).

The conserved region R1 encompasses the left border of pFiD188 and the related linear replicons (Fig. 2). Although some genes had no functional homologs, the R1 region comprised genes putatively involved in natural transformation, plasmid replication, and partitioning. *pFi_002* probably codes for a DNA-processing chain A (DprA) protein belonging to the Smf superfamily that is required for natural transformation, a process inherent to almost all bacteria. DprA has been suggested to be involved in the protection of incoming exogenous DNA prior to homology-dependent recombination into the genome (Mortier-Barrière et al. 2007). The gene pair *pFi_009* and *pFi_010* is conserved in the other rhodococcal linear plasmids and codes for the 5' terminal and telomere-associated proteins that play a role in telomere patching during replication of linear plasmids (Chen 2007). *pFi_009* is predicted to contain a *racA* domain that is active in polar chromosome and plasmid segregation and functions in association with a *Soj/ParA* ATPase (Wu and Errington 2003), which is encoded by *pFi_013*. Generally, the ATPase activity of *ParA* is regulated by *ParB* and the corresponding gene pair is thought to be essential for plasmid partitioning (Surtees and Funnell 2003). However, no putative *ParB*-coding gene was identified on pFiD188 or on several other linear plasmids from actinobacteria, including pBD2, pREL1, pRHL2, and pROB01. Because the functional significance of this lone *parA* gene is unclear, plasmid partitioning in actinobacteria might be mediated by another mechanism (Parschat et al. 2007).

The conserved region R3 encompasses a region with extensive homology to the tight adherence (*tad*) locus (*pFi_020* to *pFi_026*) (Fig. 2), initially identified as an essential virulence factor in *Actinobacillus actinomycetenscomitans*, the causative agent of localized juvenile periodontitis but present in many

archaeal and bacterial species (Tomich et al. 2007). The *Tad* proteins form a macromolecular transport system required for the assembly of adhesive pili necessary for biofilm formation, colonization, and pathogenesis (Tomich et al. 2007). Biofilm formation is essential in natural bacterial populations to ensure their survival under harsh conditions and it is also an important mechanism for the colonization of target tissues by pathogens, which is the first phase of pathogenicity (Danhorn and Fuqua 2007). *R. fascians* forms pellicles on the surface of liquids (Fig. 3A) and extensively colonizes leaf surfaces (Cornelis et al. 2001) (Fig. 3B). Thus, although the conservation of region R3 in rhodococcal linear plasmids might point to a general role in bacterial ecology, it cannot be ruled out that this region might also contribute to the *R. fascians* virulence by promoting attachment to the plant.

The third conserved region, R6, encodes mainly conserved hypothetical proteins (Fig. 2); thus, its function in the plasmid biology is unknown. The regions R4 (the most elaborate homology region between pBD2, pREL1, and pROB01) and R5 are not conserved on pFiD188 or pRHL2; R5 is not present on pROB01, either (Fig. 2). The gene products encoded by these two regions are involved in lipoprotein processing and heavy metal and arsenate resistance, respectively (Sekine et al. 2006). In *R. fascians* D188, heavy metal resistance (cadmium) is carried on a separate replicon, the circular plasmid pD188 (Desomer et al. 1988).

The conserved region R2 is involved in conjugative plasmid transfer.

The conserved R2 region contains mostly hypothetical proteins with predicted transmembrane domains (TMD) (Fig. 2). However, *pFi_086*, the middle gene of a putative three-gene operon, is homologous to cutinases, a distinct class of extracellular plant cell-wall-degrading enzymes that have been identified and studied mainly in fungi. Cutinases are serine esterases that function in the hydrolytic degradation of the cuticula by targeting mostly primary alcohol esters of the cutine polymer (Carvalho et al. 1999; Schäfer 1993). Despite extensive screening, cutinase activity does not seem to be widespread among bacteria (West et al. 2009), and analysis of bacterial genomic sequences reveals that cutinase homologs seem to be largely restricted to the gram-positive *Corynebacterinae* (pfam PF01083). Hardly any information is available on the role of cutinases in these organisms but hypothetical functions have been postulated in virulence, saprophytic growth, in vivo sur-

Fig. 2. Schematic representation of the composite structure of rhodococcal linear plasmids, based on protein similarity plots generated by the Artemis Comparison Tool (threshold similarity 90%). pFiD188 (*Rhodococcus fascians*), pBD2 (*R. erythropolis* BD2), pREL1 (*R. erythropolis* PR4), pRHL2 (*R. jostii* RHA1), and pROB01 (*R. opacus* B4). The open reading frame range of conserved (R) and unique (U) regions and the size of the linear plasmids are indicated.

vival, bacterial lipid metabolism, or enzymatic manipulation of the bacterial cell envelope during growth, cell division, or autolysis (West et al. 2009). Interestingly, analysis with the Alien Hunter application predicted that the cutinase operon on pFiD188 would be acquired through HGT.

Because endophytic populations are established by *R. fascians* (Cornelis et al. 2001), we postulated that the *pFi_086*-encoded protein would be required to breach through the cuticula and the epidermal barrier of its host. Indeed, the plasmid-free strain D188-5 only forms discrete endophytic populations (Cornelis et al. 2001), suggesting that the linear plasmid carries functions that greatly enhance penetration of and dissemination into the plant tissues. The role of the cutinase gene in virulence was evaluated by generating a cutinase deletion mutant, D188- Δ *cuti*, by means of exchange recombination. Unexpectedly, infection of unwounded excised tobacco (*Nicotiana tabacum*) leaves with the D188- Δ *cuti* mutant resulted in leafy galls that were similar to those induced

by the wild-type strain (Fig. 4A and B), not supporting the hypothesis that the function of the cutinase of *R. fascians* is analogous to that of fungi.

Interestingly, five of the genes in region R2 encode proteins that might possibly be involved in conjugation. *pFi_092* putatively codes for an ATP/GTP-binding protein of the VirB4 family, *pFi_093* is a polytopic integral membrane protein, and *pFi_098* is similar to DNA translocases of the FtsK/SpoIIIE family. Together, these proteins might constitute a functional DNA translocase complex (Barre 2007). *pFi_094* is homologous to genes encoding lytic transglycosylases and could play a role in the local opening of the peptidoglycan layer (Grohmann et al. 2003). *pFi_102* putatively encodes a prepilin-type IV peptidase, one of the players in the formation of type IV pili known to be involved in the interbacterial contact required for conjugation in gram-positive bacteria (Grohmann et al. 2003). Indeed, *R. fascians* forms cellular protrusions that resemble type IV pili during conjugation (Fig. 4C). These data suggested that the R2 region of pFiD188 might code for a membrane-associated conjugation complex. To validate this hypothesis, we tested the transfer frequency of the mutant linear plasmids from D188- Δ *cuti* and from a *pFi_102* insertion mutant into the plasmid-free acceptor strain D188-5. As a control, D188- Δ *attR* (Maes et al. 2001) was chosen because it is resistant to kanamycin (Km) and has a single interrupted gene, like the test strains D188- Δ *cuti* and D188-*pFi_102*. The pFi transfer frequency from D188- Δ *cuti* and D188-*pFi_102* was 60- to 100-fold lower than that of D188- Δ *attR*, indicating that both gene functions are important for efficient conjugation (Fig. 4D). Introduction of complementation constructs containing *pFi_086* into D188- Δ *cuti* largely restored the transfer frequency, thus supporting this finding for the cutinase gene (Fig. 4D). The occurrence of cutinase genes restricted to members of the Corynebacterinae strongly implies that cutinases in these organisms function primarily in cell wall metabolism and facilitate conjugation, most probably by local loosening of the mycolic acid cell wall that borders the peptidoglycan layer.

The unique region U1 is the main pathogenicity region.

Of the three unique regions of pFiD188, U1 is the largest and contains the previously identified virulence loci *fas*, *att*, and *hyp* (Crespi et al. 1992; Maes et al. 2001; Rajanons et al. 2011) (Fig. 2). Interestingly, the *fas* operon (*pFi_077* to *pFi_082*) that is the central virulence locus and encodes the enzymatic machinery for cytokinin production (Pertry et al. 2009, 2010) has a low G+C content (58.8%) and is located in one of the regions identified with the Alien Hunter application as possibly being acquired through HGT. In this region, four additional genes (*pFi_075*, *pFi_076*, *pFi_083*, and *pFi_084*) colocalize with the *fas* locus; therefore, their gene products might be implicated in cytokinin production or virulence. *pFi_075* and *pFi_076* encode *S*-adenosylmethionine-dependent methyltransferases and they are conserved in the *fas* locus of *Streptomyces turgidiscabies*, another plant-pathogenic actinomycete capable of inducing leafy galls, as part of a pathogenicity island thought to be acquired through HGT (Joshi and Loria 2007). Interestingly, mutants in *pFi_075* and *pFi_076* are nonpathogenic (Pertry 2009).

In addition to these known virulence loci, region U1 also contains a biosynthetic gene cluster (*pFi_055* to *pFi_062*) that putatively produces a γ -butyrolacton-type autoregulator typically involved in cell-to-cell signaling and control of secondary metabolite production (Kato et al. 2007). Possibly, this autoregulator is involved in the regulation of cytokinin (Pertry 2009) or auxin biosynthesis (Vandeputte et al. 2005) in *R. fascians*.

Fig. 3. Biofilm formation by *Rhodococcus fascians* D188 populations. **A**, Detail of a pellicle formed on the liquid-air interphase of a bacterial culture. The inset shows a macroscopic view of the pellicle (indicated by *). **B**, Epiphytic biofilm on a tobacco leaf.

Finally, the region U1 contains a putative toxin-antitoxin system (pFi_041 and pFi_042), originally identified as a mechanism to ensure plasmid inheritance or stability (Van Melder and De Bast 2009). However, pFiD188 is not essential for growth, because a plasmid-free derivative could be isolated easily (Desomer et al. 1988). Moreover, this system is not conserved in the related rhodococcal linear plasmids, all arguing against such a function in *R. fascians*. Nevertheless, pFiD188 has proven to be extremely stable both under culture conditions and in plant tissues (D. Vereecke, *personal communication*). In the latter, the presence of pFiD188 in the bacteria is essential for the continuous production of plant growth regulators that are instrumental for niche maintenance (Vereecke et al. 2000). Therefore, it cannot be ruled out that pFi_041 and pFi_042 are involved in plasmid stability during the epi- and endophytic phases of the *R. fascians* life cycle. However, in several bacterial species, chromosomal homologs of toxin-antitoxin genes have been identified that cleave mRNA or phosphorylate proteins in response to nutritional stress, thereby inducing a transient state of cell cycle arrest that allows the bacteria to

persist under starvation or other adverse conditions (Hayes and Low 2009). In *Mycobacterium tuberculosis*, such mechanisms have been implicated in the apparent metabolic dormancy of cells that mediate persistent infections (Zhu et al. 2006). Because *R. fascians* and *M. tuberculosis* share persistence strategies (Vereecke et al. 2002), it is tempting to assume that pFi_041 and pFi_042 encompass a mechanism involved in successful survival in plant tissues.

Altogether, the genes present in the U1 region apparently seem to be either genuine virulence factors or involved in pathogenicity regulation. Therefore, this region is considered to be the main pathogenicity island on pFiD188.

The putative nonribosomal peptide synthetase located in the unique region U2 is not essential for virulence.

Most genes within the unique U2 region have no significant homologs and their role in the *R. fascians* biology remains to be determined (Fig. 2). Nevertheless, the largest gene cluster of the unique U2 region consists of 13 genes (*pFi_111* to *pFi_123*) with the same transcriptional orientation; their low

Fig. 4. Conjugation-related aspects of *Rhodococcus fascians*. **A**, Leafy gall formation of strain D188 on excised tobacco leaves. **B**, Leafy gall formation of mutant strain D188- $\Delta cuti$ on excised tobacco leaves. **C**, Pili-like structures connecting *R. fascians* cells during conjugation. **D**, Efficiency of transfer of pFiD188 from D188- $\Delta cuti$, complemented D188- $\Delta cuti$, and D188-*pFi_102* compared with that of D188- $\Delta attR$ as a control. Different letters indicate statistically significant differences in the conjugation frequency according to Student's *t* tests ($P < 0.05$).

G+C content (58.5%) and Alien Hunter analysis implies that they might have been acquired via HGT. Based on homology to nonribosomal peptide synthetases (NRPS), this cluster was designated the *nrp* locus. NRPS are large multienzyme complexes organized in modular subunits that can each incorporate a single substrate and act together in a template-based mode to achieve a specific N-to-C-terminal stepwise assembly of the peptide chain (Finking and Marahiel 2004). The semi-autonomous modules contain highly conserved core motifs involved in recognition, activation, thiolation, and, optionally, modification of the substrate (Konz and Marahiel 1999) (Fig. 5A).

In Nrp5, Nrp7, Nrp8, and Nrp9, distinct functional domains could be identified but a modular arrangement was absent in all except Nrp5 (Fig. 5B). Therefore, it is not possible to predict the chemical structure of the produced peptide based on their deduced amino acid sequence. The other *nrp* genes might participate in the modification of the NRPS peptide.

Because coexpression with virulence determinants often indicates involvement in pathogenicity, we first monitored the expression of *nrp5* via a β -glucuronidase (*gus*) reporter strain grown under conditions that activate the *R. fascians* virulence genes (Materials and Methods). However, a comparable and constitutive expression level was measured for all growth conditions tested (data not shown). To examine a possible role in virulence, we generated a deletion mutant that lacks the adenylation domain of module 3 of Nrp5, D188- Δ *nrp5*, via double homologous recombination (Fig. 5B). Different infection procedures with D188- Δ *nrp5* induced symptoms on tobacco that were indistinguishable from those induced by strain D188 (Fig. 5C). These observations imply that the *nrp* locus does not encode an essential virulence factor but alternative functions in the *R. fascians* pathology might be related to host range, ecological fitness, or bacterial competitiveness.

The *stk* operon of the unique U3 region is putatively involved in phosphate-dependent cell wall modification but is not implicated in symptom induction.

Whereas one end of the five related linear rhodococcal replicons is conserved, the other end is unique for each plasmid (Fig. 2). In pFiD188, this region mainly encodes (conserved) hypothetical proteins but, interestingly, it also harbors the *stk* operon (*pFi_176* to *pFi_180*), which is homologous to a conserved gene cluster essential for proper cell division found in all sequenced actinomycetes to date (Fig. 6A). In these clusters, regulatory proteins active in serine/threonine phosphorylation/dephosphorylation events occur together with proteins implicated in cell wall biosynthesis and morphogenesis. The colinearity and the absence of a comparable cluster in the genomes of other prokaryotes suggest that this locus is specific for a cell division-associated regulatory pathway, corresponding to the high G+C branch of the gram-positive bacteria (Molle and Kremer 2010). Thus, the occurrence of the *stk* operon on pFiD188, which is dispensable for bacterial growth and survival, implies that it has acquired a different function in *R. fascians*.

The first gene of the *stk* operon, *stk1* (*pFi_176*), encodes a serine/threonine kinase (Jones and Dyson 2006). The hydrophathy profile of the Stk1 protein predicts the presence of four N-terminal TMD, creating a putative extracellular N-terminal sensor module. Typically, the extracellular modules of the sensor kinases of the conserved actinobacterial clusters contain PASTA domains involved in β -lactam binding (Yeats et al. 2002) but such motifs are absent from the putative sensor domain of Stk1, suggesting that it probably responds to a different type of ligand.

The gene product of *stk2* (*pFi_177*) has two TMD and is homologous to members of the protein phosphatase Mg^{2+} - or Mn^{2+} -dependent family that catalyzes the dephosphorylation of

Fig. 5. The *nrp* locus of pFiD188. **A**, Structural organization of the domains in a typical NRPS module. **B**, Schematic representation of the *nrp* locus. The condensation (C), adenylation (A), thiolation (T), epimerase (E), and thioesterase (Te) domains of Nrp5, Nrp7, Nrp7, Nrp8, and Nrp9 are indicated. The white *nrp* genes do not contain any domains. The deleted region in D188- Δ *nrp5* is marked by two arrowheads. **C**, Symptoms induced by D188 and D188- Δ *nrp5* on excised tobacco leaves.

phosphoserine- and phosphothreonine-containing proteins (Zhang and Shi 2004). The *stk3*-encoded protein (pFi₁₇₈) has one TMD and a C-terminal forkhead-associated domain, known to bind phosphothreonine-containing peptides and mediating phosphorylation-dependent protein-protein interactions (Liang and Van Doren 2008). Gene *stk4* (pFi₁₇₉) encodes a protein with two functionally distinct domains. The N-terminal part of Stk4 is characterized by 12 TMD and shows similarity to RodA/FtsW proteins that belong to the “shape, elongation, division, and sporulation” family of integral membrane proteins conserved in bacteria with a peptidoglycan cell wall (Letek et al. 2008). The C-terminus of Stk4 is homologous to the transpeptidase domain of class B penicillin-binding proteins (PBP). These high-molecular weight PBP function in the assembly of the cell wall by cross-linking peptidoglycan strands via their peptide side chains and are responsible for septum formation during cell division and maintenance of the cell shape during elongation. Interestingly, in other prokaryotes, RodA/FtsW and Class B PBP operate as a functional couple in which RodA/FtsW is proposed to recruit the PBP to their appropriate location in the cell membrane (Letek et al. 2008; Sauvage et al. 2008). Most PBP are anchored to the membrane via a single TMD. The composite nature of Stk4 strengthens the functional coupling between the PBP and the RodA/FtsW domain and might ensure the firm anchoring of the PBP to the membrane at the location dictated by RodA/FtsW. The last gene of the operon, *stk5* (pFi₁₈₀), encodes a soluble hypothetical protein.

Taken together, we postulate that cross-interaction between Stk1 and Stk2 could translate environmental signals into cellular responses through changes in the phosphorylation state of Stk3. Phosphorylated Stk3 could then modulate the PBP activity of Stk4 and, hence, the peptidoglycan biosynthesis or modulation, leading to morphological cell changes that might be important in the interaction with or survival in the plant host. This hypothesis was partially substantiated by biochemical analysis of Stk1 and Stk2. To prevent purification difficulties, an N-terminal maltose-binding protein (MBP)-tagged version of the Stk1 protein without the TMD was produced in *Escherichia coli* (MBP-Stk1_{sol}). In contrast to other *stk*-like genes (Jin and Pancholi 2006), heterologous expression of *stk1* did not affect the cell morphology of *E. coli* (data not shown). MBP-Stk1_{sol} exhibited autokinase activity in the presence of Mn²⁺ and Mg²⁺ as cofactors, with a preference for Mn²⁺; no autophosphorylation was detected in the presence of Ca²⁺ ions. A truncated Stk1 (Stk1_{trunc}), lacking the

ATP-binding subdomain, yielded a nonfunctional kinase (Fig. 6B).

The phosphatase activity of Stk2 was assessed by heterologous production of an N-terminal MBP-tagged version of the protein without the TMD in *E. coli* (MBP-Stk2_{sol}). MBP-Stk2_{sol} hydrolyzed the model substrate *p*-nitrophenyl phosphate (pNPP) into *p*-nitrophenol in the presence of Mn²⁺ only (Fig. 6C). To test whether Stk1 and Stk2 cross-reacted, we incubated purified MBP-Stk1_{sol} with [γ-³²P]ATP under optimal conditions for autokinase activity. The subsequent incubation of radiolabeled MBP-Stk1_{sol} with purified Stk2_{sol} led to the

Fig. 6. The *stk* operon of pFiD188. **A**, Structure of *stk*-like loci in actinobacteria. Genes coding for serine/threonine protein kinases (red), penicillin-binding proteins (green), RodA/FtsW proteins of the “shape, elongation, division, and sporulation” family (yellow), protein phosphatases (blue), forkhead-associated domain-containing proteins (purple), and hypothetical proteins (gray) are shown. **B**, Stk1, a functional protein kinase. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified maltose-binding protein (MBP)-Stk1_{sol} and MBP-Stk1_{trunc} fusion proteins from *Escherichia coli* (left). In vitro autophosphorylation of MBP-Stk1_{sol} and MBP-Stk1_{trunc} in the presence of different divalent cations at various concentrations (right). M, molecular weight marker. **C**, Stk2, a functional phosphatase. Hydrolysis of *p*-nitrophenyl phosphate by purified MBP-Stk2_{sol} in the presence of different concentrations of MnCl₂: ○, 0 mM MnCl₂; □, 0.1 mM MnCl₂; △, 0.5 mM MnCl₂; ◇, 1 mM MnCl₂; ●, 2 mM MnCl₂; ■, 10 mM MnCl₂. **D**, Cross-reaction between the Stk proteins. SDS-PAGE of the purified fusion proteins (left) and autoradiogram of the protein gel (right). Proteins that are combined in the enzymatic reactions are indicated above each lane. Dephosphorylation of autophosphorylated MBP-Stk1_{sol} by MBP-Stk2_{sol} (lanes 1 and 2); transphosphorylation of MBP-Stk3 by MBP-Stk1_{sol} (lane 3), but not by MBP-Stk1_{trunc} (lane 5); MBP-Stk4_{sol} is not a substrate for MBP-Stk1_{sol} (lane 4). MBP-Stk1_{sol} (arrow); MBP-Stk3 (asterisk); MBP-Stk4_{sol} (hash). **E**, Leafy gall formation on excised tobacco leaves.

almost complete disappearance of the signal corresponding to the autophosphorylated kinase (Fig. 6D), indicating that the kinase activity of Stk1 is controlled through dephosphorylation by its cognate protein phosphatase Stk2. Finally, to check the intermolecular phosphorylation capacity of Stk1_{sol}, N-terminal MPB-tagged versions of Stk3 and of the PBP part of Stk4 were produced in *E. coli*. Incubation of autophosphorylated MBP-Stk1_{sol} with MBP-Stk3 and MBP-Stk4_{sol} revealed that Stk3 but not the C-terminal part of Stk4 was a substrate for Stk1 (Fig. 6D). No phosphorylation occurred when Stk1_{sol} was replaced by Stk1_{trunc} (Fig. 6D).

Assessment of the expression of *stk1* and *stk4-gus* reporter strains revealed that both genes were constitutively expressed under all conditions tested (data not shown) and the symptoms induced by an *stk1* and *stk4* mutant on tobacco were comparable with those provoked by the wild-type strain D188 (Fig. 6E). Nevertheless, the presence of the locus in a unique region of the virulence plasmid of *R. fascians* hints at a specialized, still-unknown function for the *stk* locus in the interaction with its host. Possibly, the *stk* operon might be involved in the reported modification or complete loss of the bacterial cell wall of the endophytic population (Cornelis et al. 2001; Lacey 1961). Alternatively, because *R. fascians* is a nonmotile bacterium, the *stk* locus could function in polar peptidoglycan production, allowing hyphal and, possibly, directional growth toward a host. Indeed, apical growth in the actinomycete genus *Streptomyces* occurs in a phosphorylation-dependent way (Flärdh 2010) and elongated *R. fascians* cells have been observed on leaves (Cornelis et al. 2001).

Concluding remarks.

In contrast to the virulence plasmid of the animal pathogen *R. equi*, the structural organization of pFiD188 of *R. fascians* is largely similar to that found in the linear plasmids pBD2, pREL1, pRHL2, and pROB01 of *R. erythropolis* strains BD2 and PR4, *R. jostii* RHA1, and *R. opacus* B4, respectively, strongly implying a common origin. Just like the related environmental rhodococcal linear plasmids, pFiD188 has a composite structure with conserved regions involved in plasmid maintenance and unique regions implicated in the pathogenic and plant-associated life style of *R. fascians*. Interestingly, the gene products of these unique regions of pFiD188 are often highly similar to proteins of other nonpathogenic actinomycetes. Thus, in *R. fascians*, the evolution toward interaction with a plant host and disease elicitation might have occurred through a mechanism that reoriented the function of core actinobacterial traits, combined with acquisition of genuine virulence factors and genes involved in adaptation to the plant niche, possibly via HGT. These different classes of gene origin are illustrated by the *stk*, *fas*, and *nrp* loci. However, many genes in the unique regions of pFiD188 are found solely in *R. fascians* or encode conserved hypothetical proteins. Therefore, the availability of the sequence of pFiD188 combined with ongoing extensive transposon-mediated mutagenesis (O. Vandeputte, *personal communication*) offers new opportunities and challenges to unravel the pathogenicity strategies of *R. fascians*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

An overview of the *R. fascians* strains and plasmids used is given in Supplementary Table S2. *R. fascians* strains were grown at 28°C in yeast extract broth (YEB) (Sambrook and Russell 2001) supplemented, when required, with chloramphenicol (Cm) at 25 µg ml⁻¹, Km at 50 µg ml⁻¹, phleomycin (Phleo) at 1 µg ml⁻¹, or streptomycin (Sm) at 100 µg ml⁻¹. The *E. coli* DH5α (Hanahan 1983) was used for cloning purposes

and to propagate a D188 genomic cosmid library (Desomer et al. 1991). Cells were grown at 37°C in Luria-Bertani medium (Sambrook and Russell 2001) supplemented, when required, with carbenicillin (Cb) at 100 µg ml⁻¹ or Km at 50 µg ml⁻¹.

Identification of the plasmid ends.

R. fascians cells were grown in 5 ml of YEB for 2 days at 28°C, diluted 50-fold in 100 ml of YEB, and allowed to grow overnight. Culture aliquots of 6 ml were centrifuged, embedded in LMP agarose, and lysed using lysozyme, *N*-lauroylsarcosine, and proteinase K as described (Kalkus et al. 1998). Proteinase K was omitted for the preparation of nonproteolytically treated plasmid. The plugs were washed with TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) in which they were stored at 4°C until further processing.

For the isolation of the linear plasmid ends, restriction with *KpnI* was carried out in situ in the proteolytically and nonproteolytically treated agarose plugs. The restriction digest was loaded on 1% agarose gels (Pulsed Field Certified Agarose; Bio-Rad, Hercules, CA, U.S.A.) in 0.5× Tris-borate-EDTA buffer and resolved by contour-clamped homogeneous electric field electrophoresis at 6 V cm⁻¹ for 18 h at 14°C with a field angle of 120 degrees and a ramped pulse time from 1 to 30 s (CHEF MAPPER; Bio-Rad). The fragments showing a modified electrophoretic mobility were cloned into pBluescript.

Generation of template DNA for sequencing and gap closure.

For sequencing pFiD188, a first source of template DNA was a genomic cosmid library of *R. fascians* D188 (Desomer et al. 1991). Additional DNA fragments were generated through cloning of restriction fragments that had been identified as overlapping with cosmid borders via DNA gel blot hybridization analysis. By using a genome-walking method optimized for GC-rich template DNA (Gröning et al. 2010) and vector-ette polymerase chain reaction (PCR) (Arnold and Hodgson 1991), sequences of unknown regions adjacent to known DNA regions were amplified and cloned into pCRBlunt for subsequent sequencing.

Intriguingly, the DNA fragment that bridged both final contigs could not be cloned in *E. coli* because it was highly unstable; eventually, it was obtained by inverse PCR and direct sequencing (Ochman et al. 1988). Therefore, genomic DNA of strain D188 was treated with *SphI* (left contig) or *SacI* (right contig). After heat inactivation and self-ligation, the DNA was relinearized with *KpnI* (left contig) or *EcoRV* (right contig). With two outward sequence-specific primers for both contigs, the sequence of the unknown fragment was determined.

Sequencing and annotation.

The EZ-Tn5 <TET-1> transposon insertion kit (Epicentre Biotechnologies, Madison, WI, U.S.A.) was used to randomly insert primer-binding sites to facilitate sequencing of large DNA fragments (>5 kb). The DNA sequence of cloned fragments was determined for both strands with automated dideoxy-sequencing systems (ALF DNA sequencer [GE Healthcare, Little Chalfont, U.K.] and ABI377 DNA sequencer [Applied Biosystems, Foster City, CA, U.S.A.]). Subsequently, the DNA of the sequences was interpreted via computer assistance with Institut Pasteur biological software.

The obtained sequence was annotated by means of online Xbase bacterial genome annotation that uses the Glimmer gene prediction software (Delcher et al. 2007). For identification of protein-coding genes, the genome sequence was translated in six frames to generate potential protein products of open reading frames longer than 90 bp, considering ATG and GTG as potential initiation codons when preceded by a putative ribo-

some-binding site in agreement with the consensus sequence GAPuPuNGAPuTC (Crespi et al. 1994). The potential protein sequences were compared with the nonredundant Swiss-Prot/TrEMBL databases by means of the BLASTP program and an *E* value of 10^{-6} as cut-off (Altschul et al. 1997). When the initiation codons of the potential protein sequences could be identified without ambiguity by manual inspection, they were included in the gene list.

The Alien Hunter application was used to predict the occurrence of regions acquired via HGT (Vernikos and Parkhill 2006; Sanger Institute website).

Scanning electron microscopy.

Samples were fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), post fixed in 2% OsO_4 in H_2O overnight at 4°C , dehydrated in an ethanol series, and dried in liquid CO_2 with a critical point dryer. The samples were mounted on stubs with double-sticky adhesive, gold-coated with a sputter coater, and viewed with a JSM 840 microscope (Jeol, Tokyo).

Generation of recombinant *R. fascians* strains.

For the functional analysis of the cutinase, *nrp*, and *stk* loci, mutants were isolated via double homologous exchange recombination or disruptive single homologous recombination (Crespi et al. 1994). Therefore, the suicide plasmids pSPI Δ cuti, pCRDVprep, pUCIF Δ nrp5, pBLIFstk1, and pBLIFstk4 were constructed. For the mutation of *pFi_086*, a 1.9-kb *Bgl*III and a 1.7-kb *Sma*I-*Bgl*III fragment of the cutinase locus were cloned flanking the Km resistance gene (Taylor and Rose 1988) into pSP72. Then, the Cm resistance (*cmr*) gene was introduced as a 2.5-kb *Xba*I fragment (Desomer et al. 1992), generating the final construct pSPI Δ cuti that carries a 474-bp deletion in *pFi_086*. For the mutation of *pFi_102*, a 530-bp internal fragment was amplified via PCR with the primers PREP-FW and PREP-REV (Supplementary Table S3) and cloned into pCRBlunt, generating pCRDVprep. For the generation of the *nrp5* mutant, a 5-kb *Bam*HI fragment was cloned into pUC18 and then a 2.3-kb *Eco*NI fragment was deleted. The final construct pUCIF Δ nrp5 was obtained after introduction of the *cmr* as a 2.5-kb *Xba*I fragment. For the *stk1* gene mutant, a 1.0-kb *Sal*I fragment was cloned into pBluescript. For the mutation of *stk4*, an 880-kb internal fragment was amplified via PCR with the primers STK4-FW and STK4-REV and cloned into pBluescript as an *Xba*I-*Bam*HI fragment. Both *stk* cloning intermediates were finalized by introduction of the *cmr* gene as a 2.5-kb *Xba*I fragment, yielding the final constructs pBLIFstk1 and pBLIFstk4.

For expression analysis, three additional suicide plasmids (pSPIF Δ nrp5gus, pSPIF Δ stk1gus, and pSPIF Δ stk4gus) were constructed. Fragments of 770 bp (*nrp5*), 2,405 bp (*stk1*), and 2,388 bp (*stk4*) were amplified by PCR (primers NRP5GUS-FW/REV, STK1GUSFW/REV, and STK4GUS-FW/REV) and cloned first into pGUS1 as *Hind*III-*Pst*I (*nrp5*) or *Hind*III-*Sph*I (*stk1* and *stk4*) fragments. Subsequently, the *gus* fusions were isolated from these cloning intermediates as *Hind*III-*Xba*I fragments and cloned into pSP72. Finally, the *cmr* gene was introduced as an *Xba*I fragment, yielding the final constructs.

After introduction of these plasmids into strain D188 via electroporation (Desomer et al. 1990) and selection on Cm (pBLIFstk1, pBLIFstk4, pSPIF Δ nrp5gus, pSPIF Δ stk1gus, and pSPIF Δ stk4gus) or Km (pCRDVprep), correct recombinations into the respective loci were verified by DNA gel blot hybridization with gene-specific probes. For the generation of D188- Δ cuti and D188- Δ nrp5 via exchange recombination, the first recombination was selected on Cm. For D188- Δ cuti, the second recombination was screened on Km accompanied by the loss of Cm resistance. For D188- Δ nrp5, the second recombination

was evaluated by the loss of the Cm resistance. Both recombination events were verified by DNA gel blot hybridization.

For the complementation of D188- Δ cuti, two replicating plasmids were constructed (pRFDVp*Fi_087* to pRFDVp*Fi_086* and pRFDVp*Fi_087* to pRFDVp*Fi_085*), for which a 3.1-kb *Apo*I fragment and a 3.6-kb *Apo*I/*Pst*I fragment, respectively, were first cloned into pBluescript and then retrieved as *Hind*III/*Xba*I fragments to be cloned into the shuttle vector pRF37 to yield the final constructs. The plasmids were introduced into D188- Δ cuti by selection on Phleo.

Conjugation.

The conjugation experiments were done with 1:1 culture ratios on nitrocellulose filters (pore size 0.45 μm , diameter 25 mm; Millipore, Bedford, MA, U.S.A.) as described (Desomer et al. 1988). Serial dilutions of the conjugation mixtures were plated on Sm-containing YEB medium to count the number of acceptors, whereas undiluted samples were plated on Sm+Km-containing YEB medium to select for transconjugants. The transfer efficiency was expressed as the number of Km-resistant transconjugants divided by the number of recipient cells in the mating mixture.

Expression analysis.

To evaluate the inducibility of the expression of *nrp5*, *stk1*, and *stk4*, the *gus* reporter strains were grown in MinA medium (0.1% NH_4SO_4 , 0.025% MgSO_4 , and 0.001% thiamine) under inducing and control conditions as described (Temmerman et al. 2000). Expression was monitored with the substrate 4-methylumbelliferyl- β -D-glucuronide (Temmerman et al. 2000).

Plant infection methods.

Sterile *N. tabacum* (L.) W38 seed were germinated on solid Murashige and Skoog (MS) basal mineral medium supplemented with 1% sucrose. For the leaf inoculation assay, leaves of 3- to 4-week-old plants were excised and placed on MS medium, and a drop of bacterial culture was applied to the leaf surface without prior wounding. Phenotypes were scored 3 weeks after inoculation.

Cloning, heterologous expression, and purification of Stk proteins.

For the enzymatic analyses of the Stk1, Stk2, Stk3, and Stk4 proteins, plasmids were generated producing N-terminal MBP fusions. Gene fragments of 1.27, 0.99, 1.46, 0.88, and 0.94 kb, without the predicted TMD, were amplified via PCR with the primers STK1SOL-FW/REV, STK1TRUNC-FW/REV, STK2SOL-FW/REV, STK3-FW/REV, and STK4SOL-FW/REV, respectively. The PCR fragments were cloned as *Bam*HI-*Hind*III (*stk1*_{sol}), *Eco*RI-*Pst*I (*stk2*_{sol}), or *Eco*RI-*Bam*HI fragments (*stk1*_{trunc}, *stk3*, and *stk4*_{sol}) into pMALc2X. The resulting plasmids (pMAL Δ stk1_{sol}, pMAL Δ stk1_{trunc}, pMAL Δ stk2_{sol}, pMAL Δ stk3, and pMAL Δ stk4_{sol}) were introduced into the *E. coli* DH5 α strain via heat shock transformation and selection on Cb. The MBP fusion proteins were purified from *E. coli* according to Riggs (2000).

In vitro kinase and phosphatase assays.

Autophosphorylation reactions were done for 30 min at room temperature with 5 μg of purified MBP-Stk1_{sol} or Stk1_{trunc} in the presence of 10 μM [γ - ^{33}P]ATP (EasyTides, NEN Radiochemicals; Perkin-Elmer, Norwalk, CT, U.S.A.) as described (Jin and Pancholi 2006). The kinase buffer was supplemented with MnCl_2 , MgCl_2 , or CaCl_2 at varying concentrations (ranging from 0 to 10 mM). For transphosphorylation, 5 μg of purified MBP-Stk3 or MBP-Stk4_{sol} was added to the autophosphorylation reaction.

The phosphatase activity of MBP-Stk2_{sol} (5 µg) was assayed by hydrolysis of 20 mM pNPP as described (Jin and Pancholi 2006). The enzyme reaction was carried out at different concentrations of MnCl₂, MgCl₂, or CaCl₂ (ranging from 0 to 10 mM) at room temperature.

To demonstrate the interaction between Stk1 and Stk2, auto-phosphorylated MBP-Stk1_{sol} was separated from unincorporated [γ -³³P]ATP on a Bio-Spin 30 Chromatography Column (Bio-Rad), resuspended in phosphatase buffer, and incubated with 5 µg of purified MBP-Stk2_{sol}.

Other molecular techniques.

Plasmid and genomic DNA isolation, DNA cloning, DNA gel blot and colony hybridization, *E. coli* heat shock transformation, and other general manipulations were done according to Sambrook and Russell (2001). Primers for cloning or sequencing purposes were designed via the Primer3 program. PCR reactions were done with Phusion DNA Polymerase (Finnzymes, Espoo, Finland).

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