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The Type III Secretion System of *Xanthomonas fuscans* subsp. *fuscans* Is Involved in the Phyllosphere Colonization Process and in Transmission to Seeds of Susceptible Beans[▽]

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Understanding the survival, multiplication, and transmission to seeds of plant pathogenic bacteria is central to study their pathogenesis. We hypothesized that the type III secretion system (T3SS), encoded by *hrp* genes, could have a role in host colonization by plant pathogenic bacteria. The seed-borne pathogen *Xanthomonas fuscans* subsp. *fuscans* causes common bacterial blight of bean (*Phaseolus vulgaris*). Directed mutagenesis in strain CFBP4834-R of *X. fuscans* subsp. *fuscans* and bacterial population density monitoring on bean leaves showed that strains with mutations in the *hrp* regulatory genes, *hrpG* and *hrpX*, were impaired in their phyllospheric growth, as in the null interaction with *Escherichia coli* C600 and bean. In the compatible interaction, CFBP4834-R reached high phyllospheric population densities and was transmitted to seeds at high frequencies with high densities. Strains with mutations in structural *hrp* genes maintained the same constant epiphytic population densities (1×10^5 CFU g^{-1} of fresh weight) as in the incompatible interaction with *Xanthomonas campestris* pv. campestris ATCC 33913 and the bean. Low frequencies of transmission to seeds and low bacterial concentrations were recorded for CFBP4834-R *hrp* mutants and for ATCC 33913, whereas *E. coli* C600 was not transmitted. Moreover, unlike the wild-type strain, strains with mutations in *hrp* genes were not transmitted to seeds by vascular pathway. Transmission to seeds by floral structures remained possible for both. This study revealed the involvement of the *X. fuscans* subsp. *fuscans* T3SS in phyllospheric multiplication and systemic colonization of bean, leading to transmission to seeds. Our findings suggest a major contribution of *hrp* regulatory genes in host colonization processes.

The phyllosphere (i.e., the environment of leaf surfaces) (21) provides important niches for microbes such as fungi and bacteria with diverse lifestyles, including epiphytes, saprophytes, and pathogens. While colonizing leaves, microorganisms are exposed to a harsh environment subjected to multiple stresses, including desiccation, UV irradiation, and nutrient limitation (4, 28). *Xanthomonas fuscans* subsp. *fuscans* (40) and *Xanthomonas axonopodis* pv. phaseoli (44, 45) are causal agents of common bacterial blight of bean (*Phaseolus vulgaris* L) (46). During colonization of the bean phyllosphere, *X. fuscans* subsp. *fuscans* forms aggregates (biofilms) that protect it from stresses and maintain population sizes (22). Under conditions of low relative humidity (RH) that limit bacterial multiplication, *X. fuscans* subsp. *fuscans* can achieve a complete biological cycle on its susceptible host, from the sown seed to the harvested seed, without causing any macroscopic symptoms (10). Consequently, such an asymptomatic pathogenic life cycle during which bacteria not only survive but also multiply to colonize their host is of great pathogenic and ecological importance. It could provide a discrete inoculum for future outbreaks of common bacterial blight of bean and a means of survival for the bacteria under conditions that are not favorable for its multiplication.

Pathogen transmission is one of the most important parameters for fitness (14, 30). It combines the ability to survive outside the host prior to infection, multiplication on the host, dispersion, and transmission to new ecological niches, including host seeds. Three seed infection pathways have bee described for seed-borne pathogens (29). Seeds can be internally contaminated via the host xylem, as occurs for viruses, some fungi, and a few bacteria. This can result in the contamination of the seeds, often through the hilum (1). Seeds may also become infested via the stigma, where bacteria move through the stylar tissues to the embryo; this was recently demonstrated for bacterial fruit blotch of watermelon (47). An external infection occurs via flowers and fruits as a consequence of contact of the seed with bacterial populations on symptomatic tissue or during threshing with residues carrying large bacterial populations (50). The molecular determinants involved in active mechanisms of bacterial transmission to seeds in the absence of symptoms remain unknown.

The *hrp* genes are one of the major pathogenicity determinants of most plant pathogenic bacteria. These genes form a cluster, conserved in plant and animal pathogenic bacteria, that encodes proteins which form a molecular syringe, the type III secretion apparatus (18, 37). This type III secretion system (T3SS) allows the secretion and the injection of bacterial virulence proteins, called effectors, directly in the host cell cytoplasm. Surprisingly, it

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Source or reference
Plasmids		
pVO155	Derivative plasmid of pUC19 containing the promoterless *gus* (*uidA*) gene; Km^r Amp^r	36
pRK600	Cm^r, *tra*RK2, *ori*ColE1	16
pIJ3225	*hrp* cluster of *X. campestris* pv. campestris cloned in pLAFR1	3
Wild-type strains		
CFBP4834-R	*X. fuscans* subsp. *fuscans* CFBP4834-R wild-type strain; yellow strain producing the typical fuscous pigment; Rif^r	22
ATCC 33913	*X. campestris* pv. campestris ATCC 33913 wild-type strain; Rif^r	11
85.10	*X. axonopodis* pv. vesicatoria wild-type strain; Rif^r	6
306	*X. citri* subsp. *citri* wild-type strain	11
K-12	*Escherichia coli* wild-type strain	12
DH5α	*E. coli* F ϕ980d*lacZ*:M15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* (*lacZYA-argF*)U169	19
C600	*E. coli* C600 wild-type strain; Rif^r	34
Mutants		
4834HRCJ	CFBP4834-R *hrcJ*(420)[a]::pVO155; Rif^r Km^r	This study
4834HRCR	CFBP4834-R *hrcR*(320)::pVO155; Rif^r Km^r	This study
4834HRCT	CFBP4834-R *hrcT*(799)::pVO155; Rif^r Km^r	This study
4834HRCV	CFBP4834-R *hrcV*(1501)::pVO155; Rif^r Km^r	This study
4834HRPB2	CFBP4834-R *hrpB2*(298)::pVO155; Rif^r Km^r	This study
4834HRPG	CFBP4834-R *hrpG*(625)::pVO155; Rif^r Km^r	This study
4834HRPX	CFBP4834-R *hrpX*(459)::pVO155; Rif^r Km^r	This study
33913HRCU	ATCC 33913 *hrcU*::pVO155; Rif^r Km^r	Gift from M. Arlat
33913HRPX	ATCC 33913 *hrpX*::pVO155; Rif^r Km^r	Gift from M. Arlat

[a] Numbers in parentheses indicate the position from the start codon of pVO155 insertion in the target gene in *X. citri* subsp. *citri* 306.

has been shown that this T3SS is also necessary for leaf-associated colonization of bean by *Pseudomonas syringae* pv. syringae (20). No similar studies have been undertaken for other pathogens with an epiphytic growth phase or for other steps of host colonization, such as transmission to seeds.

The objectives of the work presented here were to characterize the *hrp* cluster of *X. fuscans* subsp. *fuscans* and to determine the role of *hrp* genes in the survival and the multiplication of this bacterium in the bean phyllosphere and in the transmission to bean seeds.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Xanthomonad strains were grown at 28°C in 10% TSA medium (1.7 g $liter^{-1}$ tryptone, 0.3 g $liter^{-1}$ soybean peptone, 0.25 g $liter^{-1}$ glucose, 0.5 g $liter^{-1}$ NaCl, 0.5 g $liter^{-1}$ K_2HPO_4, and 15 g $liter^{-1}$ agar) supplemented with the appropriate antibiotics. *Escherichia coli* cells were cultivated at 37°C in Luria-Bertani medium (10 g $liter^{-1}$ tryptone, 5 g $liter^{-1}$ yeast extract, 5 g $liter^{-1}$ NaCl, 15 g $liter^{-1}$ agar) and, for in planta studies, on 100% TSA medium (17 g $liter^{-1}$ tryptone, 3 g $liter^{-1}$ soybean peptone, 2.5 g $liter^{-1}$ glucose, 5 g $liter^{-1}$ NaCl, 5 g $liter^{-1}$ K_2HPO_4, and 15 g $liter^{-1}$ agar) supplemented with rifamycin. Antibiotics were used at the following final concentrations: rifamycin, 50 mg $liter^{-1}$; kanamycin (Km), 25 mg $liter^{-1}$; rifampin (Rif), 50 mg $liter^{-1}$; and chloramphenicol (Cm), 12.5 mg $liter^{-1}$. For in planta studies, media were supplemented with 50 mg $liter^{-1}$ cycloheximide and 10 mg $liter^{-1}$ propiconazole to inhibit fungal growth. To prepare inocula, strains were grown for 48 h in appropriate media supplemented with appropriate antibiotics. Bacterial cells were scraped from plates and suspended in sterile distilled water. Suspensions were calibrated to 1×10^8 CFU ml^{-1} and adjusted to the desired final concentrations with sterile distilled water.

Molecular biology techniques. Chromosomal DNA was extracted with the Nucleospin tissue kit (Macherey-Nagel Hoerdt, France). Plasmid preparations were performed with the Wizard Plus Minipreps DNA purification systems (Promega). Restriction enzymes, DNA ligase, and GoTaq DNA polymerase (Promega) were used according to the manufacturer's recommendations. PCRs were done in 20-μl volumes containing 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 0.4 U μl^{-1} GoTaq polymerase (final concentrations) and 4 μl of a boiled bacterial suspension (1×10^7 CFU ml^{-1}). PCR conditions were 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C; and 7 min at 72°C. Southern hybridizations were performed using gene- and vector-specific PCR fragments as probes. Probes were labeled using the PCR digoxigenin labeling mix (Roche Applied Science, France). Genomic DNA was digested with BamHI and transferred to Hybond N^+ nylon membranes (Amersham) according to supplier's instructions. Blots were hybridized for 16 h at 42°C in 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 20% sodium dodecyl sulfate (SDS), 2% blocking reagent, and 0.1% *N*-lauryl-sarcosine, followed by washes in 2× SSC–0.1% SDS and 0.1× SSC–0.1% SDS at 68°C. The probes were detected using the Fab fragments of an antidigoxigenin antibody conjugated with alkaline phosphatase and nitroblue tetrazolium chloride–5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt solution according to the supplier's instructions (Roche Applied Science, France).

Construction of *hrp* gene disruptions in *X. fuscans* subsp. *fuscans*. For specific inactivation of CFBP4834-R genes, a plasmid integration mutagenesis strategy was used as previously described (36). For each target gene, primers were designed based on the consensus sequence from sequenced genomes of xanthomonads. Sequences of oligonucleotide primers are listed in Table 2. The PCR fragment was ligated into the suicide vector pVO155 (36), and this construction was introduced into *E. coli* DH5α. PCR fragments cloned into pVO155 were sequenced to verify their identity. The plasmid was transferred into strain CFBP4834-R by triparental mating (17) using the mobilizing *E. coli* K-12(pRK600) (16).

Disruption of each target gene was verified by PCR using one primer in the plasmid (ProR or ProF) and a second primer in the genome outside the recombinant region (Table 2) and Southern blotting using the recombinant fragment as a specific probe. Single insertion was confirmed by Southern blotting using a vector-specific probe (Table 2).

For the phenotypic characterization of the mutants, the in vitro growth rate of each *hrp* mutant was compared to that of the wild-type strain CFBP4834-R. Growth curves were established by growing strains at an initial concentration of 1×10^7 CFU ml^{-1} in 100-well honeycomb microtiter plates (Thermo Electron, France) in 10% TSB (1.7 g $liter^{-1}$ tryptone, 0.3 g $liter^{-1}$ soybean peptone, 0.25 g $liter^{-1}$ glucose, 0.5 g $liter^{-1}$ NaCl, 0.5 g $liter^{-1}$ K_2HPO_4). Plates were incubated at 28°C with continuous shaking (120 rpm) over a period of 2 to 3 days. Growth measurements were done automatically every 2 h by optical density measurements at 600 nm using the Bioscreen C instrument (Labsystems, Helsinki, Fin-

TABLE 2. PCR primers used in this study to construct and validate mutations in *hrp* genes of *X. fuscans* subsp. *fuscans* CFBP4834-R

Target gene	PCR primer		Relevant characteristic	PCR fragment size (bp)
	Name[a]	Nucleotide sequence[b]		
hrcJ	HrcJ-F	GGACTAGTCCTGCTGCATGCGGGCGTGGA	SpeI site	335
	HrcJ-R	CCGCTCGAGCGGGAAAGCGGGTCGTTGTTGG	XhoI site	
	HrcJ-ext-F	CATGACGCTCATTCCTCCTGT	To be used with ProR	891
hrcR	HrcR-F	GGACTAGTCCGCTGGTGGTCATCATGCTGG	SpeI site	293
	HrcR-R2	CCGCTCGAGCGGTGTTTGAGGAGGAATTGC	XhoI site	
	HrcR-ext-R	TCACCGATAGCTCAGAACCAGG	To be used with ProF	756
hrcT	HrcT-F	GGACTAGTCCCCTCGCAGGGCGTGTCGCTG	SpeI site	329
	HrcT-R2	CCGCTCGAGCGGGTCATCTGCACATTGTTGTA	XhoI site	
	HrcT-ext-R	CGTTGGCGGCATCGTGCAAT	To be used with ProF	850
hrcV	HrcV-F	CGGGATCCCGATTCGCAAAAACGCCCCTGATT	BamHI site	454
	HrcV-R	GGTCTAGAGCCAGCAGACGCCGCAACACAT	XbaI site	
	HrcV-ext-F	TGGCCAACGAGAACGAGACAAG	To be used with ProR	789
hrpB2	HrpB2-F	GGACTAGTCCATGACGCTCATTCCTCCTGT	SpeI site	357
	HrpB2-R	CCGCTCGAGCGGGCATCAACTTGATCTGCT	XhoI site	
	HrcB2-ext-R	GGAAAGCGGGTCGTTGTTGG	To be used with ProF	896
hrpG	HrpG-F	GGACTAGTCCTGCGAGCTGCTGATCTTCGAT	SpeI site	467
	HrpG-R	CCGCTCGAGCGGTTGTAGATGTGCTGCTCCATGGT	XhoI site	
	HrpG-ext-F	CACCAACCAGCATCCTGCTC	To be used with ProR	1,680
hrpX	HrpX-F	GGACTAGTCCGCCTACAGCTACATGATCACCAA	SpeI site	206
	HrpX-R2	CCGCTCGAGCGGCTTCGGCCAGCAGTTCGT	XhoI site	
	HrpX-ext-R	TTACCGCTGCAAGGTCTCCATCGG	To be used with ProF	446
	ProR	TTCACGGGTTGGGGTTTCTACA	pVO155 primer	
	ProF	GAGATCCCCAGCCCGCCTAATG	pVO155 primer	

[a] For each target gene, the two first primers indicated were designed to construct the mutation. The third primer, which needs to be used with ProF or ProR, was designed for the validation of the mutation and for the specific identification of mutated strains.
[b] Underlined sequences indicate the relevant restriction site.

land). Noninoculated wells were used as aseptic controls. The experiment was repeated three times for each strain.

The stability of the constructions was verified in vitro by testing the Km resistance of the bacteria. Liquid cultures at initial concentration of 1×10^6 CFU ml^{-1} of each strain were cultured in 10% TSB without antibiotic selection pressure at 28°C under constant agitation (120 rpm), and 24 h later they were diluted 10-fold and grown up again; this step was repeated for four days. Finally, bacterial populations were plated on selective (10% TSA supplemented with rifamycin and Km) and nonselective (10% TSA) media and population densities were compared. Three independent cultures of every strain were analyzed, and the experiment was repeated three times. The stability of the mutation was also verified in planta by comparison of the bacterial population densities on selective and nonselective media the day of inoculation and at the last sampling date. To confirm stability of the construction versus antibiotic resistance acquisition and to check cross-contamination among treatments, the identity of 10 colonies per plant that developed on the nonselective medium was verified by PCR using specific primers (Table 2).

A functional complementation experiment with our 4834HRCT mutant was conducted by providing in *trans* the pIJ3225 plasmid, which is pLAFR1 carrying the *hrp* cluster of *Xanthomonas campestris* pv. campestris strain 8004 on a 29,200-bp EcoRI-EcoRI DNA fragment (3).

Plant material. In planta experiments were conducted with a variety of dry bean (*P. vulgaris* cv. Flavert) susceptible to common bacterial blight and pepper (*Capsicum annuum* cv. ECW10R). Seeds were sown in 10- by 10- by 18-cm pots (one seed per pot) containing soil substrate (Neuhaus humin substrat S NF 11-44-551; Proveg, La Rochelle, France). Peppers and beans were grown in growth chambers with 16 h of light (OSRAM; 2/3 metal halide arc discharge lamp type HQi-BT 400W and 1/3 high-pressure sodium lamp type NAV-T 400W) at 25°C (28°C for pathogenicity tests) and 8 h of darkness at 20°C (22°C for pathogenicity tests) and under high (95%) RH. For experiments on phyllosphere colonization and transmission to seeds, the RH was decreased to 50% from 2 days after inoculation. For all experiments, plants were watered three times per week, and once a week water was supplemented with 0.3 g $liter^{-1}$ nitrogen-phosphorus-potassium fertilizer (18:14:18). Plant inoculations were carried out under quarantine at UMR PaVé, Centre INRA, Beaucouzé, France.

Pathogenicity and hypersensitivity tests. Pathogenicity tests on bean were performed by grazing the surface of a young trifoliate leaf with cotton gauze soaked in a suspension calibrated at 1×10^7 CFU ml^{-1}. One leaf per plant and three plants per strain were inoculated. Symptoms were recorded daily for 11 days following inoculation. These tests were repeated at least three times for every bacterial strain.

Hypersensitivity tests were performed on pepper by infiltrating bacterial suspensions adjusted to 1×10^8 CFU ml^{-1} into leaves of 3-week-old plants. The presence or absence of a necrosis localized at the point of inoculation was scored 2 days after inoculation. Every strain was infiltrated three times on a plant, and three plants were inoculated per strain.

Dynamics of bacterial population densities on bean leaves. Plants at the first trifoliate stage (32) were spray inoculated until runoff with bacterial suspensions at 1×10^6 CFU ml^{-1} and with sterile distilled water as a control. The environmental conditions used for these experiments and the absence of wounding did not favor disease expression. Spray inoculation of plants is, however, satisfactory for studying bacterial colonization and dispersal. For every strain, the first trifoliate leaf of five plants was collected 3 h and at 1, 4, and 11 days after inoculation. Each leaf was weighed and ground individually (Stomacher 80; Seward, London, United Kingdom) for 2 min at maximum power in 5 ml of distilled water. Every sample and appropriate dilutions were spiral plated (Spiral Biotech, Bethesda, MD) on selective medium to enumerate the inoculated strain. Samples from control plants were plated on 100% TSA to quantify bacterial indigenous population densities. Primary leaves were imprinted on appropriate media with appropriate antibiotics at every sampling date. To avoid cross-contamination, plants receiving a similar treatment were grouped in the growth chamber and were separated by polypropylene walls from other treatments. In each experiment, treatments were randomly distributed, and experiments were repeated at least three times.

Inoculations of beans at flower bud stage and analyses of bacterial transmission to seeds. Bean plants at the flower bud stage (32) were spray inoculated until runoff with bacterial suspensions at 1×10^5 CFU ml^{-1}. Bacterial population

densities in flower buds from 5 plants were quantified at 3 h after spray inoculation, and those in seeds from 10 plants per experiment were quantified at 6 weeks after inoculation. The same experimental design as for phyllosphere colonization experiments was used. Samples (flower buds or seeds) were bulked for each plant. Bulks of flower buds were weighed and ground (Stomacher 80; Seward, London, United Kingdom) for 2 min at maximum power in 5 ml of distilled water. Asymptomatic pods were aseptically dissected under a laminar flow hood such that the seeds did not come in contact with the external portion of the pod or with any instruments that had contacted the external pod surface. Seeds were weighed and soaked overnight at 4°C in 2 ml of sterile distilled water per g of seeds (5.56 seeds g^{-1}). Samples were then vigorously shaken. To quantify population densities of strains with mutations in *hrp* genes and to look for reversion events, aliquots of 500 μl of samples were spread plated and appropriate dilutions were spiral plated on 10% TSA-rifamycin or 10% TSA-rifamycin-Km medium. For every sample, the identity of 10 colonies grown on 10% TSA-rifamycin was confirmed by PCR with appropriate primers (Table 2). For other strains, aliquots of 500 μl of samples and appropriate dilutions were plated on appropriate medium to quantify bacterial population densities.

To compare the occurrences of the vascular and the floral pathways in bacterial transmission to seeds, two different inoculation methods were used. On a first set of plants, direct flower bud inoculation was performed by depositing 20 μl of an inoculum of 1×10^6 CFU ml^{-1} per flower bud on three groups of flower buds per plant, taking every possible precaution to avoid dispersion of the inoculum on leaves. After inoculum drying, inoculated flower buds were enclosed in transparent cellophane bags to avoid any subsequent contamination of leaves by contact with inoculated flowers. On a second set of plants, three groups of flower buds per plant were protected with transparent cellophane bags before plants were inoculated by spraying the phyllosphere with an inoculum of 1×10^5 CFU ml^{-1}. Bags remained in situ until sampling at harvest time. Three hours after inoculations, bacterial population densities were quantified on leaves bulked for each plant when flower buds were inoculated by depositing drops of inoculum and on the third trifoliate leaf for spray-inoculated plants. Bacterial population densities were also quantified in inoculated and protected flower buds. At harvest (6 weeks after inoculation) bacterial population densities in pods and seeds were quantified as described above for leaf populations. Ten plants per strain and per treatment were analyzed on the day of inoculation and 30 plants per strain and per treatment at harvest. The same experimental design as for phyllosphere colonization experiments was used.

The dynamics of population densities of CFBP4834-R and 4834HRCV were determined in reproductive organs of plants inoculated by depositing 20 μl of an inoculum of 1×10^6 CFU ml^{-1} per flower bud. Flower buds and pods were sampled at 3 h and at 1, 2, 3, 4, 15, 28, and 35 days after inoculation on five plants per strain and per sampling date. Sample analyses were performed as described above.

Statistical analyses. Statistical analyses were performed using Statbox Pro software (Grimmer Logiciels, Optima France). Log-transformed data were analyzed with Kruskal-Wallis and Mann-Whitney tests. Comparisons of transmission frequencies were based on Pearson's χ^2 test. To compare paired population densities quantified on selective and nonselective media, Wilcoxon's signed-ranks test for two groups was used (41).

Nucleotide sequence accession numbers. The *hrp* genes in CFBP4834-R have been assigned NCBI accession numbers EU215387, EU215388, and EU215389.

RESULTS

Bacterial colonization of the bean phyllosphere and transmission to bean seeds are functions of the type of interaction. In order to characterize the role of *hrp* genes in the survival and the multiplication of *X. fuscans* subsp. *fuscans* in the bean phyllosphere, we first determined standard bacterial behaviors by the dynamics of bacterial population densities in null (*E. coli* C600), compatible (*X. fuscans* subsp. *fuscans* CFBP4834-R), and incompatible (*X. campestris* pv. campestris ATCC 33913) interactions. Despite the same initial inoculum concentration (1×10^6 CFU ml^{-1}), *E. coli* C600 population densities quantified in leaves at 3 hours after inoculation were about 0.01-fold those of CFBP4834-R and ATCC 33913 (Fig. 1), showing that *E. coli* C600 was impaired in its adhesion to bean

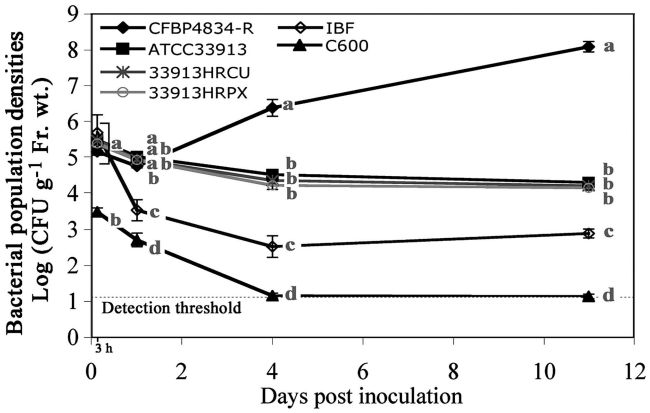

FIG. 1. Colonization of bean by wild-type strains *X. fuscans* subsp. *fuscans* (CFBP4834-R), *X. campestris* pv. campestris (ATCC 33913), and *E. coli* C600; by strains with mutations in *hrp* genes (33913HRCU and 33913HRPX); and by indigenous bacterial flora (IBF). Bacterial population densities were determined on bean leaves sampled at 3 h and 1, 4, and 11 days after spray inoculation (1×10^6 CFU ml^{-1}). Means and SEMs were calculated for five leaves per sampling date. Mean population densities followed by different letters are significantly ($P < 0.05$) different on the basis of the Mann-Whitney test.

leaves or was affected early by the environmental conditions of the phyllosphere. Moreover, *E. coli* C600 was not able to multiply in the phyllosphere and was not recovered after a few days. Only *X. fuscans* subsp. *fuscans* CFBP4834-R was able to multiply on bean leaves, reaching high population densities of around 1×10^8 CFU g^{-1} of fresh weight at 11 days after the bacteria were inoculated. Throughout the experiment, population densities of *X. campestris* pv. campestris ATCC 33913 remained stable at 1×10^5 CFU g^{-1} of fresh weight and were significantly ($P < 0.05$) higher than those of the indigenous bacterial flora. No symptoms were observed on any aerial parts of beans during experiments.

X. fuscans subsp. *fuscans* CFBP4834-R, *X. campestris* pv. campestris ATCC 33913, and *E. coli* C600 were also separately inoculated on beans at the flower bud stage to determine their rate of transmission to the new generation of seeds. No seeds of the 30 inoculated plants were contaminated with *E. coli* C600 (Table 3). *X. fuscans* subsp. *fuscans* CFBP4834-R was transmitted to the seeds at high frequency, whereas the frequency of *X. campestris* pv. campestris ATCC 33913 transmission to seeds was significantly ($P < 0.05$) lower, with much lower population densities on contaminated seeds than for *X. fuscans* subsp. *fuscans* CFBP4834-R. Thus, three different behaviors were recorded: (i) in the null interaction, there was no bacterial survival and no bacterial transmission to seeds; (ii) in the incompatible interaction, bacteria survived in the phyllosphere and were transmitted to seeds at low frequencies and with low population sizes; and (iii) in the compatible interaction, bacteria multiplied on leaves and were transmitted to seeds at high frequencies and with high bacterial population sizes.

Characterization of the T3SS of *X. fuscans* subsp. *fuscans* strain CFBP4834-R. As the *X. fuscans* subsp. *fuscans* genome is not yet sequenced, specific primers of *hrp* genes were designed based on consensus sequences obtained by comparison of *hrp* cluster sequences of *Xanthomonas citri* subsp. *citri* strain

TABLE 3. Frequencies of bacterial transmission to bean seeds and mean bacterial population densities after spray inoculation (1×10^5 CFU ml^{-1}) of bean at the flower bud stage

Strain	Frequency[a]	Mean bacterial population density (SEM) on contaminated harvest, log CFU g^{-1} (fresh wt)
CFBP4834-R	0.87	6.63 (0.33)
ATCC 33913	0.23*	1.91 (0.47)
C600	0*	ND[b]
4834HRCJ	0.17*	2.18 (0.72)
4834HRCR	0.40*	1.71 (0.24)
4834HRCT	0.17*	0.96 (0.14)
4834HRCV	0.37*	2.29 (0.39)
4834HRPB2	0.27*	1.81 (0.28)
4834HRPG	0.07*	1.85 (0.38)
4834HRPX	0.07*	1.31 (0.17)
33913HRCU	0.13*	1.15 (0.27)
33913HRPX	0.23*	1.70 (0.53)

[a] A total of 30 plants were analyzed. *, significantly different from value for the wild-type strain CFBP4834-R by χ^2 test.
[b] ND, not determined.

306 (11), *X. axonopodis* pv. glycines strain 8ra (24), *X. axonopodis* pv. vesicatoria strain 85-10 (42), *Xanthomonas oryzae* pv. oryzae strain KACC10331 (25), and *X. campestris* pv. campestris strain ATCC 33913 (38). This allowed the identification and the sequencing of a complete set of *hrp* genes in CFBP4834-R. Sequence analysis of the Hrp proteins of CFBP4834-R showed that they were more similar to the Hrp proteins of *X. citri* subsp. *citri* (strain 306) and *X. axonopodis* pv. glycines (strain 8ra) than to those of *X. axonopodis* pv. vesicatoria (strain 85-10) or to those of *X. campestris* pv. campestris (strain ATCC 33913) (Fig. 2). In contrast, the sequence of the surface-exposed domain of the pilin HrpE was more closely related to that of *X. campestris* pv. campestris (strain ATCC 33913) than to that of *X. citri* subsp. *citri* (strain 306).

In order to evaluate the role of the T3SS both in colonization processes in the absence of symptoms and in transmission of *X. fuscans* subsp. *fuscans* to bean seeds, we constructed strains with mutations in the *hrpB2*, *hrcJ*, *hrcR*, *hrcT*, *hrcV*, *hrpG*, and *hrpX* genes (Table 1). PCR amplifications using specific primers (Table 2) confirmed that pVO155 unique insertions were at the correct positions. Southern blot hybridizations confirmed the single plasmid insertion event. Population densities of each strain with mutations enumerated on nonselective medium were not ($P > 0.05$) higher that those enumerated on selective medium, showing that all constructions were stable both in vitro and in planta. Growth rates of the wild-type CFBP4834-R and of every strain with a mutation in *hrp* genes were similar in 10% TSB, indicating that the mutation of a given *hrp* gene did not impair the in vitro growth of the corresponding strain. Furthermore, no reversion was observed in vitro after 54 generations without selection pressure (data not shown).

When inoculated onto bean plants, none of the strains with mutations in *hrp* genes was able to cause any water-soaking symptoms compared to the wild-type strain (data not shown). Moreover, following infiltrations into resistant pepper leaves carrying the *BsT* and *Bs1* resistance genes, none of the strains with mutations in *hrp* genes induced a hypersensitive response (HR), the typical necrotic lesion associated with plant resistance. In contrast, the wild-type *X. campestris* pv. campestris strain ATCC 33913 carrying the *avrBs1* gene (11) induced the expected classical HR, and the wild-type strain CFBP4834-R of *X. fuscans* subsp. *fuscans* showed the same weak spotty HR as described by Escolar and colleagues (13). Pathogenicity of the 4834HRCT strain with a mutation in the *hrcT* gene was restored by complementation (data not shown). Together, these results showed that *X. fuscans* subsp. *fuscans* strain CFBP4834-R had a functional T3SS.

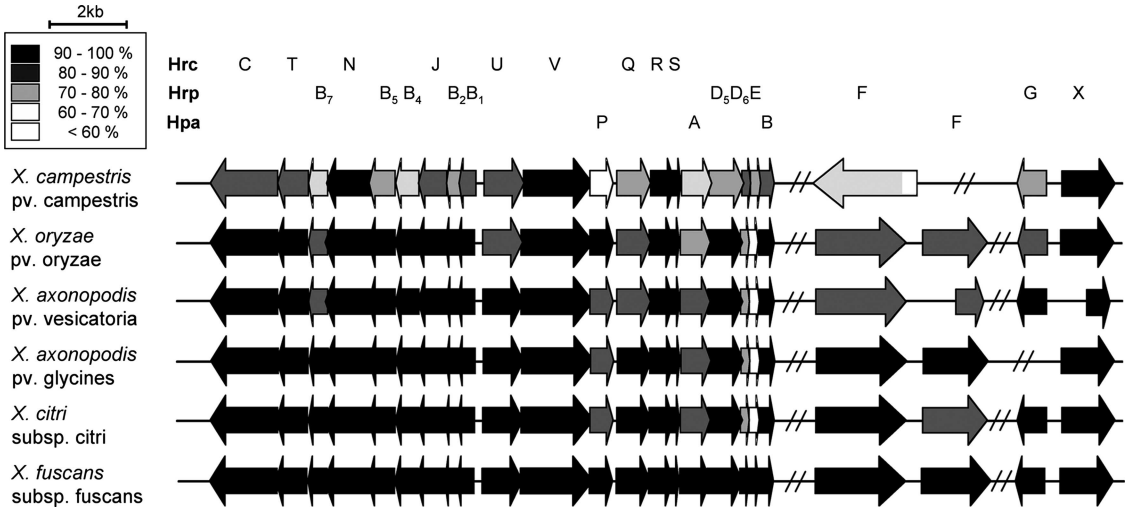

FIG. 2. Schematic overview of the *hrp* genes from different sequenced xanthomonads in comparison with *X. fuscans* subsp. *fuscans hrp* genes. Genes of the following strains are shown: *X. campestris* pv. campestris strain ATCC 33913 (11), *X. axonopodis* pv. vesicatoria strain 85-10 (42), *X. citri* subsp. *citri* strain 306 (11), *X. axonopodis* pv. glycines strain 8ra (24), *X. oryzae* pv. oryzae strain KACC10331 (25), and *X. fuscans* subsp. *fuscans* strain CFBP4834-R. Arrows indicate the sizes, positions, and orientations of the *hrp*, *hrc*, and *hpa* genes. The identity of each protein sequence with its homolog in *X. fuscans* subsp. *fuscans* is presented by use of a black/gray color scale. *X. fuscans* subsp. *fuscans* amino acid sequences were compared using the NCBI BLAST website http://www.ncbi.nlm.nih.gov/BLAST/with default parameters.

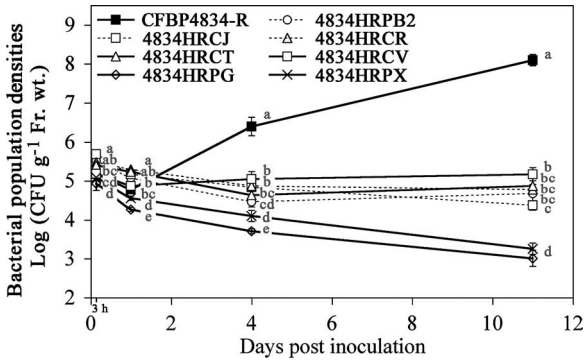

FIG. 3. Colonization of bean by *X. fuscans* subsp. *fuscans* strain CFBP4834-R and strains with mutations in *hrp* genes. Bacterial population densities were determined on bean leaves sampled at 3 h and 1, 4, and 11 days after spray inoculation (1×10^6 CFU ml^{-1}). Means and SEMs were calculated for five leaves per sampling date. Mean population densities followed by different letters are significantly ($P < 0.05$) different on the basis of the Mann-Whitney test.

Impact of mutations in *hrp* genes of CFBP4834-R on bacterial multiplication and bacterial survival in the phyllosphere. Individual inoculation of strains with mutations in *hrp* genes and the wild-type strain resulted in three different behaviors during the asymptomatic colonization of the bean phyllosphere (Fig. 3). First, in the compatible interaction, CFBP4834-R population densities increased from the first day after inoculation and reached 1×10^8 CFU g^{-1} of fresh weight at 11 days after inoculation. Mean CFBP4834-R population densities at 11 days after inoculation were significantly higher ($P < 0.05$) than those determined on the first day after inoculation. Second, strains (4834HRPB2, 4834HRCJ, 4834HRCR, 4834HRCT, and 4834HRCV) with mutations in structural *hrp* genes (*hrpB2*, *hrcJ*, *hrcR*, *hrcT*, and *hrcV*) were not able to multiply efficiently throughout the experiment, and their population densities stabilized at 1×10^5 CFU g^{-1} of fresh weight, as observed previously in the incompatible interaction for ATCC 33913 (Fig. 1). For these strains, their mean population densities on the 11th day after inoculation were not significantly ($P > 0.05$) higher than those determined on the first day after inoculation. To gain insight into the milieu colonized by the strains, we imprinted the surface of leaves on agar medium. Imprinting of leaves inoculated by strain 4834HRCV with a mutation in the *hrcV* gene (Fig. 4) did not show any substantial differences in surface populations compared to leaves inoculated with the wild-type strain CFBP4834-R. Surface populations illustrated by number of colonies sometimes seemed even higher than what was observed for the wild-type strain. Similar results were obtained for other strains with mutations in structural *hrp* genes. Third, the behavior of strains (4834HRPX and 4834HRPG) with mutations in the regulatory *hrp* genes (*hrpX* and *hrpG*) was the most affected, with a decrease of their population densities throughout the experiment. Their mean population densities determined on the first day after inoculation were significantly ($P > 0.05$) higher than those determined 11 days after inoculation. Imprinting of leaves inoculated by strain 4834HRPG, with a mutation in the *hrpG* gene (Fig. 4), showed a lower number of colonies on leaves than for the

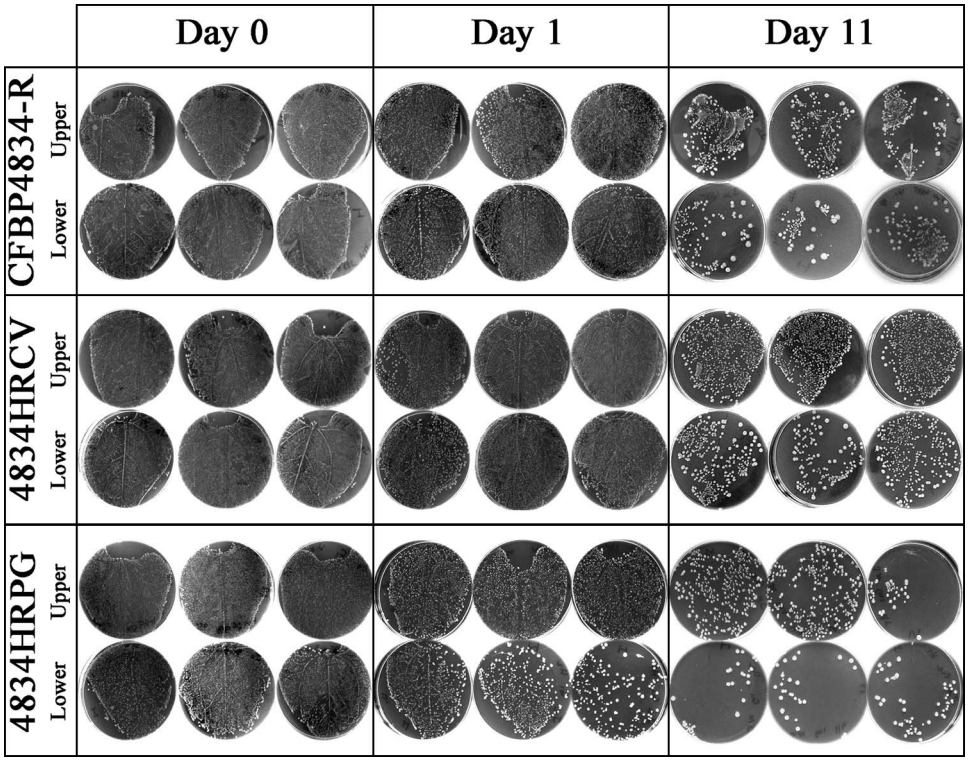

FIG. 4. Imprinting of bean leaves (upper and lower surfaces) during phyllosphere colonization by *X. fuscans* subsp. *fuscans* strain CFBP4834-R and strains mutated in the *hrcV* (4834HRCV) or *hrpG* (4834HRPG) gene after spray inoculation (1×10^6 CFU ml^{-1}). Three leaves per strain and per sampling date are presented. Leaves were sampled at 3 h after inoculation (day 0) and 1 and 11 days later.

wild-type strain CFBP4834-R and strains with mutations in structural *hrp* genes (Fig. 4).

The dynamics of the bacterial population densities of two strains of *X. campestris* pv. campestris, one with a mutation in a structural *hrp* gene (*hrcU*) and a second with a mutation in a regulatory *hrp* gene (*hrpX*), were quantified in the bean phyllosphere in comparison with the wild-type strain ATCC 33913. Strains of *X. campestris* pv. campestris with mutations in *hrp* genes behaved similarly to their parental strain in the bean phyllosphere throughout the 11 days of the experiment (Fig. 1). Their population densities were not significantly different ($P < 0.05$) from each other and from those of ATCC 33913 at any sampling date. This absence of differences among population densities indicated that a functional T3SS did not play any role in *X. campestris* pv. campestris survival in the nonhost phyllosphere.

Analysis of transmission to bean seeds for strains with mutations in *hrp* genes. The rates of transmission of each strain of *X. fuscans* subsp. *fuscans* CFBP4834-R with mutations in *hrp* genes to the bean seeds were significantly ($P < 0.05$) altered compared to those of transmission of the wild-type strain (Table 3). The strains with mutations in *hrp* genes also showed significantly ($P < 0.05$) lower population densities on contaminated seeds (around 1×10^2 CFU g^{-1} of fresh weight) than the wild-type strain CFBP4834-R (4.3×10^7 CFU g^{-1} of fresh weight). Strains (4834HRPG and 4834HRPX) with mutations in regulatory genes (*hrpG* and *hrpX*) appeared to be the most altered in their ability to be transmitted to the bean seeds, with very low frequencies (0.07 for each) and low final population densities on seeds. CFBP4834-R strains with mutations in *hrp* genes were transmitted to bean seeds at frequencies similar to those for *X. campestris* pv. campestris ATCC 33913 in the incompatible interaction with bean. ATCC 33913 strains with mutations in *hrp* genes were transmitted to bean seeds at frequencies (around 0.20) similar to those for their parental strain. This result indicates that in this incompatible interaction, a functional T3SS is not required for transmission to seeds.

Analysis of pathways used by CFBP4834-R and strains with mutations in *hrp* genes for transmission to seeds. To monitor the bacterial transmission to seeds by the vascular pathway, reproductive organs were protected before spray inoculation of leaves. Using this inoculation method, only wild-type strain CFBP4834-R of *X. fuscans* subsp. *fuscans* was able to be transmitted to pods and seeds with high frequencies (0.85 and 0.63, respectively), whereas none of 4834HRPG, 4834HRCT, and 4834HRCV strains were able to be transmitted to pods or seeds. The mean CFBP4834-R population density on contaminated pods was 4.47×10^3 CFU g^{-1} (standard error of the mean [SEM], 3.08 CFU g^{-1}) of fresh weight, and that on contaminated seeds was 158 CFU g^{-1} (SEM, 2.07 CFU g^{-1}) of fresh weight.

To monitor bacterial transmission to seeds by the floral pathway, an inoculum was deposited directly in flower buds. These experiments showed that CFBP4834-R was also able to be transmitted to both pods and seeds with high frequencies (0.9). CFBP4834-R strains with mutations in regulatory *hrp* gene (4834HRPG) and in structural *hrp* genes (4834HRCT and 4834HRCV) showed high transmission rates to pods, with frequencies of 0.67, 0.52, and 0.89, respectively. These strains could be transmitted to seeds with lower frequencies (0.1, 0.2, and 0.04, respectively) than CFBP4834-R. Under our asymptomatic conditions, transmission to seeds by contact with pod symptoms was prevented, and great care was taken to avoid any seed contamination with pod tissue while collecting seeds.

The lower bacterial transmission to pods is not a consequence of a lower initial colonization of the strains with mutations in *hrp* genes in flowers compared to CFBP4834-R. The dynamics of flower contamination following flower bud inoculation were similar ($P > 0.05$) for CFBP4834-R and 4834HRCV until the fourth day after inoculation. Precisely, the mean population densities of CFBP4834-R were 2.33×10^5, 4.05×10^5, 3.58×10^6, and 1.43×10^6 CFU g^{-1} of fresh weight, and those of 4834HRCV were 2.16×10^5, 1.63×10^6, 3.48×10^6, and 3.16×10^5 CFU g^{-1} of fresh weight, at days 1 to 4 after inoculation, respectively. On the 15th, 28th, and 35th days after inoculation, population densities were significantly ($P < 0.05$) lower on pods colonized by 4834HRCV (2.26×10^4, 2.29×10^4, and 1.28×10^5 CFU g^{-1} of fresh weight, respectively) than on pods colonized by CFBP4834-R (1.94×10^7, 2.77×10^7, and 3.51×10^8 CFU g^{-1} of fresh weight, respectively). Populations were 3 orders of magnitude lower for the strain with a mutation in the *hrcV* gene than for the wild type at each sampling date.

DISCUSSION

In this study, we showed that a classical pathogenicity determinant, the T3SS encoded by the *hrp* gene cluster, is involved in *X. fuscans* subsp. *fuscans* early and late colonization processes but not in its survival on its host. The T3SS is also required for a high frequency of pathogen transmission to the seeds. However, low rates of bacterial transmission to seeds are independent of a functional T3SS, as is bacterial survival on nonhost plants. Previous studies have demonstrated that alteration of the T3SS in other bacterial pathogens abolishes pathogenicity (8, 24, 27, 52) and in planta multiplication (7, 27). Our experimental approach focused on the measurement of phyllosphere colonization processes and transmission to seeds by wild-type *X. fuscans* subsp. *fuscans* and strains with mutations in *hrp* genes in low-RH conditions with low initial bacterial population densities (below 1×10^6 CFU g^{-1} of fresh weight). These conditions do not favor disease expression for *X. fuscans* subsp. *fuscans* (22) and were used to reproduce the most commonly observed situation in a temperate climate for a bacterial plant pathogen, i.e., asymptomatic colonization of the host. Indeed, the interactions between a susceptible host and a virulent plant pathogenic bacterium may only rarely result in disease symptoms (21). We show here that the absence of disease manifestation does not compromise active host colonization by *X. fuscans* subsp. *fuscans*, including the phyllosphere and the reproductive organs. This behavior of *X. fuscans* subsp. *fuscans* is not restricted to survival; it is therefore different from that of *X. campestris* pv. campestris on bean in these environmental conditions.

During bean phyllosphere colonization, *X. fuscans* subsp. *fuscans* colonizes both external and internal compartments of the plant (22, 51). It is probable that efficient asymptomatic bean colonization by *X. fuscans* subsp. *fuscans* was mainly due to an endophytic colonization. Indeed, we observed with leaf

imprinting that the number of colonies of *X. fuscans* subsp. *fuscans* that could be removed by printing the leaf surface on agar medium decreased in the days following spray inoculation whereas, the total population densities of *X. fuscans* subsp. *fuscans* in the phyllosphere significantly increased, suggesting an important endophytic colonization or the aggregation of *X. fuscans* subsp. *fuscans* in biofilms that were tightly adherent to the leaf surface. Jacques and collaborators (22) already demonstrated that *X. fuscans* subsp. *fuscans* aggregates in biofilms on the bean leaf surface. Endophytic colonization of the leaf parenchyma may be particularly well supported by the presence of more nutrients in the internal compartment of leaves than on the leaf surface (31).

Strains with alterations in the T3SS were impaired in leaf colonization. Leaf imprinting showed that similar numbers of colonies were recovered from the surface of the leaf for strains with mutations in structural *hrp* genes and the wild-type strain, whereas strains with mutations in *hrp* genes established total phyllosphere population densities that were 1,000-fold lower than those of the wild-type strain. This means that the decrease probably concerned mainly the endophytic compartment. Interestingly, similar conclusions were obtained for other phytopathogens such as *P. syringae* and *Erwinia amylovora*. *P. syringae* pv. tomato *hrp* mutants are impaired in the endophytic colonization of their host (7). Furthermore, it has been shown that structural *hrp* genes are induced inside leaf tissue and not on the leaf surface (9) and that the elicitor of *hrp* genes via PrhA in *Ralstonia solanacearum* is a nondiffusible plant cell wall component (2).

We demonstrated that *X. fuscans* subsp. *fuscans* strains deficient in the regulatory genes *hrpG* and *hrpX* were more affected than strains deficient in the T3SS structural genes in their colonization capacities, suggesting that HrpG and HrpX regulate additional genes beyond the T3SS in *X. fuscans* subsp. *fuscans*. Moreover, the population size of 4834HRPG, a strain mutated in the *hrpG* gene, was slightly but significantly lower than that of the wild type at 3 h after leaf inoculations, suggesting that the strain was impaired in leaf adhesion. This could have affected its potential for later leaf colonization. Interestingly, we also showed that this strain (4834HRPG) was also hypermotile (data not shown). It has been shown for *Ralstonia solanacearum* that HrpG positively regulates genes involved in attachment and protection response functions (43). Those authors proposed that HrpG serves as a molecular switch between saprophytic and pathogenic lifestyles (43). Indeed, there is an opposite regulation in the phytopathogenic bacterium *Erwinia amylovora* between the virulence-associated T3SS and the flagellar system (9). Moreover, it was also demonstrated that nutrient acquisition in *X. campestris* pv. campestris could involve plant carbohydrate scavenging by TonB-dependent receptors and that some TonB-dependent receptors could be under the regulation of the *hrpG* gene but also are independent of a functional T3SS (5). It is therefore tempting to hypothesize that nutrient acquisition during saprophytic development of these plant pathogenic bacteria is dependent on HrpG regulation but also independent of a functional T3SS. Alternatively, as a consequence of the positive regulation of bacterial adhesion by HrpG (43), alterations in the leaf colonization process for strains with mutations in *hrp* genes could also result from altered capacities to aggregate in adherent biofilms on the leaf surface. Surprisingly, *X. fuscans* subsp. *fuscans* strains deficient in the regulatory genes *hrpG* and *hrpX* were still able to be transmitted to seeds. This perhaps is linked to the very different chemical and physical natures of these two environments (phyllosphere and flower buds). This is also coherent with other reports dealing with the pleiotropic phenotype of strains with mutations in *hrp* regulatory genes (35, 43).

We confirmed that the translated sequences of the *X. fuscans* subsp. *fuscans hrp* genes shared strong homology with Hrp proteins of other *X. axonopodis* pathovars sensu Vauterin et al. (22, 45). The percentage of identity corroborated the predicted phylogeny of xanthomonads, namely, that *X. fuscans* subsp. *fuscans* was more closely related to *X. citri* subsp. *citri* and *X. axonopodis* pv. glycines than to *X. axonopodis* pv. vesicatoria and was more closely related to *X. oryzae* pv. oryzae than to *X. campestris* pv. campestris (39, 44). However, the Hrp pilus subunit, HrpE, seemed to have evolved differently than other Hrp proteins. We found that the surface-exposed domain of the *X. fuscans* subsp. *fuscans* HrpE was more closely related to that of *X. campestris* pv. campestris than to that of *X. citri* subsp. *citri*. A structure in three domains is proposed for HrpE: (i) a domain containing the T3S signal in the N terminus, (ii) a surface-exposed domain, and (iii) a polymerization domain in the C terminus (48). Weber and Koebnik (49) showed that the C terminus is subjected to purifying selection and the surface-exposed domain to positive selection, corresponding to an evolutionary adaptation of this surface structure to avoid recognition by the plant defense system (49). Curiously, we found poor homology (30%) with that of *X. axonopodis* pv. phaseoli, which is also a bean pathogen and was until recently considered to belong to same species and pathovar (22, 45).

From both ecological and agricultural perspectives, transmission of a pathogen to the next generations of its host is a major critical step. We are not aware of any other study designed to look explicitly at the effect of loss of a major class of pathogenicity determinants on the transmission of a pathogen to the seeds of its host. One pathway for bacterial transmission to seeds (i.e., seed pollution) was suppressed in our experimental approach by avoiding contact of seeds with symptoms (the environmental conditions did not allow symptom development) or contaminated pod tissue (by delicate extraction of seeds from pods). The two remaining pathways for bacterial transmission to seeds are the vascular pathway, in which bacteria colonize reproductive organs through the vascular system, and the floral pathway, in which bacteria colonize the pistil and the ovary (29) to finally reach and contaminate the seeds. We found that alteration of the T3SS drastically decreased transmission to seeds by *X. fuscans* subsp. *fuscans*. This could be the result of low bacterial population densities of strains with mutations in *hrp* genes but also of transmission pathways not available for such strains limiting contamination of seeds. Our experiments involving leaf inoculation associated with protection of flower buds showed that strains with mutations in *hrp* genes were completely unable to use the vascular pathway for transmission to seeds. For the wild-type strain, vascular transmission to seeds was responsible for the contamination of seeds for more than 50% of the plants. By direct flower bud inoculations, *X. fuscans* subsp. *fuscans hrp* strains with mutations in *hrp* genes could be transmitted to seeds through the stylar tissues. Floral transmission to seeds via the stylar tissue

was demonstrated for *Acidovorax avenae* subsp. *citrulli* in watermelon blossoms (26) and for a bacterial biocontrol agent (15). Floral transmission to seeds is, however, less efficient for strains with mutations in *hrp* genes than for the wild-type strain. This was not a consequence of a low bacterial installation on flower buds, as population densities remained similar for the wild-type strain and 4834HRCV for at least 4 days following inoculation of flower buds.

On the basis of our results, we propose different stages to describe colonization of aerial plant parts by bacteria. First, bacteria adhere on the plant surface after arrival. This step was not possible for *E. coli* on bean leaves, based on the 100-fold-lower population densities for *E. coli* immediately following inoculation. Second, bacteria survive in the phyllosphere. This survival is certainly limited to the surface of the leaf. Indeed, based on the comparison of the population densities quantified in the phyllosphere and the number of colonies found on the leaf surface by leaf imprinting for *X. campestris* pv. campestris, strains mutated in structural *hrp* genes, and the wild-type strain of *X. fuscans* subsp. *fuscans*, we conclude that the survival of *X. campestris* pv. campestris and of strains mutated in structural *hrp* genes was restricted mainly to the leaf surface. This basic epiphytic competence depends partially on the master *hrp* regulators (HrpG and HrpX) but not on a functional T3SS. On the basis of the model proposed by Jones and Dangl (23), it may be hypothesized that bean defense reactions induced after recognition of some plant-associated molecular patterns of these strains could act to limit internal colonization by *X. campestris* pv. campestris and strains of *X. fuscans* subsp. *fuscans* with mutations in *hrp* structural genes. Meanwhile, the *X. fuscans* subsp. *fuscans* wild-type strain may inject effectors through its T3SS to suppress the plant defense reactions or prevent its recognition. Strains of *X. fuscans* subsp. *fuscans* with mutations in *hrp* structural genes and *X. campestris* pv. campestris are able to colonize new organs (data not shown) and contaminate reproductive organs such as flowers and seeds. The frequencies of such events and the associated population densities, however, are low. It could be hypothesized that *X. campestris* pv. campestris transmission to seeds operates by a kind of saprophytism via the floral structures because of the abundance of nutrients in these organs (33). Furthermore, Ngugi and Scherm (33) suggest that there are no inducible defense responses in flowers. Third, in the case of a compatible interaction, an increase in the density of the bacterial population colonizing the host is dependent on a functional T3SS even in the absence of symptoms. This colonization is mainly endophytic and leads to an efficient transmission of the pathogen to the seeds.

Together, the results reported in this study indicate that *hrp* genes are implicated in early and late stages of host phyllosphere colonization by *X. fuscans* subsp. *fuscans* and in transmission to host seeds. This new finding opens questions about the genes regulated by the master regulators HrpG and HrpX and about the physical role of the T3SS in these processes.

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