Transmission of Plant-Pathogenic Bacteria by Nonhost Seeds without Induction of an Associated Defense Reaction at Emergence^{∇†}

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An understanding of the mechanisms involved in the different steps of bacterial disease epidemiology is essential to develop new control strategies. Seeds are the passive carriers of a diversified microbial cohort likely to affect seedling physiology. Among seed-borne plant-pathogenic bacteria, seed carriage in compatible situations is well evidenced. The aims of our work are to determine the efficiency of pathogen transmission to seeds of a nonhost plant and to evaluate bacterial and plant behaviors at emergence. Bacterial transmission from flowers to seeds and from seeds to seedlings was measured for *Xanthomonas campestris* pv. *campestris* in incompatible interactions with bean. Transmissions from seeds to seedlings were compared for *X. campestris* pv. *campestris*, for *Xanthomonas citri* pv. *phaseoli* var. *fuscans* in compatible interactions with bean, and for *Escherichia coli*, a human pathogen, in null interactions with bean. The induction of defense responses was monitored by using reverse transcription and quantitative PCR (RT-qPCR) of genes representing the main signaling pathways and assaying defense-related enzymatic activities. Flower inoculations resulted in a high level of bean seed contamination by *X. campestris* pv. *campestris*, which transmitted efficiently to seedlings. Whatever the type of interaction tested, dynamics of bacterial population sizes were similar on seedlings, and no defense responses were induced evidencing bacterial colonization of seedlings without any associated defense response induction. Bacteria associated with the spermosphere multiply in this rich environment, suggesting that the colonization of seedlings relies mostly on commensalism. The transmission of plant-pathogenic bacteria to and by nonhost seeds suggests a probable role of seeds of nonhost plants as an inoculum source.

The process of the microbial colonization of germinating seeds and seedlings shapes traits that are important for successful biological control and has therefore been subjected to intense research (for a review, see reference 38). This contrasts strikingly with the situation for seed-borne plant-pathogenic bacteria and their hosts, for which little is known at this step, with efforts having been devoted mainly to interactions with fully developed plants. However, pathogen transmission is one of the most important parameters for fitness (16, 32). It combines the ability to survive outside the host prior to infection, multiply on the host, and disperse and transmit to new ecological niches, including host seeds. Seeds are passive carriers of pathogens that are transmitted when the seeds are sown and emerge (30). Seed accounts for the movement of plant pathogens across vast distances and may even be responsible for the emergence of diseases in new areas. Seed-borne bacterial pathogens are of particular concern because strategies for the management of bacterial diseases are limited, and none are efficient (20). Moreover, seeds harbor a diverse microbial community, including human pathogens such as *Salmonella enterica* serovars and *Escherichia coli* O157:H7 (24, 37). Information is lacking so far with regard to the ability of seed-borne

plant-pathogenic bacteria to multiply on germinating nonhost seeds and colonize seedlings. Some human pathogens establish themselves endophytically in plants and may be inherited from generation to generation (57).

Bacterial spermosphere-colonizing traits have been identified mainly for organisms used for biological disease control; in contrast, only few studies focused on interactions encountered in the spermosphere and seedling environments between plant-pathogenic bacteria and the plant. The colonization of germinating seeds and seedlings represents a critical step in the establishment of bacterial diseases. One observation made previously by Hirano et al. (23) suggested that *Pseudomonas syringae* pv. *syringae* does not behave as a parasite during bean germination, as its type III secretion system (T3SS) does not seem to play a major role during seed germination and seedling colonization. Indeed, similar dynamics of population sizes were observed for wild-type *P. syringae* pv. *syringae* and its T3SS mutants on seedlings in field trials.

The molecular bases of interactions between plant-pathogenic bacteria and plants have been studied extensively in the phyllosphere environment. The plant immune system is based on the recognition of pathogen-associated molecular patterns (PAMPs), resulting in the activation of defense responses (41). Three main pathways controlling the activation of plant defenses are described. The salicylic pathway controls defenses that are active against pathogens with a biotrophic lifestyle, whereas jasmonate and ethylene pathways control defenses that are active against necrotrophic pathogens (27). Most Gram-negative bacterial pathogens harbor a T3SS that allows

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the delivery of effectors directly into the plant cytoplasm, leading to a hypersensitive reaction (HR) in incompatible interactions and symptom development in compatible ones (5, 21, 35). In compatible interactions, defense responses are suppressed by T3SS effectors (3, 14). Alternatively, T3SS effectors such as AvrBS1 to AvrBS4 of *Xanthomonas axonopodis* pv. *vesicatoria* were also described to enhance epiphytic fitness and bacterial transmission in the field (63).

The floral transmission of bacteria to seeds is a permissive pathway compared to transmission through the vascular system (12). Floral transmission allows the contamination of seeds by a cohort of diverse bacteria, including biocontrol agents (17, 52). T3SS mutants of *Xanthomonas citri* pv. *phaseoli* var. *fuscans* are strongly impaired in their ability to transmit to seeds, but transmission through the floral pathway is not completely abolished for these strains (12). None of the five adhesins identified in the *X. citri* pv. *phaseoli* var. *fuscans* genome are involved in floral transmission to bean seeds. Conversely, all of them are involved in transmission through the vascular pathway, limiting or enhancing transmission efficiency and associated bacterial population sizes (11).

Xanthomonads are plant-pathogenic bacteria, and most of them are seed borne. Seed carriage is well documented for pathogens on their host, but information is lacking concerning carriage by a nonhost seed. Recently, Darsonval and colleagues (12) showed that plant-pathogenic bacteria may transmit to nonhost plant seeds. Indeed, following spray inoculation of the canopy of mother plants, bean seeds were contaminated by the incompatible bacterium *X. campestris* pv. *campestris*. However, the risk of generation of a primary inoculum focus at emergence associated with this nonhost carriage was not evaluated. Black rot, caused by the bacterium *X. campestris* pv. *campestris*, is considered the most serious disease of crucifer crops worldwide (46). Plants, such as bean, that are not in the crucifer family are not susceptible. Conversely, bean is a susceptible host for *X. citri* pv. *phaseoli* var. *fuscans* (1) (formerly *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* [60] or *X. fuscans* subsp. *fuscans* [47]), which is responsible for the common bacterial blight of bean (62). Both bacteria are seed borne on their hosts, and in natural settings, their transmission by nonhost seeds had not yet been reported.

In this paper we aimed to understand colonization processes of germinating bean seeds and seedlings by *X. citri* pv. *phaseoli* var. *fuscans*, *X. campestris* pv. *campestris*, and *Escherichia coli*, representing a compatible, an incompatible, and a null interaction, respectively. In an attempt to evaluate the risk of primary inoculum generation associated with nonhost carriage, we monitored the efficiency of the transmission of incompatible bacteria from flowers to seeds and from seeds to seedlings. We also compared the dynamics of bacterial population sizes in regard to defense reactions developed by the plant during germination and seedling development. The role of the T3SS and adhesion in seedling colonization was examined with the use of mutants in structural and regulatory genes of the T3SS and in *yapH*, a gene encoding a nonfimbrial adhesin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study are described in Table 1. The pathogenicity of wild-type strains on their respective susceptible hosts was routinely checked. Xanthomonad strains

TABLE 1. Strains used in this study

Strain	Relevant genotype or characteristic(s)	Reference or source
CFBP4834-R	<i>X. citri</i> pv. <i>phaseoli</i> var. <i>fuscans</i> wild-type strain; Rif ^r	25
ATCC 33913	<i>X. campestris</i> pv. <i>campestris</i> wild-type strain; Rif ^r	13
C600	<i>E. coli</i> wild-type strain; Rif ^r	42
4834HRPG	CFBP4834-R <i>hrpG</i> ::pVO155; Rif ^r Km ^r	12
4834HRCR	CFBP4834-R <i>hrcR</i> ::pVO155; Rif ^r Km ^r	12
4834YAPH2	CFBP4834-R <i>yapH2</i> ::pVO155; Rif ^r Km ^r	11
33913HRCU	ATCC 33913 <i>hrcU</i> ::pVO155; Rif ^r Km ^r	Gift from M. Arlat

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 K₂HPO₄, and 15 g liter⁻¹ agar) supplemented with the appropriate antibiotics. *Escherichia coli* cells were cultivated at 37°C in LB 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 K₂HPO₄, and 15 g liter⁻¹ agar) supplemented with rifamycin (Rif). Antibiotics were used at the following final concentrations: Rif at 50 mg liter⁻¹ and kanamycin (Km) at 25 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 on appropriate media supplemented with appropriate antibiotics. Bacterial cells were scraped from agar plates and suspended in sterile distilled H₂O. Suspensions were turbidimetrically calibrated to 1 × 10⁸ CFU ml⁻¹ and adjusted to the desired final concentrations with sterile distilled H₂O.

Plant material. *In planta* experiments were conducted with a susceptible cultivar of dry bean (*Phaseolus vulgaris* cv. Flavert). Seeds were kindly provided by Vilmorin (La Ménétré, France) and were considered free of *X. citri* pv. *phaseoli* var. *fuscans* following an analysis of about 100,000 seeds per lot with standard tests (44). When necessary, seeds were incubated in sterile germination boxes (17.5 by 11.5 by 15.5 cm) on moistened sterile paper in growth chambers at 25°C for 48 h. Seedlings were transplanted in 10- by 10- by 18-cm pots (1 seed per pot) containing soil substrate (Neuhaus humin substrat S NF 11-44-551; Proveg, La Rochelle, France). Beans were grown in growth chambers as previously described (12) under 16 h of light at 25°C and 8 h of darkness at 22°C and under high (95%) relative humidity. Plants were watered three times per week, and once a week, water was supplemented with 0.3 g liter⁻¹ nitrogen-phosphorous-potassium fertilizer (18:14:18).

Bean inoculations. Seeds were contaminated by two means. First, the contamination of seeds on mother plants was performed by spray inoculation of beans at the flower bud stage with 1 × 10⁷ CFU ml⁻¹ inoculum as previously reported (12). Mature pods were harvested 5 weeks later, and seeds were manually excised from pods. Seeds were stored at room temperature. Second, seeds were directly inoculated with an inoculum of 1 × 10⁶ CFU ml⁻¹ by soaking in 2 ml inoculum per seed during 30 min and vacuum infiltrated for 3 min. Seeds were dried for 2 h in a sterile environment at room temperature and directly used.

Leaves and seedlings were vacuum infiltrated with an inoculum of 1 × 10⁶ CFU ml⁻¹ for 3 min by soaking in 500 ml of inoculum per plant. Plants were dried for 2 h at room temperature. Plant inoculations were carried out under quarantine at UMR PaVé, Site INRA, Angers, France.

Monitoring of bacterial population sizes. To quantify bacterial population sizes, seeds were soaked overnight at 4°C in 2 ml of sterile distilled H₂O per seed. The samples were then shaken with a vortex for 30 s at maximal speed three times. Seedlings and leaves were weighed and ground (Stomacher 80; Seward, London, United Kingdom) individually for 2 min at maximum power in 5 ml of sterile distilled H₂O without any preliminary treatments. Every sample and appropriate dilutions were spiral plated (Spiral Biotech, Bethesda, MD) onto selective medium to quantify the inoculated strain. Samples from control plants were plated onto 100% TSA to quantify bacterial indigenous population sizes. Five samples were analyzed per treatment and per sampling date. Experiments were repeated at least twice.

To quantify the contamination rate of a seed lot, seed bulks were soaked overnight at 4°C in 2 ml of sterile distilled H₂O per g of seeds. Samples were analyzed as indicated above. For seedling lots, bulks of 4-day-old seedlings without cotyledons were ground in 5 to 20 ml of sterile distilled H₂O and analyzed as indicated above. The contamination rate (*p*) of a lot was calculated

TABLE 2. Primers used for qPCR experiments

cDNA	Gene reference no.	Function	Primer sequence	Fragment size (bp)
UBI	CV543388 ^a	Ubiquitin	GAGGATGGTCGCACCTGGCT CCCTCCTTGTCCTGAATCTTA	184
EF1- α	GI151368189 ^b	Elongation factor 1 α	CAAGGATCTCAAGCGTGGTTTCG TGGGAGGTGTGGCAATCAAGC	150
JAR1	GI171655005 ^b	Jasmonate-amino synthetase	AAGAACCGGGACACTATGTGA AATGTTCCCTCCGAACAAC	174
PR3	GI62703651 ^b	PR3 protein (chitinase)	TAGGAGTGGTGTGGATGCTG CGCTGAGATCAGTAGGAGCA	192
ACC	CV541144 ^a	ACC oxidase	TGGCACCAAAGTTACAACCTA ATTCTGGTGCCATCCGTTTGA	270

^a cDNA library of *Phaseolus vulgaris* (22).

^b Reference numbers in NCBI, *Phaseolus vulgaris* cDNA.

from the analysis of a sample divided into N groups according to the formula $p_i = 1 - (Y/N_i)^{1/n_i}$ (31), where n is the number of individuals in each group (bulked for analysis) and Y is the number of healthy groups. Preliminary experiments allowed the approximation of the contamination rate of a seed lot to define the adequate group sizes using most-probable-number tables (54). It was expected that each contaminated group contained at most one contaminated individual. To calculate the efficiency of bacterial transmission from seeds to seedlings, groups contained the same number of individuals. The efficiency of transmission was expressed as a ratio of the number of contaminated seeds to the number of contaminated seedlings (55). Experiments were repeated twice ($i = 2$) with the following sampling: $N_1 = 15$, $n_1 = 100$, $N_2 = 10$, and $n_2 = 40$.

In experiments of *X. campestris* pv. *campestris* transmission to and by bean seeds, the identity of *X. campestris* pv. *campestris* colonies was confirmed on 3 colonies per sample by PCR using specific primers for *X. campestris* pv. *campestris* (45). PCRs were done with 20- μ l volumes containing 200 μ M deoxynucleoside triphosphates (dNTPs), 1.5 mM MgCl₂, 0.5 μ M each primer, 0.4 U μ l⁻¹ GoTaq polymerase (Promega), and 4 μ l of a boiled bacterial suspension (1×10^7 CFU ml⁻¹). PCR conditions were 5 min at 94°C; 35 cycles of 20 s at 94°C, 20 s at 60°C, and 20 s at 72°C; and 7 min at 72°C.

Bacterial adhesion to bean seeds. Bacterial adhesion to bean seeds was tested as previously described (11). Briefly, columns were filled with 6 g of bean seeds and fully saturated with 12 ml of the inoculum at 1×10^5 CFU ml⁻¹. The inoculum was removed after a 2-h contact time between the culture and the seeds. Seeds in columns were washed twice with 12 ml of sterile distilled H₂O. Bacterial population sizes adherent on seeds were monitored by seed maceration. The ratio of the number of attached cells to the number of inoculated cells indicated irreversible adhesion capacities (15). Experiments were repeated at least twice with three samples per treatment.

Bacterial adhesion to polypropylene. Bacterial adhesion under static conditions was quantified by a crystal violet (CV) incorporation assay with 96-well polypropylene microtiter plates (Microwell; Nunc, Denmark) using a method described previously by O'Toole and Kolter (43), modified as described previously by Darsonval et al. (11). Three wells per strain were filled with 200 μ l MME minimal medium [K₂HPO₄ at 10.5 g liter⁻¹, KH₂PO₄ at 5.4 g liter⁻¹, (NH₄)₂SO₄ supplemented with 20 mM glutamate at 1 g liter⁻¹, 1 mM MgSO₄, and Casamino Acids at 0.15 g liter⁻¹] (2) and inoculated with 1:20 dilutions of the inoculum calibrated at 1×10^7 CFU ml⁻¹. Plates were incubated without shaking at 28°C for up to 3 days. Bacterial growth was confirmed by measuring the optical density at 600 nm (OD₆₀₀) of the supernatants by using a μ Quant Platemeter reader (Bio-Tek Instruments, Inc.). To quantify surface-attached bacteria, surface-attached cells in wells were stained for 15 min at room temperature with 200 μ l per well of a 1% (wt/vol) CV solution. The CV solution was then discarded, and the wells were thoroughly washed with sterile distilled H₂O. Finally, the dye incorporated into attached cells was solubilized in 200 μ l of 95% ethanol per well, and the OD₆₀₀ was determined by using the microplate reader. These tests were repeated at least twice for every bacterial strain.

RT-qPCR. RNA was extracted from 50 to 250 mg of plant tissue according to a method described previously by Venisse et al. (61) and treated for 1 h at 37°C with DNase according to the manufacturer's recommendations (Ambion; Ap-

plied Biosystems, Courtaboeuf, France) to remove DNA. Samples were incubated for 10 min at 75°C to inactivate DNase, and RNA was ethanol precipitated, dissolved in double-distilled H₂O, and calibrated at 0.5 μ g μ l⁻¹. The quality (A_{260}/A_{280} and A_{260}/A_{230} ratios) and quantity of RNA were checked with a Nanodrop instrument (Thermo Scientific). The absence of contamination by DNA was checked by quantitative PCR (qPCR) with EF1- α primers (Table 2) using 1/80 dilutions of calibrated RNA. Samples were duplicated, and cDNA was added in a copy of each sample to check the absence of PCR inhibitors. Reverse transcription (RT) was performed with 2 μ g of RNA according to a protocol described previously by Venisse et al. (61), final cDNA was dissolved in 30 μ l of double-distilled H₂O, and 1/16 dilutions were used for qPCR experiments. Quantitative amplifications were performed in a 25- μ l volume with 6.25 μ l of SYBR green (qPCR MasterMix Plus; Eurogentec, Angers, France) and 5 to 10 pmol of primers (Table 2). The concentration of primers was optimized to obtain a PCR efficiency ranging between 90% and 100%. Amplification conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. An additional melt curve was realized in each experiment to check the specificity of the amplification. Relative changes in gene expression were calculated by using the $\Delta\Delta C_T$ method (29), normalized to the two internal controls UBI and EF1- α (Table 2) according to a method described previously by Vandesompele et al. (59) and relative to a calibrator (control samples treated with H₂O at every sampling date and in every RT-qPCR). The three main signaling pathways of defense responses were investigated through PR-3 (chitinase), representing the salicylic acid pathway (48); ACO (ACC oxidase) for the ethylene pathway (8); and JAR-1 (jasmonate resistant 1 in *Arabidopsis thaliana*) for the jasmonate pathway (53). When available, annotated *P. vulgaris* cDNA sequences were used to design primers (22); in the other cases, annotated *A. thaliana* sequences were blasted against expressed sequence tag (EST) libraries to find homologous *P. vulgaris* sequences (Table 2). Experiments were performed with three independent duplicated samples of plant material per treatment. Each sample was made of the aerial part of one seedling without cotyledons or primary leaf pieces from one plant. The mean of values from three water-treated samples was used for calibration at each sampling date, and the geometric mean of values obtained for internal controls was used to normalize each sample.

Assays for POX and GLU activities. Enzymes were extracted as described previously by Venisse et al. (61), by grinding 0.8 to 1 g of plant material into 5 ml of 50 mM sodium acetate buffer (pH 5.5) containing 1 mM polyethylene glycol, 1 mM phenylmethylsulfonyl fluoride, 8% (wt/vol) polyvinylpyrrolidone, and 0.01% (vol/vol) Triton X-100. Homogenates were centrifuged at $16,000 \times g$ at 4°C for 20 min, and supernatants were centrifuged at $10,000 \times g$ at 4°C for 10 min. Peroxidase (POX) activity was determined according to the method of Chance and Maehly (9) and were expressed in μ mol tetraguaiacol mg⁻¹ protein⁻¹ min⁻¹. β -1,3-Glucanase (GLU) activity was assayed according to a method described previously by Wirth and Wolf (64), modified as described previously by Venisse et al. (61), and calculated from the differences in the absorbances at 600 nm between the sample incubated 45 min at 37°C and the sample not incubated. The protein content in crude enzyme extracts was determined according to the method of Bradford (4), using Coomassie protein assay reagent (Pierce, Rockford, IL). Experiments were performed with at least two

FIG. 1. Colonization of bean seedlings by *X. citri* pv. phaseoli var. fuscans wild-type strain CFBP4834-R and mutant strains 4834HRCR and 4834HRPG, *X. campestris* pv. *campestris* ATCC 33913, and *E. coli* C600. Bacterial population sizes were quantified on bean seeds 2 h and 24 h after vacuum infiltration (1×10^6 CFU ml $^{-1}$) of seeds and on primary leaves at 7, 9, 11, and 14 days. The physiological stage of the plant at each sampling time is illustrated on the right of the graphs. Population sizes of indigenous bacterial flora (IBF) were quantified at each sampling date. Means and SEM were calculated for data from four independent experiments with five individuals per sampling date and per treatment. Mean population sizes followed by different letters are significantly ($P < 0.05$) different on the basis of a Mann-Whitney U test.

independent duplicated samples. Each sample was made of the bulk of three to eight seedlings (aerial part without cotyledons) or the bulk of primary leaf pieces from three plants.

Statistical analyses. Statistical analyses were performed by using Statview 5.0 (SAS Institute Incorporated). Data expressed as means of log $_{10}$ -transformed population sizes were analyzed with Mann-Whitney U tests (51).

RESULTS

Bacterial seed transmission in compatible and incompatible interactions. Seed contamination by an incompatible pathogen could represent an epidemiological concern if the bacterium is able to multiply on germinating seeds, colonize seedlings, and disperse to neighboring host plants. To evaluate the risk at emergence of the generation of a primary inoculum associated with nonhost carriage, we measured the efficiency of transmission of pathogenic bacteria from seeds to seedlings of a nonhost plant. Bean seeds contaminated by the incompatible pathogen *X. campestris* pv. *campestris* ATCC 33913 were produced following spray inoculation of beans at the flower bud stage. A 100- to 1,000-fold increase in mean population sizes in 4-day-old seedlings compared to seeds indicated that *X. campestris* pv. *campestris* ATCC 33913 multiplied in this environment (mean *X. campestris* pv. *campestris* ATCC 33913 population sizes of 5.28 log $_{10}$ CFU [standard error of the mean {SEM} = 0.43] and 4.13 log $_{10}$ CFU [SEM = 0.34] in seedlings and 2.49 log $_{10}$ CFU [SEM = 0.35] and 2.18 log $_{10}$ CFU [SEM = 0.16] in seeds per contaminated individual in two independent experiments, respectively). Population sizes reached up to 1.8×10^4 CFU per contaminated seed and 2.4×10^6 CFU per contaminated seedling. The two harvested seed lots showed

contamination rates of 0.6% and 1.4%, and 4-day-old seedlings showed corresponding contamination rates of 0.4% and 3%, respectively. In these experiments transmission efficiencies ranged from 1.5:1 to 0.5:1 (number of contaminated seeds needed to obtain one contaminated seedling), evidencing an efficient transmission of a plant-pathogenic bacterium from seeds to seedlings of a nonhost plant.

To characterize the behavior of bacteria representing incompatible versus compatible and null interactions with a plant at emergence, we analyzed their dynamics of population sizes during seedling development. Bean seeds were inoculated by vacuum infiltration with *X. campestris* pv. *campestris* (incompatible interaction), *X. citri* pv. phaseoli var. fuscans (compatible interaction), and *E. coli* (null interaction). Two hours after inoculation, population sizes of the different strains were similar on seeds, except for those of *E. coli* C600, which were significantly ($P < 0.01$) lower than the others (Fig. 1). As early as 24 h after inoculation, the multiplication of *E. coli* C600 allowed it to reach population sizes similar to those of *X. citri* pv. phaseoli var. fuscans and *X. campestris* pv. *campestris*. Until 14 days after germination, *X. citri* pv. phaseoli var. fuscans strain CFBP4834-R did not multiply significantly better than *X. campestris* pv. *campestris* strain ATCC 33913 and *E. coli* C600 on bean seedlings ($P > 0.05$) (Fig. 1). Therefore, whatever the type of interaction, bacteria behaved similarly at emergence. Similar results were repeatedly monitored and were also obtained with higher inoculum concentrations (data not shown). During the experiments, no symptoms were ever observed on seeds and seedlings following seed contamination with *X. citri*

FIG. 2. Adhesion capacities of bacterial strains on bean seeds. *X. citri* pv. phaseoli var. fuscans strains CFBP4834-R, 4834HRCR, 4834HRPG, and 4834YAPH2; *X. campestris* pv. campestris ATCC 33913; and *E. coli* C600 suspensions at 1×10^5 CFU ml $^{-1}$ were incubated during 2 h with bean seeds. Bars represent the ratios of numbers of attached cells on seeds (#Ac) versus inoculated cells (#Ic). Means and SEM were calculated for three samples per treatment and per experiment. For each treatment double bars stand for data from two independent experiments. Within an experiment, mean population sizes followed by different letters are significantly different ($P < 0.05$) on the basis of a Mann-Whitney U test.

pv. phaseoli var. fuscans strain CFBP4834-R, *X. campestris* pv. campestris, or *E. coli*. Germination rates and seedling development were not affected by any treatment (data not shown).

The ability of both compatible (*X. citri* pv. phaseoli var. fuscans) and noncompatible (*X. campestris* pv. campestris and *E. coli*) strains to multiply on bean seedlings implies that seedling colonization may not require bacterial pathogenicity determinants. Thus, we tested the impact of mutations in the T3SS on seedling colonization by *X. citri* pv. phaseoli var. fuscans. The behaviors of a mutant in a structural gene of the T3SS (secretion mutant strain 4834HRCR) and a mutant in a master regulator gene of the T3SS (regulatory mutant strain 4834HRPG) of *X. citri* pv. phaseoli var. fuscans were compared to that of the wild-type strain. Until 14 days after germination, population sizes of both mutants were not significantly different ($P > 0.05$) from those of wild-type strain CFBP4834-R on bean seedlings (Fig. 1) and were similar to those of the total indigenous bacterial flora, indicating that each bacterial population reached the carrying capacity of the seedling and that this carrying capacity is similar whatever the bacterium (Fig. 1).

Adhesion capacities on bean seeds and polypropylene. Despite the same initial inoculum concentrations (1×10^6 CFU ml $^{-1}$), *E. coli* C600 population densities quantified in seeds 2 h after vacuum inoculation were about 0.02-fold of those of xanthomonads, suggesting an *E. coli* adaptation defect in this environment. This adaptation defect could involve either an impairment in the capacities of adhesion to bean seeds or the death of a part of the population. Adhesion capacities tested in seed columns were not significantly different ($P > 0.05$) among a set of strains composed of *E. coli* C600, *X. campestris* pv. campestris ATCC 33913, and 4834YAPH2, an adhesin mutant

FIG. 3. Kinetics of the adhesion capacities of bacterial strains on a polypropylene surface. *X. citri* pv. phaseoli var. fuscans strains CFBP4834-R, 4834HRCR, 4834HRPG, and 4834YAPH2; *X. campestris* pv. campestris ATCC 33913; and *E. coli* C600 were cultured during 3 days at 28°C under static conditions in polypropylene microtiter plates from an inoculum at 5×10^5 CFU ml $^{-1}$. Crystal violet-stained surface-attached cells were quantified by solubilizing the dye absorbed by adherent cells, after the removal of suspensions, in ethanol and determining the optical density at 600 nm. Means and SEM were calculated for data from two independent experiments, each containing three replicates per treatment and per sampling date. For a given sampling date different letters refer to significantly ($P < 0.05$) different values based on a Mann-Whitney U test.

of *X. citri* pv. phaseoli var. fuscans in the *yapH2* gene (Fig. 2). However, their adhesion capacities were significantly lower ($P < 0.05$) than that of *X. citri* pv. phaseoli var. fuscans CFBP4834-R. Mutants of *X. citri* pv. phaseoli var. fuscans in T3SS genes (4834HRCR and 4834HRPG) were slightly impaired in their adhesion capacities on seeds compared to the wild-type strain (Fig. 2). Similar results were obtained in two independent experiments with three samples per treatment and experiment (Fig. 2). To confirm that the alteration in *X. campestris* pv. campestris ATCC 33913 and *E. coli* C600 adhesion capacities on bean seeds was not linked to a general lack of adhesion capacities of these strains, we compared the adhesion capacities of the set of strains on a polypropylene surface with a crystal violet incorporation assay in a time course experiment (Fig. 3). *E. coli* strain C600 adhered significantly ($P > 0.05$) more on this material than all other tested strains: *X. citri* pv. phaseoli var. fuscans CFBP4834-R and its *hrpG*, *hrcR*, and *yapH2* mutants (4834HRPG, 4834HRCR, and 4834YAPH2, respectively) and *X. campestris* pv. campestris ATCC 33913 (Fig. 3). This test confirmed that no strain was deficient in its adhesion capacities and that *X. citri* pv. phaseoli var. fuscans and *X. campestris* pv. campestris had similar (although weaker than *E. coli*) properties. Thus, both *E. coli* C600 and *X. campestris* pv. campestris ATCC 33913 shared an adhesion defect on bean seeds. It did not affect *X. campestris* pv. campestris ATCC 33913 settlement on bean seeds, as *X. campestris* pv. campestris ATCC 33913 population sizes were similar to *X. citri* pv. phaseoli var. fuscans CFBP4834-R population sizes 2 h after inoculation (Fig. 1).

FIG. 4. Relative normalized expressions of the *PR-3*, *JAR-1*, and *ACO* genes in seedlings following seed inoculation. Seeds were inoculated with 1×10^6 CFU ml^{-1} of *X. citri* pv. phaseoli var. fuscans strain CFBP4834-R, *X. campestris* pv. campestris strain ATCC 33913, acibenzolar-S-methyl (Bion/WG 50; 400 mg liter^{-1}) as a positive control, and water as negative control (calibrator). Dotted bars represent a 4-time [$\log_2(4) = 2$] induction or repression relative to the negative control. The mean of water treatment values was used for calibration at each sampling date, and the geometric mean of values obtained for internal controls (*UBI* and *EF1- α*) was used to normalize each sample. The physiological stage of plants at the sampling time is illustrated on the right of the graphs. Means and SEM were calculated for data from three independent experiments, each containing three independent samples of plant material per treatment and two replicates per sample.

Plant defense responses. The similar multiplication of bacteria on seedlings, whatever the type of interaction, and the presence of a functional T3SS or not suggested an absence of plant defense reactions. To test this hypothesis, we assayed the three main pathways of plant defense signaling responses in seedlings during the first steps of germination using an RT-qPCR approach. Seeds were inoculated with *X. citri* pv. phaseoli var. fuscans CFBP4834-R and *X. campestris* pv. campestris ATCC 33913. We controlled that plant defenses were inducible using acibenzolar-S-methyl (Bion/WG 50, 50% acibenzolar-S-methyl in water dispersible granules; Syngenta), which is known to be a plant defense activator (28). Relative quantifications of salicylic acid, jasmonate, and ethylene pathway inductions were tested with the *PR-3*, *JAR-1*, and *ACO* genes, respectively, by using water as a calibrator and the

FIG. 5. Relative expressions of the *PR-3*, *JAR-1*, and *ACO* genes following infiltration of 2-day-old seedlings (a) and leaves (b). Plants were inoculated with 1×10^6 CFU ml^{-1} of *X. citri* pv. phaseoli var. fuscans strain CFBP4834-R, *X. campestris* pv. campestris strain ATCC 33913, acibenzolar-S-methyl methyl (Bion/WG 50; 400 mg liter^{-1}) as a positive control, and water as a negative control (calibrator). Dotted bars represent a 4-time [$\log_2(4) = 2$] induction or repression relative to the negative control. The mean of water treatment values was used for calibration at each sampling date, and the geometric mean of values obtained for internal controls (*UBI* and *EF1- α*) was used to normalize each sample. The physiological stage of plants at the sampling time is illustrated at the right of the graphs. Means and SEM were calculated for three independent samples of plant material per treatment and two replicates per sample.

EF1- α and *UBI* genes as internal controls. Seed infiltration leading to seedling colonization was used to mimic the natural behavior of bacteria originating from contaminated seeds, while seedling or leaf infiltration was used to force the bacteria into the intercellular spaces of the parenchyma. Two days after seed infiltration, almost a 3-time induction [$\log_2(3) = 1.58$] of the *PR-3* gene was monitored in seedlings for acibenzolar-S-methyl (positive control), while no induction could be recorded following seed inoculation with the incompatible bacterium *X. campestris* pv. campestris ATCC 33913 (Fig. 4). However, *PR-3* gene expression was induced in the 2 days following the infiltration of 2-day-old seedlings with the same strain and with acibenzolar-S-methyl (Fig. 5a). Furthermore, a 32-time increase [$\log_2(32) = 5$] and an 8-time increase [$\log_2(8) = 3$] of *PR-3* expression were observed 2 days after leaf infiltration

FIG. 6. β -1,3-Glucanase (GLU) and peroxidase (POX) activities in protein extracts from bean seedlings after seed infiltration (1×10^6 CFU ml⁻¹) with *X. citri* pv. phaseoli var. fuscans strains CFBP4834-R, 4834HRCR, and 4834HRPG; *X. campestris* pv. campestris strains ATCC 33913 and 33913HRCU; *E. coli* C600; acibenzolar-*S*-methyl (Bion/WG 50; 400 mg liter⁻¹); and distilled water (control). The physiological stage of plants at the sampling time is illustrated at the right of the graphs. Means and SEM were calculated for two independent duplicated samples. Each sample was made of the bulk of three to eight seedlings (aerial part without cotyledons) or the bulk of primary leaf pieces from three plants.

with *X. campestris* pv. campestris ATCC 33913 and with the positive control, respectively, while an 8-time decrease [$\log_2(1/8) = -3$] in the expression of this gene was observed for leaves infiltrated with *X. citri* pv. phaseoli var. fuscans CFBP4834-R (Fig. 5b). This modification of *PR-3* gene expression did not last, as it was no longer observed 4 days after leaf infiltration (data not shown). Conversely, the *PR-3* gene expression level increased slightly in seedlings 4 days after seed infiltration with *X. campestris* pv. campestris ATCC 33913 (Fig. 4). These results showed that plant defenses (salicylic acid pathway) were highly induced in leaves following the infiltration of the incompatible bacterium *X. campestris* pv. campestris while poorly induced during the colonization of bean seedlings after seed inoculation. The expression levels of *JAR-1* and *ACO* varied only slightly and mainly in response to acibenzolar-*S*-methyl. These results confirmed that these pathways were not involved in the response of bean to bacterial colonization. For this reason, proteins involved in the salicylic acid pathway were assayed to confirm RT-qPCR results by a biochemical approach.

To confirm the low level of defense response induction in

the early steps of plant development following bacterial colonization originating from seeds, we measured the enzymatic activities of two other pathogenesis-related (PR) proteins, peroxidase (POX) (EC 1.11.1.7) and β -1,3-glucanase (GLU) (EC 3.2.1.6), in seedlings following seed inoculation versus seedling infiltration. Seeds were inoculated with *X. citri* pv. phaseoli var. fuscans CFBP4834-R, *X. campestris* pv. campestris ATCC 33913, and *E. coli* C600 at 1×10^6 CFU ml⁻¹. Mutants (4834HRCR, 4834HRPG, and 33913HRCU) of *X. citri* pv. phaseoli var. fuscans CFBP4834-R and *X. campestris* pv. campestris ATCC 33913 in the T3SS regulatory (*hrpG*) gene and structural (*hrcR* and *hrcU*) genes were tested, as was the positive control acibenzolar-*S*-methyl. No POX activities were induced in any seedlings treated with the different strains until 11 days after germination. *X. campestris* pv. campestris ATCC 33913, 33913HRCU, 4834HRCR, and 4834HRPG did not induce more of a defense response in seedlings than *X. citri* pv. phaseoli var. fuscans CFBP4834-R. GLU activity increased progressively in the positive control (Fig. 6). Similar results were obtained for two independent experiments and were also obtained for a higher-inoculum dose (1×10^7 CFU ml⁻¹)

FIG. 7. β -1,3-Glucanase (GLU) and peroxidase (POX) activities in protein extracts from infiltrated bean seedlings. Enzymatic activities were assayed 2 days after the infiltration of 2-day-old bean seedlings with 1×10^6 CFU ml⁻¹ of *X. citri* pv. phaseoli var. fuscans strain CFBP4834-R, *X. campestris* pv. campestris strains ATCC 33913 and 33913HRCU, and water (negative control). Means and SEM were calculated for two independent duplicated samples. Each sample was made of the bulk of three seedlings (aerial part without cotyledons).

(data not shown). However, vacuum infiltration of 2-day-old seedlings with *X. campestris* pv. campestris ATCC 33913 induced a 2-fold increase of POX and GLU activities. The levels of these POX and GLU activities were higher than those in the negative control and higher than those in seedlings infiltrated with *X. citri* pv. phaseoli var. fuscans CFBP4834-R and mutant strain 33913HRCU, indicating that defense responses were inducible in seedlings upon the recognition of an incompatible bacterium (Fig. 7). Similarly, an increase in levels of POX and GLU activities was obtained for primary leaves of 2-week-old plants following vacuum infiltration with *X. campestris* pv. campestris ATCC 33913 compared to the controls (data not shown), confirming the defense response induction in this bean cultivar faced with *X. campestris* pv. campestris ATCC 33913. These results showed that plant defenses were inducible in seedlings following the infiltration of the incompatible bacterium *X. campestris* pv. campestris but did not naturally occur during the colonization of bean seedlings when the inoculum originated from the seed independent of its level.

DISCUSSION

Associations developing on germinating seeds mark the first point of contact between plants, pathogens, and microorganisms, with beneficial or harmful results for plant health, growth, and development. In an attempt to evaluate the potential efficiency of control strategies against seed-borne pathogenic bacteria, we designed experiments aimed at quantifying the temporal dynamics of bacterial population sizes and associated defense responses in plant. We demonstrated that bacterial multiplication occurred in seedlings with similar dynamics of population sizes whatever the type of interactions that bacteria develop with the plant. No significant defense responses were induced in the plant for the incompatible interaction until bacteria were introduced within plant tissues. These results indicate that bacterial colonization of seedlings relies mostly on surface colonization and not on parasitic relationships. The transmission of incompatible bacteria to seed-

lings from contaminated seeds suggests a probable role of seeds of nonhost plant as an inoculum source for seed-borne pathogenic bacteria. Taken together, these results suggest that plant defense responses are not naturally induced by seed-associated bacteria in early stages of plant development.

Interactions occurring in seeds and seedlings between bacterial and plant partners allowing bacteria to multiply and having no negative impact on plants defined a commensal interaction. For compatible situations, it has always been argued that seed-borne bacteria and especially xanthomonads colonize the surface of seedlings and have no early endophytic development (19). With nutrients being available in sufficient amounts on germinating seeds and seedlings (38), bacteria do not need to set up a molecular dialogue with the plant to multiply efficiently in this environment. Here, we show that bacteria colonize germinating seeds and seedlings with similar population sizes whatever the type of interaction that they develop with the plant. Indeed, similar trends for *X. citri* pv. phaseoli var. fuscans, *X. campestris* pv. campestris, and *E. coli*, representing compatible, incompatible, and null interactions, respectively, were monitored on beans from seed imbibition until they were 14-day-old seedlings. In addition, bacterial multiplication did not require a functional T3SS, suggesting that nutrients are not a limiting factor in the spermosphere. Indeed, T3SS genes are not induced in nutrient-rich medium (58). This contrasts with the phyllosphere, where nutrients are in limiting quantities (33). To multiply efficiently in this environment, bacteria have to rely on the expression of T3SS (12). Taken together, these results indicate that seedling colonization requires different bacterial determinants than phyllosphere colonization and that environmental parameters to which bacteria have to adapt are different in these two environments. Specific interactions between *X. citri* pv. phaseoli var. fuscans and bean take place later on during the colonization of the phyllosphere (11, 12), when nutrient availability is limiting for bacterial multiplication. In addition, our present work indicates that in leaves, *X. citri* pv. phaseoli var. fuscans negatively regulated the *PR-3* gene, suggesting a suppression of host defenses. This is coherent with the ability of T3SS effectors to suppress defense responses induced by PAMPs in compatible interactions (34, 40).

Regarding the incompatible situation studied in the present work, the absence of elevated defense reaction induction by *X. campestris* pv. campestris in bean seedlings after seed inoculation could result from a similar commensal interaction. Indeed, we verified that defense responses are inducible in juvenile plantlets following the infiltration of seedlings and leaves with *X. campestris* pv. campestris and acibenzolar-S-methyl. The induction of defense responses was previously observed for other plant seedlings such as cowpea and melon (6, 28). These plant defense responses are specifically induced by bacterial T3SS effectors upon recognition by the corresponding resistance genes (26). Indeed, while the *X. campestris* pv. campestris wild-type strain induces a defense reaction following the infiltration of seedlings, an *X. campestris* pv. campestris mutant in the T3SS does not. Thus, the lack of defense response induction in naturally colonized seedlings by incompatible bacteria is consecutive to a nondetection of the bacteria by the plant cells.

Attachment to seeds represents a key step for the introduction of bacteria into the rhizosphere (10) and would represent

an advantage for seed-borne pathogens. We report contrasting adhesion capacities for *X. citri* pv. phaseoli var. fuscans, *X. campestris* pv. campestris, and *E. coli*. *X. citri* pv. phaseoli var. fuscans has strong capacities of attachment to bean seeds compared to those of *X. campestris* pv. campestris and *E. coli*. We previously showed that *X. citri* pv. phaseoli var. fuscans CFBP4834-R produces at least five adhesins, some of which are involved in attachment to seeds (11). *E. coli* adhesion to bean seeds strikingly contrasts with its high capacities for adhesion to polypropylene. Polypropylene is an apolar surface shown to be particularly well suited to show slight differences in adhesion capacities among *Pseudomonas fluorescens* mutants (43). However, this material does not adequately reflect the properties of adherence to root surfaces. This is not entirely surprising, since it was previously shown for several bacteria, such as *E. coli* O157:H7 and *Vibrio cholerae*, that bacteria rely on disparate sets of genes to adhere onto both biotic and abiotic surface (36, 56). The contrasting adhesion behaviors of *E. coli* on biotic and abiotic surfaces indicate that *in vitro* adhesion assays do not simulate an *in planta* bioassay. On biotic surfaces, adhesion may depend on bacterial species, strains, and seed coat characteristics. For example, it was shown previously that *E. coli* O157:H7 and *Salmonella enterica* firmly adhere to alfalfa sprouts and seed coats (10, 18, 50, 56). Altogether, this is useful information in the context of the emergence of *E. coli* strains spreading in agricultural environments.

Could nonhost seeds serve as an inoculum source for seed-borne pathogenic bacteria? The transmission of a plant-pathogenic bacterium to seeds of a nonhost plant was demonstrated for *X. campestris* pv. campestris on bean (12). It was suggested previously that this transmission results from the lack of inducible defense responses in flowers (39). Here, we show that the subsequent epidemiological step is also feasible. A plant-pathogenic bacterium can efficiently transmit from seeds to nonhost seedlings as a consequence of saprophytic multiplication, resulting in the establishment of a primary inoculum focus. It was previously demonstrated that plant-pathogenic bacteria can survive in the phyllosphere of nonhost plants (7, 12, 49). The next epidemiological step is the dispersal of these pathogenic bacteria from this inoculum focus situated in a nonhost crop to a neighboring susceptible host crop. This remains to be demonstrated in order to evidence that nonhost carriage could serve to establish reservoirs of bacterial pathogens in the field.

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