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Naturally Occurring Nonpathogenic Isolates of the Plant Pathogen *Pseudomonas syringae* Lack a Type III Secretion System and Effector Gene Orthologues▽†

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Pseudomonas syringae **causes plant diseases, and the main virulence mechanism is a type III secretion system (T3SS) that translocates dozens of effector proteins into plant cells. Here we report the existence of a subgroup of *P. syringae* isolates that do not cause disease on any plant species tested. This group is monophyletic and most likely evolved from a pathogenic *P. syringae* ancestor through loss of the T3SS. In the nonpathogenic isolate *P. syringae* 508 the genomic region that in pathogenic *P. syringae* strains contains the *hrp-hrc* cluster coding for the T3SS and flanking effector genes is absent. *P. syringae* 508 was also surveyed for the presence of effector orthologues from the closely related pathogenic strain *P. syringae* pv. syringae B728a, but none were detected. The absence of the *hrp-hrc* cluster and effector orthologues was confirmed for other nonpathogenic isolates. Using the AvrRpt2 effector as reporter revealed the inability of *P. syringae* 508 to translocate effectors into plant cells. Adding a plasmid-encoded T3SS and the *P. syringae* pv. syringae 61 effector gene *hopA1* increased in planta growth almost 10-fold. This suggests that *P. syringae* 508 supplemented with a T3SS could be used to determine functions of individual effectors in the context of a plant infection, avoiding the confounding effect of other effectors with similar functions present in effector mutants of pathogenic isolates.**

Pseudomonas syringae is probably the most intensively studied bacterial plant pathogen for which molecular interactions with host and nonhost plants have been dissected in great detail (16, 43, 50). *P. syringae* is a member of the *Gammaproteobacteria* and comprises strains isolated from dozens of cultivated, ornamental, and wild plants. According to the current taxonomy, isolates are grouped into different pathovars based on the plant host from which they were isolated (65). The diseases that *P. syringae* strains cause range from foliar spot diseases to blights, stripes, and cankers (1). Bacteria are transmitted mainly by rain and wind, can survive for periods of time on leaf surfaces as epiphytes without causing disease, and then enter leaves either through natural openings like stomata or through wounds and finally reach high population densities in the intercellular plant spaces and cause visible disease symptoms (26). Recently, *P. syringae* isolates have been found in clouds, rain, snow, and river epilithion, suggesting that these environments are important inoculum sources (44).

P. syringae is able to cause diseases in its hosts because of its ability to suppress plant defenses elicited by microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) like flagellin (23). These defenses are called PAMP-triggered immunity (PTI) (11). Suppression of PTI is accomplished by effector proteins that are translocated from *P. syringae* into plant cells by means of a type III secretion system (T3SS) and by toxins (for example, coronatine) (38, 43). However, on some plants, effectors are directly or indirectly detected by resistance proteins that alert the plant to the presence of the invading bacterium, and effector-triggered immunity (ETI) is activated (11). Effectors that elicit ETI were previously called avirulence proteins since they abolish virulence in plants with the cognate resistance proteins. Resistance may be accompanied by programmed cell death as part of the hypersensitive response (HR), which comprises activation of a variety of plant defenses (18). The HR is visible as total leaf collapse when a high dose of pathogen is infiltrated in a leaf.

The genes coding for the structural and regulatory components of the T3SS in *P. syringae* are located in the *hrp-hrc* cluster (12). The structural components of the T3SS include the basal body embedded in the bacterial cell membrane and the pilus, a long tube that is believed to grow through the plant cell wall to reach and attach to the plant cell membrane to deliver effector proteins into the plant cell. Since many structural components of the basal body are conserved in all T3SS in various animal and plant pathogens and the components have homology to components of the flagellum, it is believed that the T3SS evolved from the flagellum in one organism and was then horizontally transferred to other organisms (56). Helper proteins encoded by some genes in the *hrp-hrc* cluster and elsewhere in the chromosome aid in pilus growth through the bacterial and plant cell wall and in docking of the pilus to the plant cell membrane (37). The *hrp-hrc* cluster is flanked on one side by the conserved effector locus (CEL), which contains

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genes coding for homologous effectors in most *P. syringae* strains, and on the other side by the exchangeable effector locus (EEL), which is variable and contains different effector genes and hypothetical genes in different strains (3). The entire region is considered to be a pathogenicity island (PAI) (22) acquired by horizontal transfer from other bacterial species (3). The CEL, the *hrp-hrc* cluster, and the EEL are present in the same genomic context in the three completely sequenced *P. syringae* strains (6, 15, 33). Phylogenetic analysis has indicated that the most recent common ancestor (MRCA) of today's *P. syringae* isolates contained the *hrp-hrc* cluster (54). Since the CEL is also present in all *P. syringae* isolates analyzed to date, it was probably also present in the MRCA. Moreover, the similar G+C contents of the *hrp-hrc* cluster, the CEL, and the rest of the *P. syringae* genome are in agreement with the ancient acquisition hypothesis (3). In contrast, the EEL varies considerably between strains and has a lower G+C content, indicating that there was relatively frequent acquisition of genes by horizontal transfer from other bacterial species at this locus (10, 14). Additional effector genes are located in different genomic contexts in the three sequenced strains (9), suggesting that these genes were also acquired by horizontal transfer. The different repertoires of effectors in different *P. syringae* strains are believed to be some of the main determinants of host range (52). Acquisition and loss of effectors through acquisition or loss of entire PAIs, nonsense mutations, and transposon insertions and by terminal reassortment (57) can be assumed to have changed the host range of *P. syringae* strains during evolution. For example, the effector gene *avrPphB* (*hopAR1*) has been shown to be lost at a high frequency from a *P. syringae* pv. phaseolicola strain during infection of bean plants containing a resistance gene that induces defenses when *hopAR1* is recognized (47). This is accomplished by excision of the PAI that carries *hopAR1*, which allows *P. syringae* pv. phaseolicola strains to instantly change from avirulent to virulent for the plant that it is infecting (47).

While most *P. syringae* strains have been isolated from symptomatic plants, there are a few examples of *P. syringae* strains that were isolated from healthy plants. One example is *P. syringae* strain cit7, which was isolated from a healthy orange tree (46). This strain became famous as the first gene-engineered microorganism released into the environment after the genes coding for its ice nucleation activity were removed (40). Other examples are *P. syringae* strain TLP2, isolated from a healthy potato leaf (41), and *P. syringae* strain 508, isolated from an apple leaf on an orchard floor (7). *P. syringae* TLP2 and *P. syringae* 508 are both examples of biocontrol strains. *P. syringae* TLP2 was found to moderately control pathogenic *P. syringae* pv. tomato strains when it was applied to tomato (*Solanum lycopersicum*) (64), and *P. syringae* 508 was found to have exceptional antimicrobial activity against the apple scab fungus *Venturia inaequalis* (7). The antifungal activity of *P. syringae* 508 is believed to be due in part to the production of the toxin syringopeptin. *P. syringae* 508 produces a particular syringopeptin with elevated antifungal and antibacterial activity (19).

Besides the biocontrol activity against *V. inaequalis*, Burr and colleagues (7) also noticed that *P. syringae* 508 was unable to cause disease in any of the plant species tested (green bean, bush bean, apple, peach, cherry, and pea) and that it was unable to cause an HR in tobacco at the dose at which all other *P. syringae* strains cause an HR or disease (10^9 CFU/ml). The ability to induce an HR, which is visible within 20 h after a bacterium has been infiltrated into a tobacco leaf as a collapsed area corresponding to the infiltration area, is an easy test to determine if a bacterium is a plant pathogen since plant-pathogenic bacteria cause an HR, while nonpathogens do not. Burr and colleagues (7) also noticed that *P. syringae* 508 DNA did not hybridize with an *hrpZ* probe, a conserved gene coding for a helper protein known to be present in all pathogenic *P. syringae* strains. Starting from this observation, we further investigated the apparent inability of *P. syringae* 508 to cause disease. We found that *P. syringae* 508 does not possess either an *hrp-hrc* cluster coding for a T3SS or orthologues of effectors of the closely related pathogenic isolate *P. syringae* pv. syringae B728a and cannot efficiently translocate the effector AvrRpt2 into plant cells. Surprisingly, we found that nonpathogenic *P. syringae* isolates like *P. syringae* 508 lacking a T3SS are common leaf colonizers in Europe and North America. These isolates most likely evolved from a pathogenic *P. syringae* ancestor through loss of its T3SS. Interestingly, adding a T3SS and one effector back to *P. syringae* 508 increased in planta growth, suggesting that the identified nonpathogenic isolates could be used to study *P. syringae* pathogenicity factors, particularly effectors. It may even be possible to engineer these isolates back into pathogens with different host ranges by adding a functional T3SS and different assortments of effector genes. This may provide an excellent tool to elucidate the basis of host range in *P. syringae*.

MATERIALS AND METHODS

Bacteria, isolation of bacteria from plants, and media. *P. syringae* isolates were either obtained from colleagues or isolated from plants at the Hahn Horticultural Garden at Virginia Tech or in the Jura and southern Alps regions of France (Table 1). For isolation from plants at Virginia Tech, leaves were surface sterilized for 10 s with 70% ethanol, patted dry on a paper towel, and then ground with a plastic pestle in 200 μl of 10 mM $MgSO_4$ in a 1.5-ml microcentrifuge tube. Twenty microliters of the bacterial suspension was plated on KB (35) plates containing 100 μg/ml of nitrofurantoin. For plants from France, strains were obtained from fresh leaves by dilution plating ground tissue on KB supplemented with cephalexin (80 μg/ml) and boric acid (1500 μg/ml). *P. syringae* isolates were generally grown on KB plates with antibiotics at the following concentrations when they were necessary to maintain plasmids: kanamycin, 50 μg/ml; and tetracycline, 15 μg/ml.

Molecular biology. Fragments of the *gap1*, *gltA*, *gyrB*, and *rpoD* genes were amplified from *P. syringae* genomic DNA extracted with a Puregene DNA purification system cell and tissue kit (Gentra Systems, Minnesota) using primers described by Hwang and colleagues (28). PCR products were sequenced at the University of Chicago Cancer Research Center DNA Sequencing & Genotyping Facility. Sequences were edited and assembled using Seqman (Lasergene, DNASTAR, Wisconsin). Evolution models were tested using Modeltest 3.7 (48) and PAUP* 4.0b10 (x86 binary portable version for Mac OS X) (58). A maximum likelihood tree was generated in PAUP* 4.0b10 using the model selected in the previous step and 2,000 bootstrap replicates.

Two integrative plasmids were constructed from pBAV208. pBAV208 is a cloning vector with an origin of transfer for transferring constructs from *Escherichia coli* to *P. syringae* by triparental mating. pBAV208 can be used either for disrupting genes in *P. syringae* or for adding genes to the chromosome of *P. syringae* by Campbell integration. It was cloned by combining the origin of transfer from a pBSL vector (2), the multiple-cloning site and the origin of replication from pBC SK+ (Stratagene, California), and the *nptII* gene for kanamycin resistance from pZERO2.1 (Invitrogen, California). The sequence of pBAV208 is available upon request. A fragment corresponding to the *P. syringae* 508 orthologue of Psy_1182 was amplified from *P. syringae* 508 genomic DNA by PCR using *Pfu* Turbo (Stratagene, California) and primers Psy_1182-F and Psy_1182-R (see Table S1 in the supplemental material), digested with the

TABLE 1. Bacterial isolates and plasmids

Isolate or plasmid	Description	Resistance	Reference(s)
Isolates			
P. syringae pv. syringae B728a	*P. syringae* isolate from *Phaseolus vulgaris* (snap bean)	Rifampin	15, 42
P. syringae pv. syringae B728a *hrcC*::*nptII*	*P. syringae* pv. syringae B728a with a disrupted T3SS	Rifampin, kanamycin	25
P. syringae pv. tomato DC3000	*P. syringae* isolate from tomato	Rifampin	13
P. syringae pv. tomato JL1065	*P. syringae* isolate from tomato	Rifampin	63
P. syringae 508	*P. syringae* isolate from a fallen apple leaf in orchard	None	7
P. syringae TLP2	*P. syringae* isolate from healthy potato leaf	None	41
P. syringae cit7	*P. syringae* isolate from healthy orange leaf	None	40
P. syringae B2-1-1	*P. syringae* isolate from unidentified plant on Virginia Tech campus	None	This study
P. syringae B1-11-10	*P. syringae* isolate from unidentified plant on Virginia Tech campus	None	This study
P. syringae cc667	*P. syringae* isolate from "old stand" garden *Primula*	None	This study
P. syringae cc1502	*P. syringae* isolate from wild *Primula farinosa*	None	This study
P. syringae cc1503	*P. syringae* isolate from wild *Primula farinosa*	None	This study
Plasmids			
pME6010	*E. coli-Pseudomonas* shuttle vector	Tetracycline	24
pLAFR3	*E. coli-Pseudomonas* shuttle vector	Tetracycline	56
pHIR11	pLAFR3 vector containing the *hrp-hrc* cluster of *P. syringae* pv. syringae 61	Tetracylcine	27
pLN18	pHIR11 derivative in which *hopPsyA* has been replaced with the *nptII* gene	Tetracycline, kanamycin	30
pBAV208	*E. coli* vector with *oriT* based on pBC SK+	Kanamycin	This study
pVT138	Integration vector containing a fragment of the *P. syringae* 508 gene homologous to the *P. syringae* pv. syringae B728a gene with the locus tag Psyr_1182	Kanamycin	This study
pVT359	pVT138 containing the *avrRpt2* gene	Kanamycin	This study
pLH12	pLAFR3 expressing *avrRpt2*	Tetracycline	63

restriction enzymes ApaI and ClaI (New England Biolabs, Massachusetts), and ligated to pBAV208 digested with the same enzymes using TaKaRa Bio USA (Wisconsin) Mighty Mix. The resulting construct, pVT138, was transferred to the *P. syringae* 508 chromosome by triparental mating. *E. coli* pVT138, *P. syringae* 508, and the helper strain *E. coli* RK600 were streaked with a loop over each other on a KB plate without selection, and after 24 h of incubation at 28°C a loopful of bacteria was streaked on a KB plate containing 100 μg/ml of nitrofurantoin and 50 μg/ml of kanamycin. The plate was incubated for 48 h, after which single colonies became visible.

avrRpt2 was added to pVT138 to construct pVT359 using the same procedure that was used to add the *P. syringae* 508 Psyr_1182 orthologue fragment to pBAV208; the only difference was that the EcoRI and SpeI enzymes and primers avrRpt2-F and avrRpt2-R (see Table S1 in the supplemental material) were used for PCR with *P. syringae* pv. tomato JL1065 DNA as the template.

For plasmid rescue of the CEL-*hrp-hrc*-EEL region of *P. syringae* 508, genomic DNA was extracted from *P. syringae* 508(pVT138) by using a Puregene DNA purification system cell and tissue kit (Gentra Systems, Minnesota) and following the manufacturer's instructions. Ten microliters of extracted DNA was digested with restriction enzyme AscI (New England Biolabs, Massachusetts) in a 20-μl (total volume) mixture for 3 h. After the enzyme was heat inactivated at 70°C for 15 min, 5 μl of the digest was mixed with 5 μl of water and 10 μl of a ligation mixture (TaKaRa Bio USA Mighty Mix) to allow self-ligation to occur, incubated at 16°C for 30 min, and transformed into *E. coli* using the heat shock procedure (51). The plasmid was extracted from an *E. coli* colony resistant to kanamycin. The insert size of the plasmid was determined by restriction digestion and was found to be approximately 17,000 bp. This plasmid was subcloned using a TOPO shotgun subcloning kit (Invitrogen, California), and colonies were sent to the University of Chicago Cancer Research Center DNA Sequencing & Genotyping Facility for plasmid extraction and sequencing. Sequences were assembled using Seqman (Lasergene, DNASTAR, Wisconsin), and gaps were closed by designing primers on contig ends and sequencing PCR products obtained with various primer combinations. The sequence obtained was annotated using GRC (http://staff.vbi.vt.edu/jcslab/gat/gat.html).

The *P. syringae* 508 genomic regions corresponding to the *P. syringae* pv. syringae B728a loci containing the effector genes *avrPto1*, *hopAE1*, and *hopH1* were amplified with primers avrPto1-loop-F and avrPto1-loop-R, primers hopAE1-loop-F and hopAE1-loop-R, and primers hopH1-loop-F and hopH1-loop-R (see Table S1 in the supplemental material) by using TaKaRa LA *Taq* (Takara Bio USA, Wisconsin) and following the manufacturer's instructions. The PCR products obtained were sequenced as described above for the plasmid insert corresponding to the *P. syringae* 508 CEL-*hrp-hrc*-EEL region.

Dot blotting was performed as described previously (61) using genomic DNA of *P. syringae* 508 and *P. syringae* pv. syringae B728a as a probe. Southern blotting was done by using the DIG system (Roche, Indiana) and following the manufacturer's instructions after genomic DNA digested with restriction enzymes EcoRI and HindIII (New England Biolabs, Massachusetts) was electrophoresed on a 0.8% 1× Tris-borate-EDTA agarose gel.

Plant infection. Bacteria used for HR tests were grown for 24 h on KB plates, scraped off the plates with a spatula, and resuspended in 10 mM $MgSO_4$. After optical densities were determined, bacteria were diluted to obtain an optical density at 600 nm (OD_{600}) of 0.1 for HR tests with 4-week-old *Nicotiana tabacum*, *Nicotiana benthamiana*, and *Arabidopsis thaliana* 'Columbia' plants grown at room temperature on light racks under conditions that included 16 h of light and 8 h of darkness per day. Bacteria were infiltrated into leaves using a blunt-end 1-ml syringe. Pictures of infiltrated leaves were taken 20 h after infection using an Olympus Camedia C-765 digital camera.

For disease assays with *N. benthamiana*, bacteria were prepared as described above for HR tests except that the OD_{600} of bacteria infiltrated into leaves was 0.00001. For disease assays with tomato and *A. thaliana*, plants were sprayed with water 1 day before infection and the humidity was kept high by putting plants in plastic bags. For disease assays with tomato, bacteria were prepared as described above for HR assays, but whole plants of cultivar Sunpride that were 3 weeks old were dipped into bacterial suspensions having an OD_{600} of 0.01 that also contained 0.02% Silwet. For *A. thaliana* disease assays, bacterial suspensions having an OD_{600} of 0.1 that also contained 0.02% Silwet were sprayed onto leaves of ecotype Columbia using plants that were 3 weeks old. Plants were kept in plastic bags for 24 after infection. To measure bacterial populations in plants, bacteria were extracted from leaf disks, which were punched out with the lid of a microcentrifuge tube. Leaf disks were then ground in 200 μl of 10 mM $MgSO_4$, bacterial suspensions were dilution plated, and colonies were counted 2 days later.

Nucleotide sequence accession numbers. The nucleotide sequences of fragments of the *gap1*, *gltA*, *gyrB*, and *rpoD* genes have been deposited in the GenBank database under accession numbers EU257726, EU257732, EU257738, and EU257744, respectively.

RESULTS

***P. syringae* 508 does not cause disease on the model plants *A. thaliana*, *N. benthamiana*, and tomato.** Burr and colleagues (7) showed that *P. syringae* 508, which was found to strongly interfere with the germination of spores of the apple scab fungus *V. inaequalis*, does not cause disease on green bean, bush bean, apple, peach, cherry, and pea and does not elicit an HR on tobacco. We extended the pathogenicity assays to three widely used *P. syringae* hosts: *A. thaliana*, *S. lycopersicum* (tomato), and *N. benthamiana*. We infected these plants with *P. syringae* 508 and pathogens of the plants at a dose at which the known pathogens cause disease symptoms within 5 days. We also included *E. coli* DH5α and a T3SS-deficient mutant of the pathogenic strain *P. syringae* pv. syringae B728a in the *N. benthamiana* assay. Figure 1 shows disease symptoms and bacterial populations which revealed that *P. syringae* 508 was unable to cause disease or to reach population levels typical of pathogens on any of the plants tested. Moreover, on *N. benthamiana P. syringae* 508 grew to the same population density as the T3SS-deficient mutant of *P. syringae* pv. syringae B728a and only slightly better than *E. coli* DH5α. However, when the concentration of the *P. syringae* 508 inoculum was increased to an OD_{600} of 0.1 in the *N. benthamiana* assay, slight water soaking (wet-looking leaf area) was observed (Fig. 2A). The water soaking observed may have been due to the production of syringopeptin, which has known phytotoxic effects (5) and which was found to be produced by *P. syringae* 508 (19).

***P. syringae* 508 and other *P. syringae* isolates that do not cause an HR on tobacco are closely related to the sequenced pathogenic strain *P. syringae* pv. syringae B728a.** After we found that *P. syringae* 508 did not cause disease on any plant tested, we were interested in determining if *P. syringae* 508 is really a *P. syringae* strain, as Burr and coworkers (7) determined based on fatty acid fingerprinting and carbohydrate assimilation tests. We sequenced fragments of the *gap1*, *gltA*, *gyrB*, and *rpoD* genes used by Hwang and coworkers (28) in their multilocus sequence typing scheme for *P. syringae*. We found that *P. syringae* 508 is in fact a member of the species *P. syringae*. The *P. syringae* strain tested by Hwang and coworkers (28) that was most similar to *P. syringae* 508 was *P. syringae* TLP2, an isolate from a healthy potato leaf, as described in the Introduction (41). We then identified three additional *P. syringae* isolates obtained from wild *Primula* species in the French Alps, which did not cause an HR on *N. tabacum*. *P. syringae* isolates were also collected from healthy plants at the Virginia Tech Hahn Horticultural Garden, and two of these isolates did not induce an HR on *N. tabacum* (Fig. 2A). The three *Primula* isolates and the two isolates obtained from plants at the Virginia Tech Hahn Horticultural Garden were found to be between 98.1 and 99% identical to *P. syringae* 508 when the DNA fragments from the *gap1*, *gltA*, *gyrB*, and *rpoD* genes were examined. It should be noted that *P. syringae* cit7, the *P. syringae* isolate obtained from a healthy orange and originally considered incapable of causing disease (40), caused an HR on tobacco (Fig. 2A) and is not part of the group that we identified as nonpathogens. Figure 2B shows a maximum likelihood tree based on the concatenated set of the four sequenced gene fragments from the nonpathogenic isolates, from the three

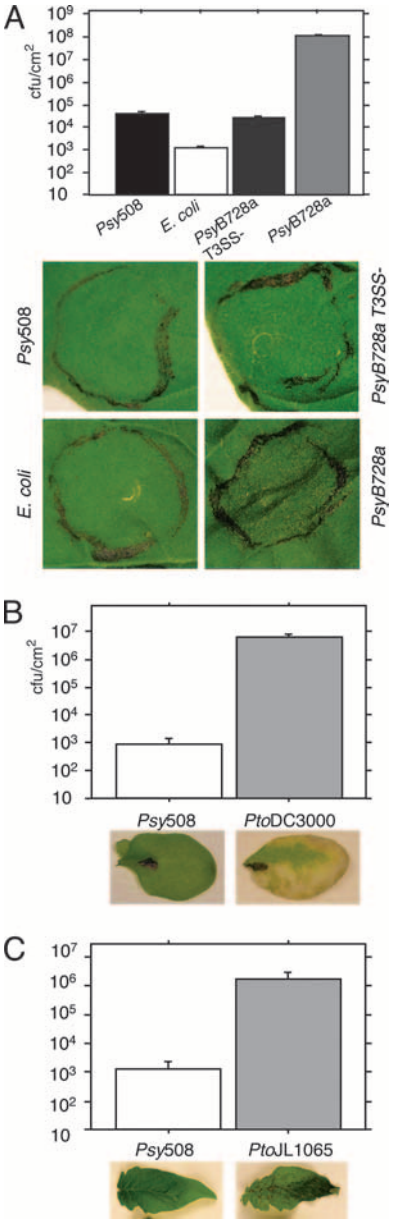

FIG. 1. *P. syringae* 508 neither causes disease nor grows to a high population density on *N. benthamiana*, *A. thaliana*, or *S. lycopersicum* (tomato). (A) *N. benthamiana* leaves were infiltrated with bacterial suspensions of *P. syringae* 508 (*Psy*508), *E. coli* DH5α, the bean and *N. benthamiana* pathogen *P. syringae* pv. syringae B728a (*Psy*B728a), and a T3SS-deficient mutant of *P. syringae* pv. syringae B728a (*Psy*B728a T3SS-) at an OD_{600} of 0.00001. (B) *A. thaliana* ecotype Columbia plants were spray inoculated with *P. syringae* 508 and the *A. thaliana* pathogen *P. syringae* pv. tomato DC3000 (*Pto*DC3000) at an OD_{600} of 0.01. (C) Tomato cultivar Sunpride plants were spray inoculated with *P. syringae* 508 and the tomato pathogen *P. syringae* pv. tomato JL1065 (*Pto*JL1065) at an OD_{600} of 0.01. Bacterial population sizes were measured either 2 or 3 days postinfection, and pictures were taken 1 week postinfection. Different types of bars indicate significantly different population sizes based on analysis of four leaf disks per strain ($P < 0.05$). Similar results were obtained in at least two independent experiments.

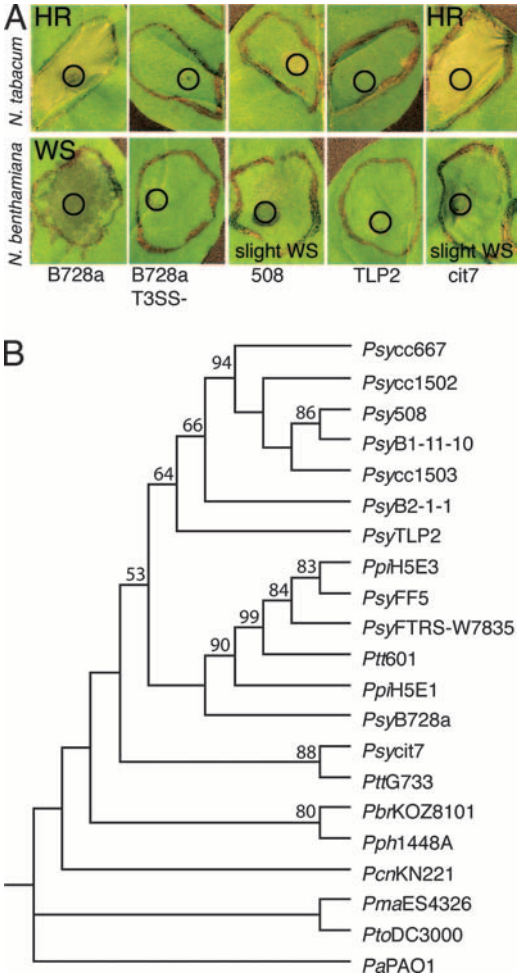

FIG. 2. *P. syringae* 508 and other closely related isolates cause neither an HR nor disease symptoms when high doses are infiltrated into leaves of *N. tabacum* and *N. benthamiana*. (A) Bacterial isolates were infiltrated into leaves of 4-week-old *N. benthamiana* and *N. tabacum* plants at an OD_{600} of 0.1, and pictures were taken 24 h later. The plant responses to *P. syringae* TLP2 are shown as an example of identical plant responses to *P. syringae* cc667, *P. syringae* cc1502, *P. syringae* cc1503, *P. syringae* B1-11-10, and *P. syringae* B2-1-1. Circles indicate where the syringe was pressed against the leaf during infiltration. WS, water soaking. (B) Maximum likelihood tree based on the concatenated sequence of four gene fragments (*gap1*, *gltA*, *gyrB*, and *rpoD*) showing that all isolates that do not cause an HR on *N. tabacum* are closely related to each other and cluster separately from the most closely related pathogenic *P. syringae* strains analyzed in previous studies (28, 53). *Psy*, *P. syringae* pv. syringae; *Ppi*, *P. syringae* pv. pisi; *Ptt*, *P. syringae* pv. aptata; *Pbr*, *P. syringae* pv. broussonetiae; *Pph*, *P. syringae* pv. phaseolicola; *Pcn*, *P. syringae* pv. coronafaciens; *Pma*, *P. syringae* pv. maculicola; *Pto*, *P. syringae* pv. tomato; Pa, *P. aeruginosa*.

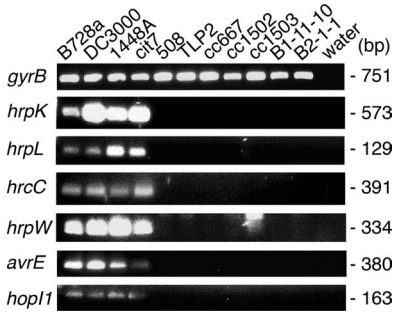

FIG. 3. Neither genes of the *hrp-hrc* T3SS cluster nor conserved effector genes are amplified from *P. syringae* 508 and other closely related isolates. Degenerate PCR primers were designed based on the three sequenced *P. syringae* isolates, *P. syringae* pv. syringae B728a, *P. syringae* pv. tomato DC3000, and *P. syringae* pv. phaseolicola 1448A. These primers and a primer pair for the housekeeping gene *gyrB* were used in PCRs performed with genomic DNA from the three sequenced *P. syringae* isolates, the HR-eliciting isolate *P. syringae* cit7, and the HR-negative isolate *P. syringae* 508 and other closely related isolates (Fig. 2A).

sequenced *P. syringae* strains, from *P. syringae* cit7, and from a selection of representative *P. syringae* isolates analyzed by Sarkar and Guttman (53) and by Hwang and colleagues (28). The pathogenic strains most similar to the nonpathogenic isolates are the completely sequenced bean pathogen *P. syringae* pv. syringae B728a and other closely related *P. syringae* pv. syringae, *P. syringae* pv. pisi, and *P. syringae* pv. aptata strains. The average DNA sequence identity between these pathogenic strains and the nonpathogenic strains is 96.6%. Interestingly, the nonpathogenic strains form a distinct cluster separated from all of the pathogenic strains in the maximum likelihood tree in Fig. 2B and in each of the four trees based on individual gene fragments (data not shown). However, the relative positions of the pathogenic and nonpathogenic strains in the *gyrB* tree are different from the relative positions in all other trees, confirming the effect of recombination on the *gyrB* locus (53). Recombination is probably also the cause of the low statistical support for the nonpathogenic cluster in the concatenated tree.

Nonpathogenic isolate *P. syringae* 508 contains neither an *hrp-hrc* cluster nor orthologues of effector genes of the closely related pathogenic strain *P. syringae* pv. syringae B728a. To understand why the nonpathogenic strains are unable to cause disease and to elicit an HR on the plant species tested, we designed a series of degenerate PCR primers based on the allele sequences in the three sequenced *P. syringae* strains of the *hrp-hrc* cluster genes *hrpK*, *hrpL*, and *hrcC*, the CEL effector genes *avrE* and *hrpW* known to be present in all pathogenic *P. syringae* strains, and the effector gene *hopI1*, which we previously found to also be present in all pathogenic *P. syringae* strains, including *P. syringae* cit7 (31). Since the three sequenced *P. syringae* strains on which the primers were based represent three of the five clades of the *P. syringae* tree constructed by Hwang and coworkers (28), these primers could be expected to anneal to orthologues in our nonpathogenic isolates closely related to *P. syringae* pv. syringae B728a. Figure 3 shows that the *gyrB* gene primers (one of the gene fragments used to construct the maximum likelihood tree in Fig. 2B) gave a PCR product for the three sequenced *P. syringae* strains, *P. syringae* cit7, and all nonpathogenic isolates. However, the *hrp-hrc* gene primers and all three effector gene primers gave products only for the three sequenced *P. syringae* strains and *P. syringae* cit7 and not for any of the nonpathogenic isolates. This suggests that the *hrp-hrc* cluster, the CEL locus, and *hopI1* are not present in any of the nonpathogenic strains except *P. syringae* cit7. To confirm that the *hrp-hrc* genes are not present in *P. syringae* 508 and *P. syringae* TLP2, we used fragments of the *hrp-hrc* cluster genes *hrpL*, *hrpS*, *hrpF*, and *hrcC* of *P. syringae*

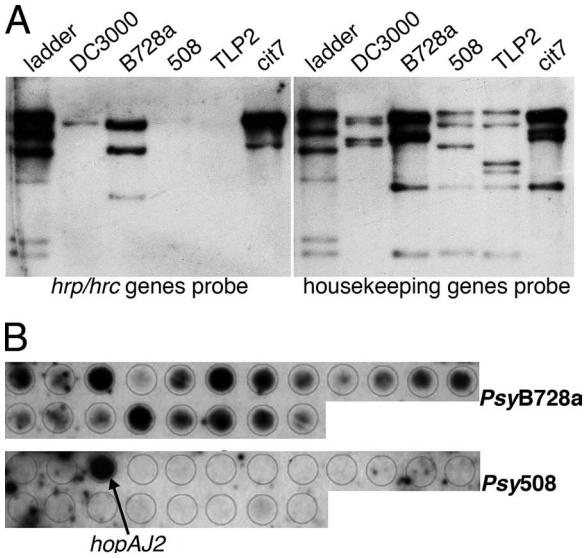

FIG. 4. Southern blot and dot blot experiments indicating that *P. syringae* 508 does not contain either an *hrp-hrc* gene cluster or any orthologues of effectors from the closely related pathogenic isolate *P. syringae* pv. syringae B728a. (A) Genomic DNA of *P. syringae* pv. syringae B728a, *P. syringae* pv. tomato DC300, *P. syringae* cit7, *P. syringae* 508, and *P. syringae* TLP2 was digested with HindIII and EcoRI and analyzed by Southern blotting using either genes of the *hrp-hrc* cluster or housekeeping genes as the probe. While *P. syringae* pv. syringae B728a, *P. syringae* pv. tomato DC300, and *P. syringae* cit7 DNA hybridized with both probes, *P. syringae* 508 and *P. syringae* TLP2 hybridized only with the housekeeping gene probe. (B) DNA of plasmids containing cloned effector genes of *P. syringae* pv. syringae B728a (*Psy*B728a) was blotted on a membrane and probed with genomic DNA of *P. syringae* pv. syringae B728a and *P. syringae* 508 (*Psy*508). While all effectors hybridized with the *P. syringae* pv. syringae B728a probe, only the *hopAJ2* gene hybridized with *P. syringae* 508. The *hopAJ2* gene of *P. syringae* pv. maculicola ES4326 was originally identified as having a type III secretion signal (21), but the full-length gene was later found not to code for a secreted protein (60).

pv. syringae B728a to probe a Southern blot containing *P. syringae* 508 and *P. syringae* TLP2 genomic DNA. In parallel, we used as a hybridization probe a mixture of the housekeeping genes *acnB*, *gap1*, *gltA*, *gyrB*, *rpoD*, and *pgi*. Figure 4A shows that *P. syringae* 508 and *P. syringae* TLP2 hybridized only with the housekeeping gene probes and not with the *hrp-hrc* gene probes, confirming that the *hrp-hrc* cluster is not present in these strains. We then used a DNA dot blot filter spotted with 20 *P. syringae* pv. syringae B728a effector genes (*avrPto1*, *avrRpm1*, *hopAJ2*, *hopH1*, *hopI1*, *hopAE1*, *hopAG1*, *hopAH1*, *avrB3*, *hopX1*, *hopZ3*, *hopAA1*, *hopH1*, *hopAF1*, *hopM1*, *hrpA1*, *hrpK1*, *hrpW1*, *hrpZ1*, and *hopAB1*) and probed it separately with *P. syringae* pv. syringae B728a and *P. syringae* 508 genomic DNA. Figure 4B shows that the *P. syringae* pv. syringae B728a probe hybridized with all effector genes, but the *P. syringae* 508 probe hybridized only with *P. syringae* pv. syringae B728a *hopAJ2*. Interestingly, the N-terminal region of the *hopAJ2* protein from amino acid 1 to amino acid 14 was originally found to be able to translocate the AvrRpt2$^{71-255}$ reporter to plant cells (21), but later the product of the full-length gene was found not to be secreted by the T3SS (60). Thus, the *hopAJ2* gene cannot be considered a T3SS effector. In a separate dot blot experiment DNA of a pathogenic *P. syringae* pv. syringae isolate from tomato also closely related to *P. syringae* pv. syringae B728a hybridized to 14 effector genes on the filter (data not shown). Moreover, we previously reported that genes with levels of DNA identity to genes in the probe as low as 85% gave a positive signal in our dot blot filters (61). Therefore, no orthologue of the *P. syringae* pv. syringae B728a effectors analyzed appears to be present in *P. syringae* 508 based on DNA-DNA hybridization.

***hrp-hrc* cluster, CEL, and EEL are largely missing from *P. syringae* 508.** The *hrp-hrc* region and the flanking CEL and EEL regions are located downstream of a gene which is annotated in *P. syringae* pv. tomato DC3000 as coding for a "conserved effector locus protein" with the locus tag PSPTO_1371 and is annotated in *P. syringae* pv. syringae B728a as coding for a "hypothetical protein" with the locus tag Psyr_1182, while the *P. syringae* pv. phaseolicola 1448A orthologue of this gene has a frameshift in the sequence encoding amino acid 27 and is annotated as a pseudogene with the locus tag PSPPH_1262 (Fig. 5). The effector gene *hopAA1-1* is located immediately

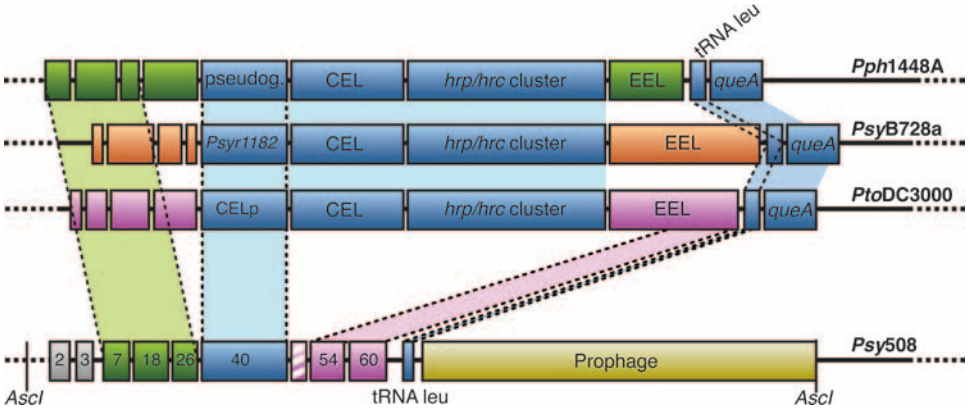

FIG. 5. CEL, the *hrp-hrc* cluster, and most of the EEL are missing from *P. syringae* 508. *P. syringae* pv. phaseolicola 1448A (*Pph*1448A) genes and their orthologues in *P. syringae* 508 (*Psy*508) are green, *P. syringae* pv. syringae B728a (*Psy*B728a) genes and their orthologues in *P. syringae* 508 are blue, and *P. syringae* pv. tomato DC3000 (*Pto*DC3000) genes and their orthologues in *P. syringae* 508 are purple. Genes conserved in all four isolates are blue, hypothetical genes in *P. syringae* 508 are white, and the prophage genome is indicated by a brown box. Individual *hrp-hrc*, CEL, EEL, and bacteriophage genes are not shown. The numbers indicate the gene identification numbers for *P. syringae* 508 genes (Table 2). AscI is the restriction enzyme that was used for plasmid rescue of this *P. syringae* 508 region. Genes are not drawn to scale.

downstream of this gene in the CEL of all three sequenced strains, followed by the other genes of the CEL, the *hrp-hrc* region, and finally the EEL. Immediately downstream of the EEL, the three sequenced *P. syringae* strains and all other *P. syringae* isolates analyzed have the $tRNA^{Leu}$ gene, followed by the *queA* gene (10, 14). Using primers based on the sequence encoding the hypothetical protein with the locus tag Psyr_1182 immediately upstream of the CEL in *P. syringae* pv. syringae B728a, we were able to amplify an orthologue from *P. syringae* 508, which shared 91% DNA identity over 804 bp with the *P. syringae* pv. syringae B728a allele. We cloned this PCR fragment into the vector pBAV208 without the origin of replication for *P. syringae* and integrated this plasmid into the *P. syringae* 508 chromosome. We then "rescued" this plasmid, including 4,039 bp upstream of the gene and 11,739 bp downstream of the gene, using restriction enzyme AscI. Sequencing and annotation of the rescued region revealed that the entire CEL locus and the *hrp-hrc* cluster are missing from *P. syringae* 508 (Fig. 5; see Table S2 in the supplemental material). One hundred sixty-five base pairs with similarity to the intergenic *P. syringae* pv. syringae B728a region between the Psyr_1182 locus and *hopAA1-1* is still present downstream of the Psyr_1182 orthologue. This short region is directly flanked by a DNA sequence with similarity to the EEL of *P. syringae* pv. tomato DC3000 and other *P. syringae* isolates and contains two EEL genes (T54 and T60 [see Table S2 in the supplemental material]) with no features of T3SS effectors. These two genes are followed by a 19-bp fragment of the $tRNA^{Leu}$ gene, which represents the end of the EEL in all pathogenic *P. syringae* strains analyzed (10, 14). Upstream of the two EEL genes is a 138-bp open reading frame (ORF) (T51 [see Table S2 in the supplemental material]) that in BLAST searches showed homology over its 20 most 3′ codons to the 20 most 3′ codons of the *avrPphE8* allele (new designation, *hopX1*) of *P. syringae* pv. phaseolicola BK378. However, this region of *P. syringae* pv. phaseolicola BK378 *avrPphE8* ($avrPphE8_{Pph\text{BK378}}$) has no homology with any of the other *avrPphE8* alleles. Thus, either these 20 codons are a result of a sequencing error and in reality not part of $avrPphE8_{Pph\text{BK378}}$ or $avrPphE8_{Pph\text{BK378}}$ has a very unusual C terminus. Moreover, the 5′ end of ORF T51 is located in the region with similarity to the noncoding region of *P. syringae* pv. syringae B728a downstream of the Psyr_1182 locus. Thus, we believe that ORF T51 should not be considered an effector or effector remnant. It is probably not even a coding sequence but is a pseudogene. This suggests that the CEL, the *hrp-hrc* cluster, and most of the EEL, including all effector genes, were deleted during the evolution of *P. syringae* 508. Just downstream of the $tRNA^{Leu}$ fragment a bacteriophage genome was found. This bacteriophage genome may have integrated into the genome at the $tRNA^{Leu}$ locus. Based on our data, it is impossible to say if this happened before, at the same time as, or after the loss of the *hrp-hrc* cluster, CEL, and EEL.

Genomic regions containing effector genes in *P. syringae* pv. syringae B728a are replaced or modified in *P. syringae* 508. After determining that the CEL, *hrp-hrc*, and EEL region was probably deleted during the evolution of *P. syringae* 508, we wanted to determine what happened to some of the effector genes that may have been present in the pathogenic ancestor of *P. syringae* 508. We aligned the *P. syringae* pv. syringae B728a genome with the *P. syringae* pv. tomato DC3000 genome using MUMmer (36) and determined the location of all *P. syringae* pv. syringae B728a effector genes in the alignment. We found that the effector genes *avrPto1* and *hopAE1* and the helper protein gene *hopH1* were located in *P. syringae* pv. syringae B728a-specific regions ("loops") that were short enough to be amplified with primers located on flanking core genome regions present in both *P. syringae* pv. syringae B728a and *P. syringae* pv. tomato DC3000 using long and accurate PCR. Using such primers with *P. syringae* pv. syringae B728a and *P. syringae* 508 as templates, we found that these loops were slightly shorter in *P. syringae* 508, suggesting that the gene contents of these loops were different in *P. syringae* 508 and *P. syringae* pv. syringae B728a (Fig. 6). We sequenced all three PCR products from *P. syringae* 508 and aligned the sequence obtained with the *P. syringae* pv. syringae B728a loops. The annotation of the *avrPto1* and *hopAE1* loops is shown in Table 2, while the sequence obtained for the *hopH1* loop was simply used in a BLASTX search against the NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi). The loop that contains the *hopAE1* gene in *P. syringae* pv. syringae B728a is nearly identical to the loop in *P. syringae* 508. The only difference is the replacement of the *hopAE1* gene and its promoter region with a 52-bp noncoding region with no homology to any sequence in the GenBank database. It is impossible to say if *hopAE1* was inserted into this region after *P. syringae* pv. syringae B728a and *P. syringae* 508 diverged or if *hopAE1* was present in their common ancestor and was deleted from an ancestor of *P. syringae* 508 after it diverged from the *P. syringae* pv. syringae B728a lineage. In the case of the *avrPto1* loop (Table 2) and the *hopH1* loop (data not shown), the differences between *P. syringae* pv. syringae B728a and *P. syringae* 508 are more substantial, and there are several gene differences. Major rearrangements may have taken place at the location of these loops since *P. syringae* 508 and *P. syringae* pv. syringae B728a diverged from their MRCA. However, the significant result is that the effector genes *avrPto1* and *hopH1* are missing from the *P. syringae* 508 loops and no other effector gene orthologue was found.

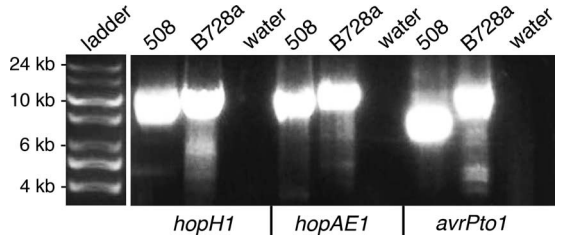

FIG. 6. Long-range PCR products obtained from *P. syringae* 508 and *P. syringae* pv. syringae B728a using primers that flank *P. syringae* pv. syringae B728a-specific regions ("loops"), which contain the effector genes *hopH1*, *hopAE1*, and *avrPto1*. Primers were designed for regions conserved in *P. syringae* pv. syringae B728a and *P. syringae* pv. tomato DC3000 located up- and downstream of effector-containing *P. syringae* pv. syringae B728a-specific regions and used in long-range PCR. Note that all three regions are slightly shorter in *P. syringae* 508 than in *P. syringae* pv. syringae B728a, indicating that there are gene differences in these regions between the two isolates.

TABLE 2. Annotation of the *P. syringae* 508 loops corresponding to the *avrPto1* and *hopAE1* loops in *P. syringae* pv. syringae B728a

Identification no.	Start position	Stop position	Length (nucleotides)	Strand	Description of best hit	Best hit accession no.	Best hit organism
avrPto1 loop (accession no. EU255788)							
T2	77	307	228	+	No hits		
T8	896	474	420	−	Hypothetical protein	YP_233221	*P. syringae* pv. syringae B728a
T13	1024	899	123	−	No hits		
T16	1084	1425	339	+	Hypothetical protein	NP_795111	*P. syringae* pv. tomato DC3000
T19	1525	2169	642	+	Phosphoglycerate mutase family protein	NP_795110	*P. syringae* pv. tomato DC3000
T29	2606	2262	342	−	Hypothetical protein	NP_795102	*P. syringae* pv. tomato DC3000
T32	2926	2603	321	−	Hypothetical protein	NP_795101	*P. syringae* pv. tomato DC3000
T37	2932	3048	114	+	No hits		
T39	3952	3224	726	−	Hypothetical protein	YP_238047	*P. syringae* pv. syringae B728a
T45	4993	3962	1,029	−	Hypothetical protein	YP_238046	*P. syringae* pv. syringae B728a
T52	5077	5400	321	+	No hits		
T57	5742	5527	213	−	No hits		
T59	5769	6104	333	+	Hypothetical protein	YP_583029	*Ralstonia metallidurans* CH34
T67	6262	6441	177	+	No hits		
hopAE1 loop (accession no. EU255789)							
T3	387	217	168	−	No hits		
T5	476	4312	3834	+	PAS:GGDEF	YP_237333	*P. syringae* pv. syringae B728a
T39	4451	5443	990	+	Regulatory protein LuxR	YP_237334	*P. syringae* pv. syringae B728a
T46	5542	6477	933	+	Hypothetical protein	YP_237335	*P. syringae* pv. syringae B728a
T55	6525	7976	1,449	+	Amidase	YP_237336	*P. syringae* pv. syringae B728a
Noncoding	7987	8039	52		—[a]		
T70	8199	8059	138	−	No hits		

[a] This region is the only region in the *P. syringae* 508 *hopAE1* loop without homology to *P. syringae* pv. syringae B728a. This region correspond to *hopAE1* and its promoter in *P. syringae* pv. syringae B728a.

***P. syringae* 508 can efficiently translocate AvrRpt2 into *N. benthamiana* and *A. thaliana* only when a T3SS is expressed ectopically from a plasmid.** *P. syringae* 508 and the other closely related nonpathogenic isolates were not able to cause disease or induce an HR, and components of the T3SS could not be amplified from their genomes. We thus wanted to test the ability of *P. syringae* 508 to elicit an HR on *N. benthamiana* when it was expressing the effector AvrRpt2. AvrRpt2 is a well-established reporter for type III secretion since it elicits a typical HR when it is recognized by intracellular plant resistance genes, and the N terminus of AvrRpt2 was repeatedly used by us in the past as a reporter to test for translocation of effector fusion proteins from *P. syringae* to *A. thaliana* (20, 21, 60). We knew that the sequenced pathogenic *P. syringae* pv. syringae B728a strain, which contains a functional T3SS and is thus able to translocate effectors into plant cells, induces characteristic HR spots on *N. benthamiana* when it is expressing AvrRpt2. A T3SS-deficient mutant of *P. syringae* pv. syringae B728a unable to translocate effectors into plant cells does not elicit any HR spots in *N. benthamiana* when it is expressing AvrRpt2 (Fig. 7A). The AvrRpt2 effector could thus be used as a reporter to test the ability of our nonpathogenic *P. syringae* isolates to translocate effectors into *N. benthamiana*. Figure 7A shows that *P. syringae* 508 did not cause significant HR spots on *N. benthamiana* when it was expressing AvrRpt2, confirming the inability of *P. syringae* 508 and its relatives to translocate effectors into host cells. However, a very small number of spots were consistently detected, while the T3SS-deficient mutant of *P. syringae* pv. syringae B728a did not elicit any HR spots when it was expressing AvrRpt2. We do not believe that these spots were an indication of T3SS-dependent translocation of AvrRpt2, but they may have been due to secretion through the flagellum (see Discussion). Also, after a similar translocation assay with *P. syringae* 508 and *A. thaliana*, no HR could be detected when *P. syringae* 508 expressing AvrRpt2 was infiltrated into leaves of *A. thaliana* ecotype Columbia (Fig. 7B). Note that *P. syringae* strain cit7, which we found to contain a T3SS based on HR tests with tobacco (Fig. 2) and based on PCR and Southern blot assays (Fig. 3 and 4), elicited a strong HR on *A. thaliana* when it was expressing AvrRpt2 (Fig. 7B).

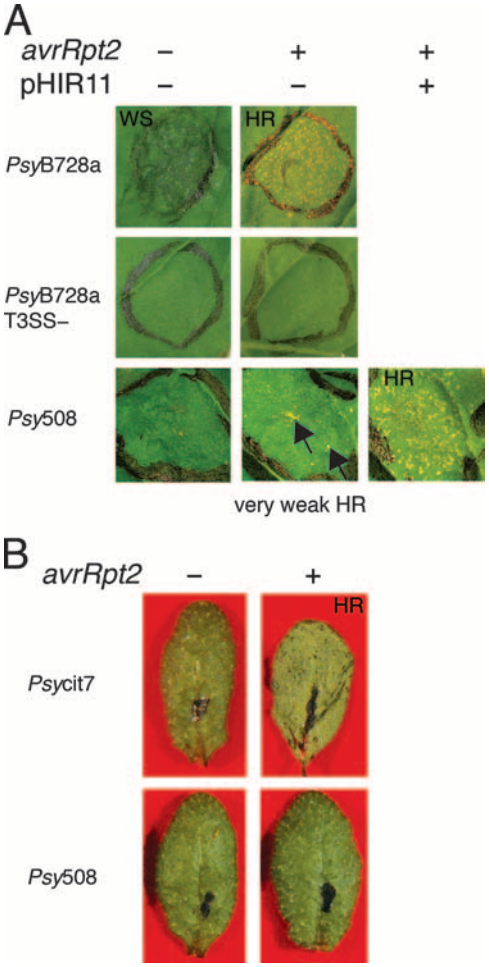

FIG. 7. *P. syringae* 508 does not efficiently translocate AvrRpt2 into cells of *N. benthamiana* and *A. thaliana*. (A) *P. syringae* pv. syringae B728a (*Psy*B728a) causes water soaking as a sign of disease when it is infiltrated into *N. benthamiana* leaves but causes HR spots when it is expressing AvrRpt2 (from plasmid pLH12). A T3SS-deficient mutant of *P. syringae* pv. syringae B728a (*Psy*B728a T3SS−) was unable either to cause disease or to induce HR spots when it was expressing AvrRpt2. *P. syringae* 508 (*Psy*508) was unable to cause disease and elicited a few individual HR spots (arrows) when it was expressing AvrRpt2 (from plasmid pVT359). Although the number of spots was low, spots were observed in 10 independent experiments. When plasmid pHIR11 encoding the T3SS of *P. syringae* pv. syringae 61 was added to the *P. syringae* 508 strain that expressed AvrRpt2, many HR spots were visible. Bacteria were infiltrated at an OD_{600} of 0.0001 since a higher dose of *P. syringae* pv. syringae B728a with and without expression of AvrRpt2 caused complete tissue collapse, making it impossible to distinguish between disease and HR. Leaves were photographed 48 h postinfection. WS, water soaking. (B) *P. syringae* cit7 (*Psy*cit7) does not cause disease in *A. thaliana* 'Columbia'. *P. syringae* cit7 expressing AvrRpt2 (from plasmid pLH12) caused an HR. *P. syringae* 508 was unable to cause disease or to elicit an HR when it was expressing AvrRpt2. Bacteria were infiltrated into *A. thaliana* leaves at an OD_{600} of 0.1. Leaves were photographed 24 h postinfection. All strains not expressing genes from plasmids contained empty vectors.

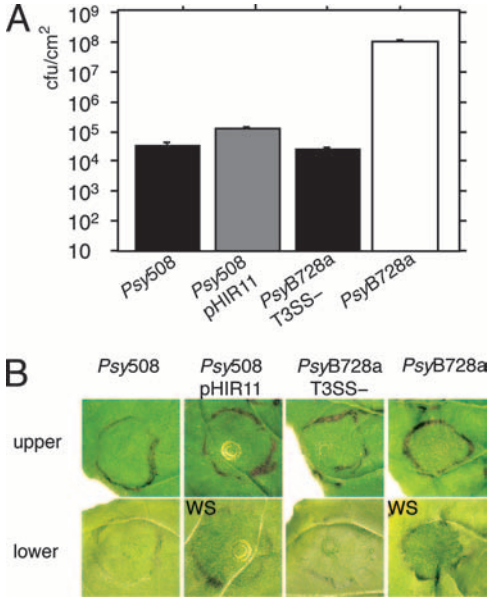

FIG. 8. Adding a T3SS and the effector gene $hopA1_{Psy61}$ to *P. syringae* 508 increases growth in planta and leads to water soaking. Plasmid pHIR11 contains the *hrp-hrc* cluster of *P. syringae* pv. syringae 61 and the effector gene $hopA1_{Psy61}$. Bacteria were infiltrated at an OD_{600} of 0.00001. (A) Addition of pHIR11 to *P. syringae* 508 (*Psy*508 pHIR11) increased bacterial population size in planta almost 10-fold by day 3 postinfection. This increase was statistically significant compared to the data for *P. syringae* 508 (*Psy*508) and for a T3SS-deficient mutant of *P. syringae* pv. syringae B728a (*Psy*B728a T3SS-). Different types of bars indicate significantly different population sizes based on analysis of four leaf disks per strain ($P < 0.05$). *Psy*B728a, *P. syringae* pv. syringae B728a. (B) Water soaking on the lower side of the leaf appeared after infection with *P. syringae* 508(pHIR11). The results for *P. syringae* pv. syringae B728a and a T3SS-deficient mutant of *P. syringae* pv. syringae B728a are shown for comparison. Pictures were taken 3 days postinfection. All strains not expressing genes from plasmids contained empty vectors. All infections were repeated at least three times, and similar results were obtained. WS, water soaking.

Huang and coworkers (27) cloned the *hrp-hrc* region of *P. syringae* pv. syringae 61, including the *P. syringae* pv. syringae 61 flanking effector gene *hopA1* ($hopA1_{Psy61}$) (originally designated *hrmA*), in a plasmid designated pHIR11. This plasmid and its derivative pLN18 (30), in which $hopA1_{Psy61}$ was replaced with a kanamycin resistance cassette, have since been used extensively for secretion assays and for HR assays when they were ectopically expressed in the nonpathogen *Pseudomonas fluorescens* 55, a *P. fluorescens* strain that naturally does not encode a T3SS (27). We wanted to determine if expressing pHIR11 in *P. syringae* 508 would allow translocation of AvrRpt2 into *N. benthamiana*. Figure 7A shows that it did. Very clear HR spots were induced by *P. syringae* 508 containing pHIR11 and expressing AvrRpt2. This demonstrates that expression of a functional T3SS in *P. syringae* 508 allows translocation of the AvrRpt2 effector into *N. benthamiana* cells. We could not perform this assay with *A. thaliana*, since most *A. thaliana* ecotypes are resistant to $HopA1_{Psy61}$ (17) and the pHIR11 derivative pLN18 does not translocate effectors into plants very efficiently (17; our unpublished data).

Ectopic expression of a T3SS and an effector in *P. syringae* 508 can reconstitute a low level of pathogenicity. Could *P. syringae* 508 and its relatives be engineered into pathogenic *P. syringae* strains by adding a T3SS and an assortment of effectors? We wondered if adding pHIR11 to *P. syringae* 508 could be a first step in engineering *P. syringae* 508 into a pathogen, i.e., if adding pHIR11 could in fact increase the growth of *P.*

syringae 508 on *N. benthamiana* because of a possible virulence effect of the $HopA_{Psy61}$ effector. We infected *N. bethamiana* with *P. syringae* 508 and *P. syringae* 508(pHIR11) using a very low dose (OD_{600}, 0.00001) and using as controls *P. syringae* pv. syringae B728a (which caused disease when it was infiltrated at an OD_{600} of 0.00001) and a T3SS-deficient mutant of *P. syringae* pv. syringae B728a (which did not cause any disease). We observed a nearly 10-fold increase in the size of the *P. syringae* 508(pHIR11) bacterial population 3 days after infection compared to the results after infection with *P. syringae* 508 without pHIR11 (Fig. 8A) and mild water soaking (Fig. 8B), which we never observed when we infiltrated *P. syringae* 508 without T3SS at this low dose. To determine if the observed increase in bacterial population size and in water soaking was the result of a virulence effect of the translocated $HopA1_{Psy61}$ effector, the result of the combined effects of the expressed T3SS and the translocated $HopA1_{Psy61}$ effector, or the result of the expression of the T3SS, we expressed the pHIR11 derivative pLN18 in *P. syringae* 508, which does not contain $hopA1_{Psy61}$, and compared the growth of the resulting strain with the growth of *P. syringae* 508(pHIR11). The growth of this strain was indistinguishable from that of *P. syringae* 508 (data not shown). However, as mentioned above, pLN18 does not translocate effectors very efficiently (17; our unpublished data). Therefore, we cannot exclude the possibility that the observed increase in virulence of *P. syringae* 508(pHIR11) compared to *P. syringae* 508 and *P. syringae* 508(pLN18) may have been due in part to an effect of the expression of the T3SS of *P. syringae* pv. syringae 61 from pHIR11 and not exclusively because of a virulence effect of the translocated $HopA1_{Psy61}$ effector. However, regardless of whether the increase in virulence of *P. syringae* 508(pHIR11) was caused by translocation of $HopA1_{Psy61}$ alone or was due to a combined effect of the expression of the *P. syringae* pv. syringae 61 T3SS and $HopA1_{Psy61}$, this result clearly shows that *P. syringae* 508 can be engineered toward virulence using appropriate virulence genes.

DISCUSSION

Although it has been extensively reported previously that some *P. syringae* strains can live epiphytically without causing disease on plants for extended periods of time, it has always been assumed that all *P. syringae* strains eventually enter plant tissue and cause disease when they find themselves on a susceptible plant under favorable environmental conditions (26). Here we show that within the species *P. syringae*, there is a group of isolates that do not contain an *hrp-hrc* cluster coding for a T3SS. Since type III secretion is an essential virulence mechanism in *P. syringae* (32) and since the identified $T3SS^-$ isolates are unable to translocate effectors into plant cells, to elicit an HR, and to cause disease on any plant species tested, it appears that these *P. syringae* strains have an entirely nonpathogenic lifestyle. One interesting question is whether syringopeptin plays a role in this nonpathogenic interaction with plants. Syringopeptin is a toxin produced by several pathogenic *P. syringae* isolates and by *P. syringae* 508 (19). It has been found to have antimicrobial activity and phytotoxic activity because it forms pores in cell membranes (5). Therefore, production of syringopeptin may simply allow *P. syringae* 508 to better compete with other microbes in the leaf environment, or its phytotoxic activity may increase nutrient and water release from plant cells. In fact, *P. syringae* 508 causes water soaking when a high dose is infiltrated into *N. benthamiana*. However, our data clearly indicate that this phytotoxic activity is not enough to cause disease since *P. syringae* 508 growth is indistinguishable from the growth of a T3SS-deficient mutant of *P. syringae* pv. syringae B728a and only slightly greater than the growth of *E. coli* on *N. benthamiana*. Moreover, no other identified $T3SS^-$ isolate causes any water soaking on *N. benthamiana* even at a high dose. The nonpathogenic lifestyle of these *P. syringae* isolates might also involve colonization of other niches, such as epilithic biofilms and other aquatic environments (44, 45).

The first T3SS is believed to have evolved in an ancestor of the genus *Chlamydia* (59). It is believed that the genes coding for the T3SS were then acquired through horizontal gene transfer by other gram-negative pathogens and symbionts. Congruence between phylogenetic trees of *P. syringae* isolates constructed using the core genome genes *gyrB* and *rpoD* and the tree constructed using the *hrpL* and *hrpS* genes of the *hrp-hrc* cluster (54) and the conserved chromosomal location of the *hrp-hrc* cluster indicate that all pathogenic *P. syringae* isolates that exist today were derived from one ancestor and that the *hrp-hrc* clusters present in pathogenic strains today were inherited vertically from that ancestor. Are the isolates that lack a T3SS descendants of an ancient *P. syringae* isolate that never acquired a T3SS, or did a T3SS-containing *P. syringae* isolate at some point lose its *hrp-hrc* cluster and give rise to today's isolates lacking a T3SS? The presence of remnants of the EEL in *P. syringae* 508 and the fact that the isolates lacking a T3SS cluster tightly together in one group with much lower diversity than members of the species *P. syringae* overall suggest that the MRCA of the $T3SS^-$ isolates lived much later than the MRCA of all *P. syringae* isolates. Therefore, loss of the T3SS in one pathogenic T3SS-containing *P. syringae* strain appears to have been the initial event in the evolution of the $T3SS^-$ isolates described here. However, more nonpathogenic isolates must be analyzed to confirm this hypothesis. In particular, it is necessary to sequence the genomic region corresponding to the CEL, the *hrp-hrc* cluster, and the EEL in additional nonpathogenic isolates to determine if all $T3SS^-$ isolates were derived from the same deletion event.

Loss of T3SSs has been reported in other species and even in one sequenced *P. syringae* strain. *P. syringae* pv. phaseolicola 1448A contains a cluster of genes coding for T3SS components with the closest homologues in *Rhizobium*, *Photorhabdus*, *Aeromonas* spp., and *Pseudomonas aeruginosa* (33). Some essential T3SS components are missing in this cluster, and thus no functional T3SS is encoded. Whether the T3SS was used by an ancestor of *P. syringae* pv. phaseolicola 1448A for plant infection or possibly to infect nonplant hosts or whether it was used in a symbiotic interaction is not known. Another example is *E. coli*. Even the laboratory strain K-12 and the majority of all other pathogenic and nonpathogenic *E. coli* isolates contain the ETT2 gene cluster coding for a nonfunctional T3SS that contains genes with homology to the *Salmonella* Spi-1, Spi-2, and Spi-3 PAIs (49). Surprisingly, although degenerate, ETT2 has been shown to have a role in virulence in some *E. coli* isolates (29).

Why do bacteria lose T3SSs, and in particular, why did the *P. syringae* T3SS$^-$ isolates lose the T3SS? Even pathogenic *P. syringae* strains spend part of their life cycle growing epiphytically without causing disease. Therefore, loss of the T3SS may have allowed *P. syringae* isolates to simply expand that part of their life cycle. It is well known that the effectors secreted by the T3SS into plants are "double agents" (4) and can, depending on the plant that is infected, be either virulence factors that subvert the plant immune system or avirulence factors that betray the presence of the bacterium to the plant immune system, eliciting ETI (11). Because of the evolution of a wide array of plant resistance genes, each *P. syringae* isolate can cause disease on only a limited number of host plants, while on a majority of plants T3SS-translocated effectors trigger ETI. Therefore, loss of the T3SS may have been advantageous in the ancestor of today's T3SS$^-$ isolates; although losing the T3SS abolished the ability to cause disease on some plants, it may have allowed this strain to grow better on all the plants on which any of its effectors elicited ETI. Of course, T3SS$^-$ isolates can be assumed to still elicit microbe-associated molecular pattern-triggered immunity (11), like all other pathogenic and nonpathogenic bacteria. This assumption is in agreement with our observation that *P. syringae* 508 growth on *N. benthamiana* in controlled infections is indistinguishable from the growth of a *P. syringae* pv. syringae B728a T3SS-deficient mutant and only slightly greater than the growth of *E. coli*. Therefore, the isolates lacking a T3SS may have adapted to an ecological niche in which moderate growth in the presence of PTI on many different plants may be more advantageous (or at least comparably advantageous) than extensive growth on a small number of susceptible plants.

How did nonpathogenic *P. syringae* isolates evolve after the T3SS was lost? Based on DNA-DNA hybridization and PCR, *P. syringae* 508 does not contain any orthologues of effectors from the closely related strain *P. syringae* pv. syringae B728a (Fig. 4) and from *P. syringae* pv. tomato DC3000 (data not shown). However, we cannot exclude the possibility that it contains some effectors with low homology to the effectors that we analyzed or to members of undiscovered effector families. Sequencing of the *P. syringae* 508 genome is necessary to definitely determine if there are any effector genes present in its genome. In the meantime, the absence of effector genes from the CEL and the EEL and from the loci that in *P. syringae* pv. syringae B728a contain *hopAE*, *hopH1*, and *avrPto1* strongly suggests that *P. syringae* 508 lost most of its effectors after it lost its ability to secrete them. This is not at all surprising since it would be disadvantageous to maintain effector genes if effectors cannot be secreted. However, it is also possible that the pathogenic ancestor of today's nonpathogenic isolates had only a very small number of effectors since it may have existed during a period in the evolution of *P. syringae* when the number of effector genes was still very limited.

All our molecular data point to the absence of a T3SS in *P. syringae* 508, but why then does expression of AvrRpt2 cause a small number of HR spots on *N. benthamiana*? We believe that the most likely explanation is very inefficient translocation of AvrRpt2 through the flagellum. The flagellum contains its own T3SS that secretes flagellin and other distal components during construction of the flagellum (34). Expression of the T3SS, in particular the sigma factor HrpL, has been found to down-regulate the flagellar system in *Erwinia amylovora* (8), and negative cross-control between these two systems has also been observed in *P. aeruginosa* (55). Considering that in a laboratory-constructed T3SS-deficient *P. syringae* mutant only one of the T3SS components is missing and HrpL can be assumed to still be expressed, the flagellar T3SS is probably down-regulated during infection in these T3SS mutants (like the T3SS-deficient *P. syringae* pv. syringae B728a mutant that we used [Fig. 7A]). Therefore, translocation of AvrRpt2 through the flagellum may be blocked in these mutants because the flagellum is not expressed. In the case of *P. syringae* 508, however, the entire *hrp-hrc* cluster, including *hrpL*, is missing. Therefore, in *P. syringae* 508 the flagellum T3SS may be highly expressed during infection and be capable of weakly translocating AvrRpt2. In fact, it has been shown that the flagellum can secrete proteins besides flagellum components (34). Another possible explanation is that *P. syringae* 508 contains a T3SS with no homology to the typical *P. syringae* T3SS. This T3SS may be able to inefficiently translocate AvrRpt2. Only genome sequencing of *P. syringae* 508 will be able to definitely tell us which of the two hypotheses is correct as mutation of the flagellum in *P. syringae* 508 may cause the disappearance of the HR spots because of pleiotropic effects.

Expression of a plasmid-borne T3SS and of the effector HopA1$_{Psy61}$ increased growth of *P. syringae* 508 in *N. benthamiana* almost 10-fold and allowed *P. syringae* 508 to cause water soaking. This suggests that *P. syringae* 508 or other nonpathogenic *P. syringae* isolates supplemented with a T3SS could be used as a tool to identify the functions of individual effectors in the context of infection. This is very important since the functions of individual effectors are often masked by other effectors with partially redundant functions when mutations in individual effectors are made in pathogenic isolates (39). Making a multiple-effector deletion mutant is one way to solve this problem, and making a multiple-effector mutant of *P. syringae* pv. tomato DC3000 has already revealed interesting virulence effects (37, 62), but it will be challenging to delete all effectors in a single isolate.

Could *P. syringae* 508 and its relatives be engineered back into pathogenic strains? The fact that growth of *P. syringae* 508 is indistinguishable from growth of a T3SS mutant of *P. syringae* pv. syringae B728a indicates that a T3SS and the right assortment of effectors could allow *P. syringae* 508 to cause disease on *N. benthamiana* and other plants. We also coinfected *N. benthamiana* with *P. syringae* pv. syringae B728a and *P. syringae* 508 and observed no reduction in the growth of *P. syringae* pv. syringae B728a but a nearly 100-fold increase in the growth of *P. syringae* 508 (data not shown). This suggests that *P. syringae* 508 does not elicit any plant defenses that cannot be overcome by the right assortment of *P. syringae* pv. syringae B728a effectors and therefore that *P. syringae* 508 can potentially be engineered into an *N. benthamiana* pathogen or a pathogen of other plants. Hence, we believe that *P. syringae* 508 may be an excellent tool to identify the minimum repertoire of T3SS effectors that cause disease in different plant species and to determine the underlying virulence mechanisms. This should ultimately be very helpful in unraveling the molecular basis of host range determination in *P. syringae*.

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