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Short title: Diversity of *hrp/hrc* cluster-deficient *P. syringae*.

Pseudomonas syringae naturally lacking the canonical type III secretion system are ubiquitous in non agricultural habitats, are phylogenetically diverse and can be pathogenic.

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Abstract

The type III secretion system (T3SS) is an important virulence factor of pathogenic bacteria, but the natural occurrence of variants of bacterial plant pathogens with deficiencies in their T3SS raises questions about the significance of the T3SS for fitness. Previous work on T3SS-deficient plant pathogenic bacteria has focused on strains from plants or plant debris. Here we have characterized T3SS deficient strains of *Pseudomonas syringae* from plant and non-plant substrates in pristine non agricultural contexts, many of which represent recently described clades not yet found associated with crop plants. Strains incapable of inducing a hypersensitive reaction in tobacco (HR^-) were detected in 65% of 126 samples from headwaters of rivers (mountain creeks and lakes), snowpack, epilithic biofilms, wild plants and leaf litter and constituted 2% to 100% of the *P. syringae* population associated with each sample. All HR^- strains lacked at least one gene in the canonical *hrp/hrc* locus or the associated conserved effector locus but most lacked all 6 of the genes tested (*hrcC, hrpL, hrpK1*, *avrE1,* and *hrpW1*) and represented several disparate phylogenetic clades. Although most HR^- strains were incapable of causing symptoms on cantaloupe seedlings as expected, strains in the recently described TA-002 clade caused severe symptoms in spite of the absence of any of the 6 conserved genes of the canonical T3SS according to PCR and Southern blot assays. The phylogenetic context of the T3SS variants we observed provides insight into the evolutionary history of *P. syringae* as a pathogen and as an environmental saprophyte.

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Introduction

The type III secretion system (T3SS) is believed to confer a major fitness advantage to a wide range of Gram-negative bacteria that are pathogens of plants or animals. By delivering effectors that suppress the defense mechanisms of hosts (Block and Alfano, 2011; Cornelis and Van Gijsegem, 2000; Hueck, 1998) it contributes to creating more favorable conditions for bacterial multiplication in the host. The functions and evolutionary origins of this secretion system have been intensively explored because of its critical role in pathogenicity (Troisfontaines and Cornelis, 2005). Three complementary analytical approaches are needed to attain a full understanding of the significance of the T3SS for bacterial fitness, pathogenicity and evolution. These approaches are: mutational analyses of model strains and population genetics and comparative genomics of strains representative of pathogen diversity. For plant pathogenic bacteria, the latter two have been hindered by the research focus on highly virulent crop pathogens and a concomitant neglect of the pathogen diversity present on wild plants and in the environment in general. This neglect of pathogen diversity is partly due to the narrow species descriptions of plant pathogenic bacteria, which include the ability to induce a hypersensitive response (HR) on tobacco (see for example the LOPAT test used to identify *Pseudomonas syringae*, (Lelliot *et al.*, 1966)). This narrow species description automatically excludes plant pathogen species members that might be missing a T3SS since induction of an HR is an indicator of a functional T3SS (He, 1996). Strains deficient in their capacity to induce HR (HR^-) have recently been described for *Erwinia pyrifoliae* (Jock *et al.*, 2003), *Pectobacterium* sp. (Kim *et al.*, 2009), and *P. syringae* (Clarke *et al.*, 2010; Kniskern *et al.*, 2011; Mohr *et al.*, 2008). All strains examined in these studies were isolated from plants (wild or cultivated) or from leaf litter in cultivated fields. The HR^- strains of *Pectobacterium* represent two species, *P. carotovorum* and *P. wasabiae*. But the *P. syringae* strains are remarkably homogenous. All HR^- strains of *P. syringae* characterized to date represent a single phylogenetic group and consistently lack the canonical *hrp/hrc* cluster (Clarke *et al.*, 2010; Kniskern *et al.*, 2011; Mohr *et al.*, 2008). Furthermore, these strains harbor a novel *hrp/hrc* cluster with limited effectiveness in expressing and/or delivering effectors to plant cells. HR^- strains of *E. pyrifoliae* and *P. syringae* were reported to be incapable of causing disease on the hosts tested, whereas the HR^- strains of *Pectobacterium* spp. maintained pathogenicity on the original host from which they were isolated. The considerable role of pectolytic enzymes in the pathogenicity of *Pectobacterium* spp. might explain why there was no loss in pathogenicity for this group of bacteria.

Recent work on the ecology of *P. syringae* in non agricultural habitats revealed the existence of nine new clades of this bacterium that had not been previously observed (Morris *et al.*, 2010). All phylogenetic descriptions of this species prior to the study of non agricultural habitats focused on strains from plants. The extensive phylogenetic diversity observed in non agricultural habitats could also be a source of genetic diversity of the T3SS and of pathotypes. Strains in these clades are clearly apt at surviving in a wide range of habitats, such as snowpack, water and epilithic biofilms. The key traits for adaptation to these specific habitats, and for enabling many strains of *P. syringae* to be fit in multiple habitats including plant tissues, have not been identified. In particular, the role that the T3SS plays in this adaptation has not been explored. Hence, we have explored the frequency of occurrence and the genetic diversity of strains of *P. syringae*, from non agricultural habitats, that are HR^- in tobacco. This work reveals that HR^- strains are frequent in non agricultural habitats and have diverse phylogenetic origins. Furthermore – and surprisingly - their incapacity to induce hypersensitivity is not necessarily indicative of their incapacity to cause disease on plants. This work provides a unique comparative foundation for identifying and studying the genetic factors that underlie evolutionary adaptation and ecological specialization of *P. syringae*.

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Materials and Methods

Origin of strains. A total of 63 strains of *P. syringae* were deployed in this study, including 40 strains unable to incite a hypersensitive reaction (HR^-) and 23 strains capable of inciting a hypersensitive reaction (HR^+) obtained from 21 samples of water, snowpack, epilithic biofilms and wild plants as described in the Supplementary Table. Six reference strains were also included. Samples were part of a survey for hypersensitive incompetent strains that included a range of environmental substrates collected from 2004 to 2010 mostly in alpine regions and outside of agricultural fields (Table 1) and processed as described previously (Morris *et al.*, 2007; Morris *et al.*, 2010; Morris *et al.*, 2008) to determine the density of *P. syringae* populations. Phenotypic traits that confirm identity as *P. syringae* were determined as described previously (absence of arginine dihydrolase and cytochrome oxidase). The 40 strains of *P. syringae* unable to incite a hypersensitive reaction used for this study were selected from that survey to represent the range of sample sites, substrates and phylogenetic diversity. Reference strains included the HR^- strains Psy508 and Psy642 (Clarke *et al.*, 2010; Mohr *et al.*, 2008) and the HR^+ strains B728A (from the stock culture of S. Hirano, University of Wisconsin, Madison), DC3000 and 1448A from J. Greenberg (The University of Chicago), and Cit7 from S.E. Lindow (University of California, Berkeley). All strains were stored at -80° C in 20% glycerol and all phenotypic and molecular tests were conducted on cultures prepared from a single transfer of the storage cultures.

Hypersensitivity, pathogenicity and growth dynamics of bacteria in plants. For hypersensitivity tests, aqueous bacterial suspensions at 10^8 colony-forming units (cfu) mL^{-1} from 48 h cultures on King's medium B (King *et al.*, 1954) were infiltrated with a needleless syringe into the leaves of tobacco (*Nicotiana tabacum* L. cv. Xanthi). Hypersensitivity was scored as negative if necrotic patches did not develop in the infiltrated zone within 48 h after infiltration. Assays were repeated three times and included an HR^+ strain as a control.

The capacity of strains to induce disease symptoms on cantaloupe seedlings was determined for strains regardless of their capacity to induce hypersensitivity on tobacco. Aggressiveness on cantaloupe plants (*Cucumis melo* var. *cantalupensis* Naud. cv. Védrantais) was quantified by injecting 50 µL of bacterial suspensions (prepared as for hypersensitivity tests) into the junction of the cotyledons of 12 plants at the cotyledon stage (ca. 10 days after sowing) as described previously (Morris *et al.*, 2010). Plants were incubated for seven days at 25°C during the daylight period (16 h) and 18°C at night (8 h) and symptoms were observed daily after inoculation. Final disease severity was noted at 7 days after inoculation on a scale of 0 to 4: 0 (no symptoms), 1 (1 cotyledon wilted or discolored), 2 (both cotyledons wilted or discolored), 3 (both cotyledons completely wilted and the upper stem of the plant collapsed) and 4 (the plant was collapsed at the base and the entire plant had fallen on the soil surface).

To determine population dynamics of strains in cantaloupe plants at the cotyledon stage, plants were inoculated and incubated in the same way as for pathogenicity tests. At each sampling time for up to 7 days after inoculation, 3-6 plants were randomly sampled from the 12 plants inoculated with each strain, weighed, and individually macerated in 10 ml of 0.1 M phosphate buffer. Aliquots of the macerate were diluted and plated on KBC medium (Mohan and Schaad, 1987), the same medium used to isolate these strains from the environment. Colonies were counted after 4 days of incubation at 22-25°C in the dark. All colonies were fluorescent under UV light as expected and there were no indications of contamination of the plants with other bacteria.

PCR detection of genes in the *hrp/hrc* cluster. PCR detection was conducted on DNA purified from 48 h cultures on KB medium from strains stored at -80° C as described above. Bacterial suspensions (10^{12} cfu mL^{-1}) prepared in sterile distilled water were used for DNA extraction with a Puregene DNA purification system cell and tissue kit (Gentra Systems, Minnesota) following the manufacturer's instructions.

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For detection of *hrp-hrc* cluster genes (*hrpK1, hrpL, hrcC*), CEL effectors (*avrE1, hrpW1*) and *hopI1*, primers described by Mohr et al. (Mohr *et al.*, 2008) were used for amplification, while for phage detection we designed forward primer PhF (5'- TCAGGCCGCTTTCAACTTGGC-3') and reverse primer PhR (5'- CCATAYGGCGCAGTGGTGG- 3') based on the sequence of a putative transcriptional regulator gene (locus tag ABX64468) located within the prophage at the *hrp/hrc* locus of Psy508 (Mohr *et al.*, 2008). To detect the presence of the *hrcC* orthologue of Psy642, colony PCR was performed as previously described. (Clarke *et al.*, 2010). Amplification conditions for detection of these eight genes are described in detail in the supplemental information section.

Southern blotting. To validate the results of PCR reactions, Southern blot analyses were performed for a set of strains representing the different profiles of absence and presence of the 6 conserved genes as described previously (Mohr *et al.*, 2008; Vinatzer *et al.*, 2006). Genomic DNA of *P. syringae* pv. *syringae* B728a, *P. syringae* 508, and hypersensitive incompetent *P. syringae* UB15, UB185, UB186, UB190, UB192, UB193, UB195, UB246, UB313, SZ30, SZ37, SZ45, SZ124, SZ131, TA043 was digested with *Hin*dIII and *Eco*RI (New England Biolabs, Germany). Digested DNA was electrophoresed on a 1% Tris-borate-EDTA agarose gel. Southern blotting, hybridization, and detection was performed following the manufacturer's instructions (Roche Diagnostics) using a mixture of digoxigenin (DIG)-labelled PCR products of the 6 genes amplified from strain B728a and the DIG-labeled PCR product of the amplification of a housekeeping gene (*gapA*) from strain B728a as probes.

Gene sequencing and phylogenetic analysis. From previous studies (Clarke *et al.*, 2010; Mohr *et al.*, 2008; Morris *et al.*, 2010), the clade identity was known for 19 strains used here relative to the phylogenetic tree reported by Morris et al (Morris *et al.*, 2010), and based on 4 housekeeping genes (*rpoD*, *gyrB*, *cts* and *gapA*). For the remaining strains, assignment to clades was based on our previous results that strains could be accurately assigned to clades based on DNA similarity in the *cts* sequence using a threshold dissimilarity of 1.8% (Morris *et al.*, 2010). The sequence of the *cts* gene was determined as previously described (Morris *et al.*, 2010). Based on the aligned and truncated (ca. 400 bp) *cts* sequences (DAMBE version 5.1.1 (Xia and Xie, 2001)), the genetic distance (dissimilarity) of each strain from each of the other 121 strains of *P. syringae* that constitute our previously reported phylogenetic tree (Morris *et al.*, 2010) was determined using MEGA, version 3.1 (Kumar *et al.*, 1994). There were no cases in which strains had ambiguous similarities (i.e., there were no cases in which the similarity criterion was met for more than one clade).

If the similarity criterion was not met, the other three housekeeping genes (*rpoD*, *gyrB* and *gapA*) were sequenced. Details of PCR amplifications are described in the supplemental information section. A phylogenetic tree was constructed with the concatenated 4 housekeeping gene sequences from these strains and those from the ensemble of *P. syringae*, *P. viridiflava* and *P. fluorescens* used in a previous work (Morris *et al.*, 2010). All strains fell into previously described clades.

Sequences of the PCR-amplified bacteriophage gene fragment were aligned with DAMBE version 5.1.1 and truncated to 302 bp.

Results

HR$^-$ strains are abundant in non-agricultural habitats and can dominate the *P. syringae* population in some cases. HR$^-$ strains were detected in 65% of the 126 environmental samples analyzed (Table 1) and were found in headwaters of rivers, snowpack, epilithic biofilms, wild plants and leaf litter. HR$^-$ strains were also found in some samples of freshly fallen rain but not in any of the samples of fresh snow fall. When detected, the HR$^-$ fraction of the total *P. syringae* population varied greatly, from 2% to 100%, and HR$^-$ strains attained population densities of up to 10^5 cfu L^{-1} of river water or 10^9 cfu g^{-1} of plant tissue, for example. In 20% of the samples, HR$^-$ strains constituted 50% or more of the total *P. syringae* population (Fig. 1). The frequency of HR$^-$ strains

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in the total *P. syringae* population was not related to the *P. syringae* population density (Fig. 1). For the samples analyzed here, *P. syringae* population density did not exceed 10^7 cfu g^{-1} or cfu L^{-1} except in one case. Strains from some of these samples were further characterized for the presence of components of the T3SS and for their pathogenicity to cantaloupe seedlings (Table 2).

HR$^-$ strains lack at least one conserved effector gene. The conserved type III effector gene *hopI1* (Jelenska *et al.*, 2007) was absent from all HR$^-$ strains. In most of these HR$^-$ strains, a varying number (or even all) of the canonical *hrp/hrc* locus genes *hrpK1*, *hrpL*, and *hrcC* and the conserved effector locus effector genes *avrE1* and *hrpW1* were also absent based on PCR detection and subsequent Southern blotting (Table 2). Interestingly, for about a dozen HR+ strains we were also unable to detect one or more of the 6 tested genes (Table 2). For the other 16 HR$^+$ strains, all 6 genes were detected with PCR. Among the HR$^-$ strains, the most frequent molecular profile was similar to that previously reported for strain Psy508 (Mohr *et al.*, 2008) and Psy642 (Clarke *et al.*, 2010) and for HR$^-$ strains from *Arabidopsis thaliana* (Kniskern *et al.*, 2011), *viz*. strains lacked all 6 conserved genes tested (Table 2). An example of the results observed with PCR detection and subsequent Southern blotting are presented in the supplementary figure.

HR$^-$ strains lacking all 6 tested *hrp/hrc* genes and conserved effector genes are found in multiple distinct clades. In all previous descriptions of HR$^-$ *P. syringae* lacking the 6 tested genes, all strains fell into a single phylogenetic group, clade 2C (Clarke *et al.*, 2010; Kniskern *et al.*, 2011; Mohr *et al.*, 2008). Clade 2C corresponds to clade SZ-030 in our previous work (Morris *et al.*, 2010)) and we will thus continue calling this clade SZ-030 here. In this present work, HR$^-$ strains lacking all 6 conserved genes of the canonical *hrp/hrc* locus were found in clade SZ-030 and in three additional phylogenetic groups, clades TA-002, UB-246 and Group 2b (Table 2). In addition to the previously-reported strains from plants (cultivated and wild plants, weeds, debris), strains lacking the canonical *hrp/hrc* locus were isolated from snow pack and headwaters of rivers.

Previous descriptions of strains in the SZ-030 clade indicated that they possess a distantly related variant of the *hrcC* gene (Clarke *et al.*, 2010), called $hrcC_{Psy642}$ from here on. The presence of $hrcC_{Psy642}$ was observed only in HR$^-$ strains lacking all the 6 conserved genes in the canonical T3SS. This included all strains in the SZ-030 clade tested here and one strain outside of this clade, strain USA-050. The sequence of a 560 bp long fragment of the USA-050 *hrcC* allele was found to be 97% similar to that of strain Psy642.

Some but not all *P. syringae* strains from French sources that lack the canonical *hrp/hrc* locus island carry identical sequences originating from a bacteriophage. Previous work reported that strain Psy508 lacking the *hrp/hrc* locus harbors a prophage downstream of the associated $tRNA^{leu}$ (Mohr *et al.*, 2008). We sought to determine if all strains lacking the canonical *hrp/hrc* locus island had this same sequence because its placement in the genome is suggestive of its potential role in the absence of this island. PCR with primers for one of the prophage genes of Psy508, a putative transcriptional regulator, revealed the presence of the prophage sequence in 21 strains lacking the *hrp/hrc* locus, including the reference strain Psy508. Strains containing the phage sequence were found in all but one of the clades that contained strains without the *hrp/hrc* locus, *viz*. clades SZ-030, TA-002 and UB-254 (Table 2). However, the phage sequence was not present in all strains lacking the *hrp/hrc* locus; it was found in 18 of 23 strains in clade SZ-030, in 1 of 12 strains in clade TA-002, in 2 of 2 strains in clade UB-246, and not in the strain from the Group 2b clade. It was not detected in any strain for which at least one gene in the canonical *hrp/hrc* cluster was detected. The nucleotide sequence of the prophage was determined. The 380 bp sequence was identical among all strains collected in France and showed 91% similarity with the prophage sequence reported for strain Psy508 isolated from the state of New York (USA) (Mohr *et al.*, 2008).

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Incapacity to induce HR is not necessarily indicative of the incapacity to cause disease or to persist in plant tissue. For many species of plant pathogenic bacteria, and in particular for *P. syringae*, the ability to induce hypersensitivity has been considered to be an indicator of the capacity to cause disease in susceptible host plants (He, 1996). Likewise, the inability to induce hypersensitivity is considered indicative of the absence or incapacitation of a functional T3SS and hence of the incapacity to cause disease. Therefore, it is not surprising that most HR^- strains were not pathogenic in an assay on cantaloupe seedlings. In all clades, with the exception of clade TA-002, HR^- strains caused at most a weak disease symptoms (score = 1) in 1 of 12 of the inoculated seedlings after 7 days of incubation. However, HR^- strains in clade TA-002 were among the most aggressive on cantaloupe of the strains tested in this study. In particular, of 15 strains characterized in the TA-002 clade, 13 were HR^- and 12 lacked all 6 genes in the canonical *hrp/hrc* locus; 8 of these strains caused compatible disease reactions on 75% or more of the cantaloupe seedlings that were inoculated (Table 2) (Fig. 3). The range of pathogenic capacity of these strains, indicated by their aggressiveness on cantaloupe, was similar to that observed among HR^+ strains in the broad host range clades Groups 2a and 2b (Morris *et al.*, 2010).

When inoculated into cantaloupe seedlings, HR^+ strains with complete canonical T3SS (CC0654, DC3000) or with a partial set of the canonical alleles (USA-032) increased their mean population size by a factor of 10 to 30 within 4 days after inoculation (Fig. 2A). For CC0654 and USA-032, this corresponded to marked disease symptoms, but for strain DC3000 only one plant manifested slight symptoms (disease score = 1) during the incubation period (Fig. 2B). HR^- strain TA-043 showed a slight population increase at 2 days after inoculation and afterwards stabilized around the initial population size although disease symptoms were expressed. For the HR^- strains SZ-030 and CC1504, population sized decreased slightly and stabilized at around 10^7 cfu $plant^{-1}$. As expected, no disease symptoms were observed for these strains. For all strains considered together, plants with no symptoms harbored population sizes up to 3 x 10^8 cfu $plant^{-1}$. Populations in plants with a disease score of 1 ranged in size from 2 x 10^7 to 1.5 x 10^8 cfu $plant^{-1}$; those with a disease score of 2 or more had populations of 5 x 10^7 to 6 x 10^8 cfu $plant^{-1}$.

Discussion

The natural occurrence of variants of bacterial plant pathogens with apparent deficiencies in their T3SS has raised questions about the significance of the T3SS for the fitness of these bacterial species. For *P. syringae*, it has been proposed strains deficient in their overall pathogenicity have adapted to reduce the fitness costs of host specific virulence in an environment where there is a series of ephemeral host plants (Kniskern *et al.*, 2011). Absence of an effective T3SS could widen the range of plant species on which strains can grow without triggering an HR. In fact, many of the HR^- strains have the same level of reproductive fitness in plants as pathogenic strains - in absence of symptoms (Clarke *et al.*, 2010; Kniskern *et al.*, 2011). For the strains tested here, induction of disease symptoms in cantaloupe was associated with increases of population densities of about 1 order of magnitude. However, HR^- strains were able to persist in plants at densities above 10^6 cfu $plant^{-1}$ (or 10^6 cfu g^{-1}, data converted to a per gram basis not shown here), which is on the high range of *P. syringae* population densities naturally observed in wild plants and symptomless crops (Morris *et al.*, 2008). Ephemeral weeds and wild plants might be part of the selective pressures leading to the successful survival of strains of *P. syringae* deficient in their T3SS. However, *P. syringae* is clearly apt at inhabiting a range of non-plant and non-living substrates, thereby suggesting that this species has considerable fitness in these substrates. The proliferation of strains deficient in the T3SS might result from selection pressures specifically related to these substrates. Here we have shown that T3SS-deficient *P. syringae* can be found not only in wild plants, but also in epilithic biofilms, snow pack and river and lake waters in relatively pristine environments and can compose the dominant fraction of the *P. syringae* population in these substrates. These observations strongly suggest that deficiency in the canonical T3SS does not confer a marked loss of fitness of *P. syringae* in the context of the ensemble of habitats it exploits under natural

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conditions. T3SSs are considered to be all-purpose tools for undermining eukaryote biology thereby assuring an advantage for the bacterium (Pallen *et al.*, 2005) not only during pathogenesis but during symbiosis (Downie, ; Marchetti *et al.*) and to foster mutualism or commensalism in the rhizosphere (Lugtenberg and Kamilova, 2009) or the mycosphere (Nazir *et al.*, 2010)). Co-existence of HR$^+$ and HR$^-$ strains would suggest that in certain environmental habitats the subversion of eukaryotic cell biology - that of the wild plant host or of the algal or fungal co-inhabitants of the same biofilm, for example - has no decisive role in the fitness of *P. syringae*. This might be because the population densities that they attain under natural conditions are not large enough to trigger detrimental host responses. However, we cannot rule out that T3SSs in some HR$^-$ strains, for example the T3SS in clade SZ-030 (Clarke *et al.*, 2010; O'Brien *et al.*, 2010), play a role in the interaction with eukaryotes and are equally effective as the canonical T3SS in suppressing host responses.

We have also observed that pathogenicity does not necessarily depend on the canonical T3SS. The surprising capacity of HR$^-$ strains to cause disease symptoms in cantaloupe was confined to one clade (TA-002), but the symptoms induced were of the same range of severity as those caused by HR$^+$ strains across diverse clades. The type III injectisome is usually one of many different munitions in the whole arsenal used to attack plant cells, and is often complimented by toxins and cell wall-degrading enzymes. Pathogenicity of strains in the TA-002 clade might involve a range of arms independent of a type III injectisome. However, no syringomycin-like broad host range toxins have been detected in these strains according to bioassays (data not shown). Alternatively (or in addition), pathogenicity of strains in the TA-002 clade might be the result of an unknown T3SS that does not elicit hypersensitivity in tobacco but that is capable of delivering effectors. Genome sequencing has led to an important broadening of the known diversity of T3SSs (Pallen *et al.*, 2005). Current efforts to analyze fully-sequenced genomes of *P. syringae* strains from non agricultural habitats, including HR$^-$ strains, will eventually reveal the full range of potential pathogenicity factors of *P. syringae*. Nevertheless, this result is troubling because it weakens the utility of the hypersensitivity test in disease diagnostics. This test has been a key criterion to separate pathogens from saprophytes. Furthermore, all papers reporting HR$^-$ strains of *P. syringae* have referred to them as 'non pathogenic'. If strains in the TA-002 clade have not been reported as the causal agent of epidemics of crop disease it might be due to their incapacity to do so under field conditions, but more likely it is due to the fact that a positive hypersensitive reaction is generally used early in the diagnostic process as a criterion for targeting candidate causal agents.

The absence of the canonical T3SS in multiple disparate clades raises interesting questions about the processes leading to the acquisition or the loss of the canonical T3SS in *P. syringae*. There is strong evidence that the canonical T3SS was acquired by the most recent common ancestor of the *P. syringae* species complex (Sawada *et al.*, 1999) and has evolved via diversifying selection involving mostly mutations (Guttman *et al.*, 2006). However, those phylogenetic studies ignored the existence of nine clades of *P. syringae*, five of which are more deeply rooted in the phylogenetic tree than the other previously described clades (Morris *et al.*, 2010), the most deeply rooted being clade UB-246. We speculate that the canonical T3SS was acquired at the time of diversification of UB-246 from all of the other clades. The most parsimonious explication for T3SS deficiency in the other clades is subsequent loss by the ancestor of the clades in which it is currently absent. However, clade TA-002 contains several strains that effectively induce hypersensitivity and for which some, but not all, of the six conserved genes were detected. This suggests a diversification of the genes beyond what could be detected with the primers used here, designed based on only three completely sequenced *P. syringae* genomes (Mohr *et al.*, 2008). For all the strains in the CC1524 clade, closely related to TA-002, we could only detect some of the six conserved genes in the canonical T3SS. Likewise, for all strains in clades UB-370, USA-102 and USA-032, which are together in another branch of the phylogenetic tree, some but not all of the six conserved genes were detected in these otherwise HR$^+$ strains. This suggests that divergence of the alleles in the canonical T3SS is more rapid in some branches than elsewhere in the phylogenetic tree of *P.*

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syringae. Our observations highlight that natural habitats likely harbor a greater diversity of alleles of the genes in the T3SS of *P. syringae* than currently observed in strains from crops thereby raising exciting questions about the nature of the drivers of this diversification.

The presence of a bacteriophage sequence in strain Psy508 near the site of the (missing) canonical *hrp/hrc* cluster (Mohr *et al.*, 2008) raised questions about the link between the presence of this phage and the absence of the canonical T3SS. Here we show that, although this prophage sequence is present only in strains lacking the canonical T3SS, it is not universally present in all strains with this deficiency. Furthermore, acquisition of the phage seems to have occurred multiple times and perhaps at distinctly different dates as indicated by sequence divergence between French and US strains. This suggests that there were multiple and very recent acquisitions of the same phage in clades TA-002, UB-246 and SZ-030 in France, and that strain Psy508 acquired a variant of the same phage or had acquired it at a much earlier date. We must also consider that some strains in which we did not detect the phage may have at one time acquired it and then lost it again.

For the two functional T3SSs described to date in *P. syringae* (Clarke *et al.*, 2010; Tampakaki *et al.*, 2010), our observations suggest an exclusivity of the type of T3SS harbored in the genomes of *P. syringae*. Likewise in *P. viridiflava,* strains harbor only one of the two types of T3SS identified by Bergelson and colleagues (Araki *et al.*, 2006). However, Pallen reported that certain bacteria (*Burkholderia* spp., for example) can accumulate different T3SSs (Pallen *et al.*, 2005). The secrets of T3SS polymorphism in *P. syringae* will be disclosed by genomic analyses that incorporate the novel strains described here.

Our results also have implications for understanding which habitats have been important in the evolutionary history of *P. syringae.* Compared to other members of the genus such as *P. fluorescens, P. syringae* has a much reduced capacity to use carbon sources (Sands *et al.*, 1970). It has been suggested that the ancestor of *P. syringae* lost a range of metabolic functions, as an adaptation to the nutritionally constrained environment characteristic of the plants compared to the more nutritionally diverse and copious environments encountered by *P. fluorescens* as a saprophyte (Mithani *et al.*, 2011). This analysis was based on genomes of *P. syringae* in the Group 1, 2 and 3 clades. In light of the new clades of *P. syringae* that our work has revealed, it would be important to consider the metabolic adaptations of the strains in the UB-246 clade, the most deeply rooted of the known *P. syringae* clades and represented solely by HR$^-$ strains. If they resemble those of *P. syringae* in general, then this would suggest that adaptation to a nutritionally constrained environment would be most probably for success as a resident of pristine fresh water habitats. We propose that loss of metabolic capacities in *P. syringae* might have originally been an adaptation for the oligotrophic conditions of certain freshwater habitats. Dissolved organic carbon sources in freshwater generally originate from plants (leaching and degradation). The ability to use sparse quantities of plant-derived carbon sources could have prepared *P. syringae* for a more intimate association with plants, but the intimate association with plants itself might not have been the initial selection pressure for adaptation to oligotrophic conditions.

The persistence of non pathogenic variants of plant and animal pathogens in the environment has been a springboard for the development of biological control for plant pathogens (Kerr, 1987; Larkin *et al.*, 1996; van Alfen, 1982) and of diseases of human and animal intestinal and urinary tracts (Kruis *et al.*, 2004; Schneitz, 2005). The diversity, abundance and ubiquity of non pathogenic strains of *P. syringae* suggest that competitive exclusion of pathogen strains by non pathogenic con-specifics as a means to control disease might be worth exploring. Competitive exclusion between ice nucleation active and inactive variants of *P. syringae* has successfully limited frost damage induced by this bacterium under experimental conditions (Lindow, 1987). Inundative biological control is the most obvious of the potential practical applications of this work but strategies for plant breeding or crop management that foster proliferation of non pathogenic strains of *P. syringae* might also have potential to mitigate disease development.

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Table 1. Characteristics of environmental samples, from alpine regions and outside of cropped fields, used in survey for populations of *Pseudomonas syringae* incapable of inducing a hypersensitive reaction in tobacco (HR-).

Substrate	Region	n° of samples[2]	n° HR$^-$ samples[3]	Size range of the total *P. syringae* population (log cfu g^{-1} and log cfu L^{-1})[4]	Previous description of sample or sampling site
Epilithic biofilm					
	France, Alps	1	1	4.9	(Morris *et al.*, 2007)
	France, southeast	3	0	1.70 - 2.43	(Morris *et al.*, 2007)
	USA, western Rockies	6	1	1.56 - 3.76	(Morris *et al.*, 2007)
Headwater					
	France, Alps	37	34	1.47 - 5.8	(Morris *et al.*, 2010; Morris *et al.*, 2008)
	France, southwest	2	1	2.65 - 3.03	(Morris *et al.*, 2010)
	New Zealand	1	1	3.45	(Morris *et al.*, 2010)
	USA, western Rocky mountains	3	1	2.69	(Morris *et al.*, 2010)
Wild plants					
	France-Italy border, Alps	10	6	2.68 - 9.00	(Morris *et al.*, 2008)
	France, southwest	2	1	3.85 - 5.73	(Morris *et al.*, 2008)
Alpine leaf litter					
	France, Alps	11	8	2.89 - 6.45	
Rain[1]					
	France, Alps	1	1	4.23	
	France, southeast	5	1	1.00 - 3.05	(Morris *et al.*, 2008)
Snow pack					
	Austrian Alps	1	0	2	
	France, Alps	35	25	1.20 - 6.95	(Morris *et al.*, 2008)
	Morocco, Atlas mountains	1	1	4.72	
Snow fall					
	France, Alps	6	0	2.18 - 5.11	(Morris *et al.*, 2008)
	USA, western Rockies	1	0	1.83	

[1]Some rain samples were collected in peri-urban zones, but outside of cropped fields.
[2]Number of total samples of each substrate that were analyzed and in which *P. syringae* was detected.
[3]Number of samples of each substrate in which HR$^-$ strains of *P. syringae* were detected. When fewer than 20 strains of *P. syringae* were isolated from the sample, all were tested for capacity to induce hypersensitivity. Otherwise, a random set of about 30 strains was tested per sample.
[4]The range of sizes of populations of *P. syringae*, expressed as cfu g^{-1} (plants, leaf litter and epilithic biofilms) or cfu L^{-1} (rain, headwaters, and meltwater from snow) observed in the different substrates.

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Table 2. Genetic and pathogenic diversity of strains of *P. syringae* according to the phylogenetic context of the strains. The presence or absence of genes was based on PCR detection.

			Disease on cantaloupe seedlings		Conserved genes in the canonical T3SS								
						CEL effectors		*hrp-hrc* cluster					
Clade[a]	Strain[b]	HR[c]	severity[d]	incidence[e]	*hopI1*	*avrE*	*hrpW*	*hrpK*	*hrpL*	*hrcC*	Psy642 *hrcC*[f]	*Phage*	*gapA*
SZ-030	Psy508[g]	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	SZ-030	-	0.08	0.08	-	-	-	-	-	-	+	+	+
	SZ-037	-	0.08	0.08	-	-	-	-	-	-	+	+	+
	SZ-045	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	SZ-051	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	SZ-124	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	SZ-142	-	0.17	0.17	-	-	-	-	-	-	+	+	+
	SZ-144	-	0.08	0.08	-	-	-	-	-	-	+	+	+
	UB-015	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	UB-185	-	0.08	0.08	-	-	-	-	-	-	+	+	+
	UB-186	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	UB-190	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	UB-192	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	UB-193	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	UB-194	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	UB-195	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	UB-313	-	0.00	0.00	-	-	-	-	-	-	+	+	+
	UB-330	-	nd[h]	nd	-	-	-	-	-	-	+	+	+
	Psy-642[g]	-	0.00	0.00	-	-	-	-	-	-	+	-	+
	SZ-093	-	0.00	0.00	-	-	-	-	-	-	+	-	+
	UB-184	-	0.00	0.00	-	-	-	-	-	-	+	-	+
	UB-218	-	0.00	0.00	-	-	-	-	-	-	+	-	+
	CC1504	-	0.00	0.00	-	-	-	-	-	-	+	-	+

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	USA-052	-	0.00	0.00	-	+	+	+	-	-	+	-	+
TA-002	TA-043	-	1.67	1.00	-	-	-	-	-	-	-	+	+
	CCE-284	-	2.17	0.83	-	-	-	-	-	-	-	-	+
	CCE-286	-	1.75	0.75	-	-	-	-	-	-	-	-	+
	CCE-291	-	1.33	0.75	-	-	-	-	-	-	-	-	+
	CCE-321	-	0.17	0.42	-	-	-	-	-	-	-	-	+
	CCE-324	-	0.50	0.50	-	-	-	-	-	-	-	-	+
	CCV-0050	-	1.17	1.00	-	-	-	-	-	-	-	-	+
	CCV-0172	-	0.17	0.17	-	-	-	-	-	-	-	-	+
	CCV-0178	-	2.91	1.00	-	-	-	-	-	-	-	-	+
	CCV-0180	-	2.50	1.00	-	-	-	-	-	-	-	-	+
	CSZ-0284	-	1.25	0.67	-	-	-	-	-	-	-	-	+
	CSZ-0297	-	2.08	0.67	-	-	-	-	-	-	-	-	+
	CC-1582	-	2.58	1.00	-	-	-	-	-	+	-	-	+
	CC-0655	+	0.50	0.42	-	+	-	-	-	+	-	-	+
	CC-0657	+	0.00	0.00	-	+	-	+	+	+	-	-	+
UB-246	SZ-131	-	0.08	0.08	-	-	-	-	-	-	-	+	+
	UB-246	-	0.00	0.00	-	-	-	-	-	-	-	+	+
CC-1524	CC-1419	-	0.00	0.00	-	+	-	-	+	+	-	-	+
	CC1418	+	0.00	0.00	-	+	+	-	+	+	-	-	+
	CC1422	+	0.00	0.00	-	+	+	-	+	+	-	-	+
	UB-169	+	0.00	0.00	-	-	-	+	-	+	-	-	+
	UB-172	+	0.00	0.00	-	-	-	+	-	+	-	-	+
Cit7	Cit7[g]	+	no known hosts[i]		+	+	+	+	+	+	-	-	+
Group 1	SZ-122	-	0.00	0.00	-	-	+	+	+	-	-	-	+
	SZ-026	+	0.25	0.25	+	+	+	+	+	+	-	-	+
	SZ-046	+	0.25	0.25	+	+	+	+	+	+	-	-	+
	SZ-049	+	0.33	0.25	+	+	+	+	+	+	-	-	+
	SZ-145	+	0.00	0.00	+	+	+	+	+	+	-	-	+
	CC-1569	+	nd	nd	+	+	+	+	+	+	-	-	+

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	DC3000[g]	+	0.00	0.00	+	+	+	+	+	+	-	-	+
Group 2a	UB-419	+	2.83	1.00	+	+	+	+	+	+	-	-	+
	SZ-035	+	0.46	0.30	+	+	+	+	+	+	-	-	+
	SZ-048	+	0.50	0.42	+	+	+	+	+	+	-	-	+
	SZ-089	+	2.17	1.00	+	+	+	+	+	+	-	-	+
	UB-197	+	0.00	0.00	+	+	+	-	+	+	-	-	+
	UB-210	+	0.33	0.08	+	+	+	-	+	+	-	-	+
	UB-421	+	2.83	1.00	+	+	+	+	+	+	-	-	+
Group 2b	USA-050	-	0.00	0.00	-	-	-	-	-	-	+	-	+
	CC0654	+	1.40	0.50	+	+	+	+	+	+	-	-	+
	B728A[g]	+	0.00	0.00	+	+	+	+	+	+	-	-	+
Group 3	1448A[g]	+	0.00	0.00	+	+	+	+	+	+	-	-	+
UB-370	UB-370	+	0.00	0.00	-	+	+	-	+	+	-	-	+
USA-102	USA-046	+	0.00	0.00	-	+	+	-	+	-	-	-	+
	USA-106	+	0.00	0.00	-	+	+	+	+	+	-	-	+
USA-032	USA-032	+	3.25	1.00	-	+	+	-	+	+	-	-	+

[a] Clades are those described by Morris and colleagues (Morris *et al.*, 2010).
[b] The origins of strains from non agricultural habitats used in this study are described in the supplementary table.
[c] Hypersensitive reaction on tobacco (cv. Xanthi).
[d] Mean intensity, on a scale of 0 to 4, of disease symptoms on 12 cantaloupe seedlings at 7 days after inoculation.
[e] The proportion of 12 cantaloupe seedlings showing disease symptoms at 7 days after inoculation.
[f] Detection of the Psy642 variant of the *hrcC* gene was based on primers different from those for detecting the canonical *hrcC*.
[g] Reference strains described previously as follows: Psy508 (Mohr *et al.*, 2008), Psy-642 (Clarke *et al.*, 2010), Cit7 (Hirano and Upper, 1990), DC3000, B728A, and 1448A (*Pseudomonas syringae* Genome Resources: http://pseudomonas-syringae.org/home.html).
[h] Not determined.
[i] The pathogenicity of Cit7 has been previously well characterized. There are no known hosts and it is considered to be non pathogenic (Hirano and Upper, 1990).

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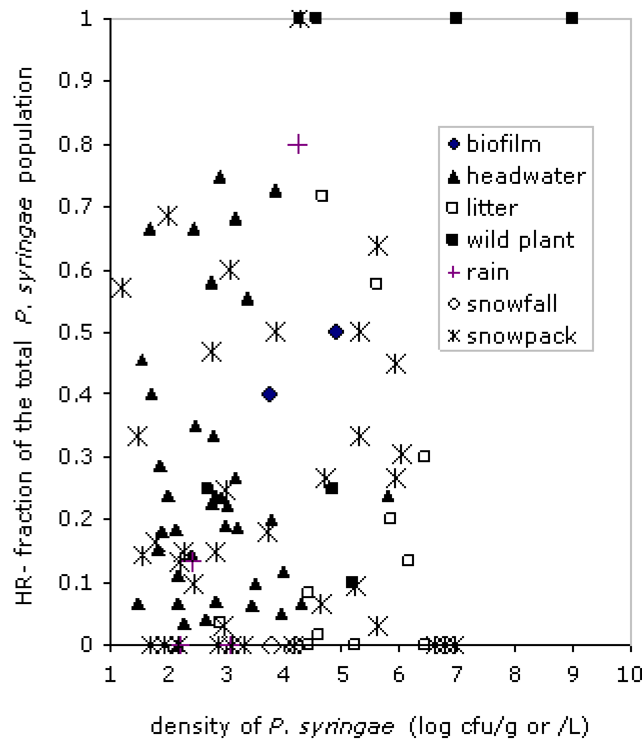

Figure 1. Fraction of HR^- strains in the total *P. syringae* populations in environmental samples in terms of the density of the *P. syringae* population in the sample. Population densities are expressed as log cfu g^{-1} for biofilms, plant and leaf litter or log cfu L^{-1} for water, rain and snow (as snowmelt). The data constitute values from 100 of the 126 samples described in Table 1. For these 100 samples, they were processed to permit quantification of the density of the HR^- component population as well as that of the total *P. syringae* population.

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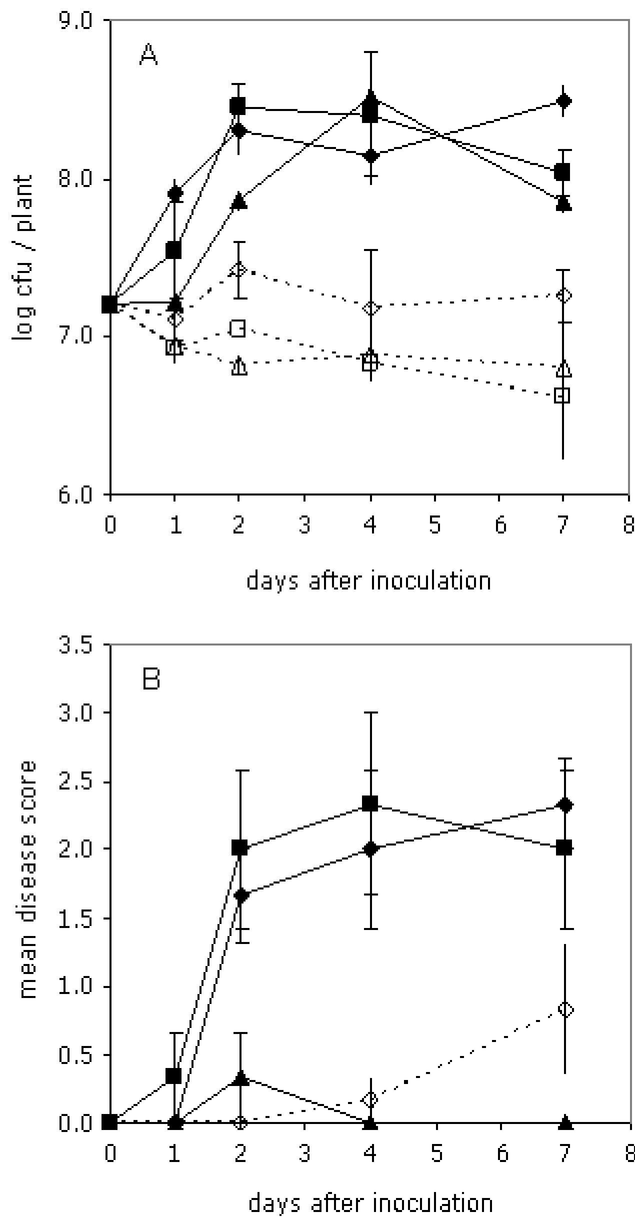

Figure 2. Population dynamics of *P. syringae* (A) and disease (B) in cantaloupe seedlings after inoculation with either HR^+ or HR^- strains. HR^+ strains are represented by solid lines and symbols (◆ CC0654; ▲ DC3000; ■ USA-032) and HR^- strains by dashed lines and open symbols (◊ TA-043; △ SZ-030; ▫ CC1504). At each sampling time, 3-6 seedlings were cut off at the soil level and individually macerated to determine population densities. Disease was rated on each of the 3-6 plants sampled. Plants inoculated with strains SZ-030 and CC1504 did not show any disease symptoms. Only one plant inoculated with strain DC3000 showed disease symptoms at 2 days after inoculation. For strain TA-043 half of the inoculated plants showed symptoms at 7 days after inoculation. Error bars indicate the standard error of the mean.

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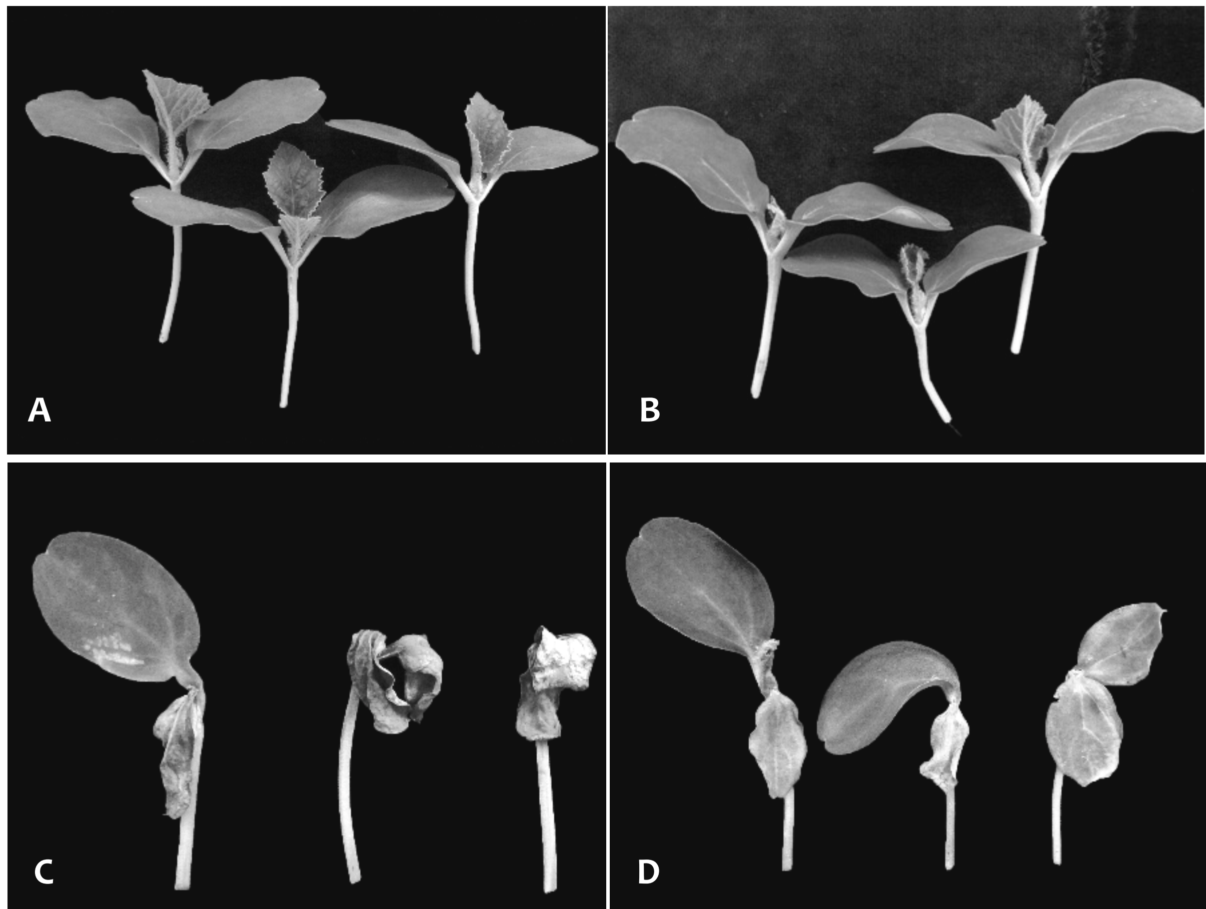

Figure 3. Reaction of cantaloupe plants at the cotyledon stage to inoculation with HR^+ and HR^- strains of *P. syringae* after 7 days of incubation: plants were inoculated with A) sterile distilled water, B) HR^- strain CC1524 in the clade of the same name C) HR^+ strain CC0094 in the Group 2b clade, or D) HR^- strain TA043 in the TA002 clade. Plants shown here were chosen at random among the 12 plants inoculated for each strain.

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Supplemental Information. Amplification conditions for PCR

To detection genes in the *hrp-hrc* cluster (*hrpK1, hrpL, hrcC*), the CEL effectors (*avrE1, hrpW1*) and the *hopI1* genes, primers described by Mohr et al. (Mohr *et al.*, 2008) were used for amplification. GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) was used for PCR performed on a Mastercycler epigradient thermocycler (Eppendorf, Hamburg, Germany), starting with 2 min at 95°C, followed by 33 cycles at 95°C for 30s, at 72°C for 1 min (this temperature was decreased by 0.5°C every cycle until the touchdown temperature of 58°C was reached), and primer extension at 72°C for 1 min. One additional cycle was carried out at an annealing temperature of 58°C, followed by final incubation for 10 min at 72°C, at the fastest ramp rate (4°C s^{-1}) from the denaturation to the annealing step.

Phage PCR, using the forward primer PhF (5'- TCAGGCCGCTTTCAACTTGGC-3') and the reverse primer PhR (5'- CCATAYGGCGCAGTGGTGG- 3'), was performed with conditions starting with 5 min at 95°C, followed by 30 cycles at 94°C for 1 min, at 72°C for 1 min (the temperature was decreased by 0.5°C every cycle until the touchdown temperature of 57°C was reached), and primer extension at 72°C for 1 min. Five additional cycles were carried out at an annealing temperature of 57°C, followed by final incubation for 10 min at 72°C. Negative controls were PCR mixtures with the addition of water in place of template DNA.

To detect the presence of the *hrcC* orthologue of Psy642, colony PCR was performed by placing a small amount of cultured bacteria from a KB plate into 15 µL of PCR reaction mixture including 7.5 µL of 2x Immomix (Bioloine, Taunton, MA, USA) and 0.75 µL each of $hrcC_{\text{Psy642}}$ forward and reverse primer (Clarke *et al.*, 2010). PCR included an initial denaturation step at 95°C for 10 minutes followed by 35 cycles of 40 seconds denaturation at 94°C, 30 seconds annealing at 58°C, and 1 minute extension at 72 °C. Amplification was concluded with a 10 minute extension at 72 °C. Strains DC3000 and Psy642 were used as the negative and positive controls respectively.

For housekeeping genes, PCR conditions were as described in our previous work (Morris *et al.*, 2008) using primers described by Yamamoto et al (Yamamoto *et al.*, 2000) for *rpoD* and *gyrB* and by Sarkar and Guttman (Sarkar and Guttman, 2004) for *cts*. The alternative primers for *gapA* described by Morris et al (Morris *et al.*, 2010) were used when those described by Sarkar and Guttman (Sarkar and Guttman, 2004) did not amplify the *gapA* gene. Amplified products were purified and sequenced as described previously (Morris *et al.*, 2010; Morris *et al.*, 2008).

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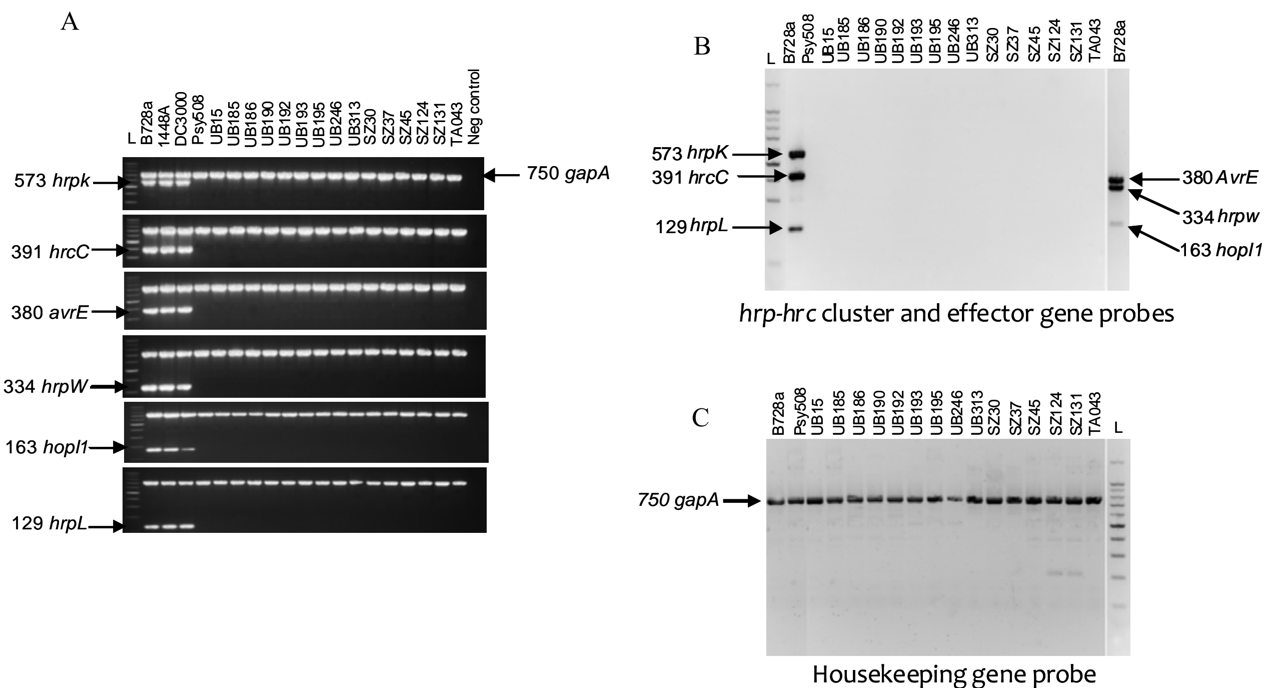
Supplemental Figure 1. (**A**) Example of PCR results for canonical *hrp-hrc* cluster (*hrpK1, hrpL hrcC,*), the conserved effector locus (*avrE1, hrpW1*), the *hopI1* effector gene, and the housekeeping gene *gapA*. Neither the genes of the *hrp-hrc* T3SS cluster nor the effector genes were amplified from HR⁻ *P. syringae* Psy508 and other closely related isolates of HR⁻ *P. syringae*. 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 as described previously (Mohr *et al.*, 2008). Lane L is the kb ladder.

(**B, C**) Example of Southern blot experiments indicating that *P. syringae* Psy508 and other HR⁻ isolates of *P. syringae* do not contain either an *hrp-hrc* gene cluster nor any orthologues of effectors from the closely related pathogenic isolate *P. syringae* pv. syringae B728a. Genomic DNA of *P. syringae* B728a, *P. syringae* Psy508, and T3SS-deficient *P. syringae* digested with HindIII and EcoRI and analyzed by Southern blotting using either genes of the canonical *hrp-hrc* cluster (*hrpK1, hrpL hrcC,*), the conserved effector locus (*avrE1, hrpW1*), the *hopI1* effector gene (**B**), and the housekeeping gene *gapA* (**C**) as the probe. While *P. syringae* B728a DNA hybridized with all six probes of the PAI as well as the *gapA* probe, T3SS-deficient *P. syringae* hybridized only with the housekeeping gene probe.

Mohr TJ, Liu H, Yan S, Morris CE, Castillo JA, Jelenska J *et al* (2008). Naturally occurring non-pathogenic isolates of the plant pathogen species *Pseudomonas syringae* lack a Type III Secretion System and effector gene orthologues. *J. Bacteriol.* **190:** 2858-2870.



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Supplementary Table 1. Sources of strains of *P. syringae* from non agricultural habitats. These sources are a subset of the samples described in Table 1.

Substrate	Site	latitude / longitude / altitude	Date	strains: HR⁻	strains: HR⁺
River water	Rioul Mounal creek, Haute-Alpes Co., France	44° 31' 58" N 06° 42' 13" E 2100 m	July 2006	UB-015	
			Oct. 2006	UB-218	UB-210
			May 2007	UB-313, 330	
		44° 32' 09" N 06° 42' 12" E 2100 m	July 2006		UB-169, 172
			Oct. 2006	UB-184, 185, 186, 190, 192, 193, 194, 195	UB-197
			May 2007		UB-419, 421
			April 2009	CCV-0050	
		44° 31' 49" N 06° 42' 38" E 1880 m	June 2009	CCV-0172, 0178, 0180	
	Ubaye River, Hautes-Alpes Co., France	44° 23' 50" N 06° 28' 54" E 1000 m	Oct 2006	UB-246	
		44° 27' 14" N 06° 23' 13" E 785 m	May 2007		UB-370
	Sauze River, Hautes-Alpes Co., France	44° 20' 40" N 06° 40' 54" E 2100 m	April 2007	SZ-030	SZ-026
			May 2007	SZ-093	SZ-089
		44° 20' 40" N 06° 40' 48" E 2100 m	April 2007	SZ-037, 045, 051	SZ-035, 046, 048, 049
			May 2007	SZ-122, 124	
		44° 20' 11" N 06° 40' 15" E 2500 m	May 2007	SZ-131, 142, 144	SZ-145
	Pisse Creek, Hautes-Alpes Co., France	44° 38' 10" N 06° 47' 19" E 2200 m	June 2009	CCE-284, 286, 291	
		44° 38' 54" N 06° 47' 33" E 1690 m	June 2009	CCE-321, 324	
	Cascade Creek, Grand Teton Park, Wyoming, USA	43° 45' 54" N 110° 45' 00" W 2200 m	Aug. 2007	USA-050, 052	USA-032, 046
	Pilgrim Creek, Grand Teton Park, Wyoming, USA	43° 55' 42" N 110° 33' 42" W 2140 m	Aug 2007		USA-106

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Snow pack	Hautes-Alpes Co., France	44° 41’ 45” N 6° 59’ 12” E 2600 m	June 2006		CC-1569
		44° 20’ 40” N 06° 41’ 57” E 2000 m	May 2009	CSZ-0284, 0297	
Epilithic biofilm	Hautes-Alpes Co., France	44° 41’ 45” N 6° 59’ 12” E 2600 m	June 2006	CC-1582	
	Palisade Falls, Gallatin Co, MT, USA	45° 28’ 18” N 110° 56’ 11” W 2150 m	Sept 2004	CC-1419	CC-1418, 1422
Primula officinalis	Lozère Co., France	44° 22’ 44” N 03° 47’ 41” E 1300 m	April 2007	TA-043	
	Mézel, Alpes-de-Hautes-Provence Co., France	43° 59’ 22” N 06° 11’ 43” E 580 m	March 2004		CC-0654, 0655, 0657
Primula farinosa	Hautes-Alpes Co., France	44° 38’ 36” N 6° 57’ 20” E 2700 m	June 2006	CC-1504	

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