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ORIGINAL ARTICLE

The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle

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Pseudomonas syringae **is a plant pathogen well known for its capacity to grow epiphytically on diverse plants and for its ice-nucleation activity. The ensemble of its known biology and ecology led us to postulate that this bacterium is also present in non-agricultural habitats, particularly those associated with water. Here, we report the abundance of** ***P. syringae*** **in rain, snow, alpine streams and lakes and in wild plants, in addition to the previously reported abundance in epilithic biofilms. Each of these substrates harbored strains that corresponded to** ***P. syringae*** **in terms of biochemical traits, pathogenicity and pathogenicity-related factors and that were ice-nucleation active. Phylogenetic comparisons of sequences of four housekeeping genes of the non-agricultural strains with strains of** ***P. syringae*** **from disease epidemics confirmed their identity as** ***P. syringae*****. Moreover, strains belonging to the same clonal lineage were isolated from snow, irrigation water and a diseased crop plant. Our data suggest that the different substrates harboring** ***P. syringae*** **modify the structure of the associated populations. Here, we propose a comprehensive life cycle for** ***P. syringae*****—in agricultural and non-agricultural habitats—driven by the environmental cycle of water. This cycle opens the opportunity to evaluate the importance of non-agricultural habitats in the evolution of a plant pathogen and the emergence of virulence. The ice-nucleation activity of all strains from snow, unlike from other substrates, strongly suggests that** ***P. syringae*** **plays an active role in the water cycle as an ice nucleus in clouds.**

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Introduction

The prevailing concepts about the life histories or life cycles of plant and animal pathogens have significant practical consequences for controlling or avoiding epidemics. Medical microbiologists long ago recognized that human pathogens can also reside and flourish in nonhost habitats, in which they can evolve and diversify independent of their activity as pathogens. This has led to the modern practices of water sanitation, surveillance of wild-animal populations, cleaning and maintenance of ventilation systems in buildings, limited entry of floral bouquets into hospitals etc. Plant pathologists have tended to focus more restrictively in defining the life cycles of plant pathogens principally by their agricultural contexts *sensu stricto* and in terms of the availability and response of host plants. Nonetheless, agricultural systems are open and most plant pathogens are not obligate parasites, suggesting that they might well be able to disperse and thrive elsewhere, yet the parts of their life cycles involving non-agricultural contexts have largely been ignored.

Pseudomonas syringae is an archetype among the plant pathogenic bacteria. Its biology, ecology and genetics have been studied extensively, but these studies are done almost exclusively in the context of

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the relationship of this bacterium with plants and agricultural habitats (Hirano and Upper, 2000; Sarkar and Guttman, 2004). Recently, strains of *P. syringae*, virulent on diverse species of crop plants, were isolated from epilithic biofilms from rivers in pristine regions outside the zones of agricultural production (Morris *et al.*, 2007). This is one of the several observations suggesting that this plant pathogen is widely spread in non-agricultural niches. *P. syringae* has a strong net upward flux from plant canopies into the atmosphere (Lindemann *et al.*, 1982), and there are several reports of isolation of *P. syringae* from clouds at several kilometers altitude (Sands *et al.*, 1982; Amato *et al.*, 2007). From clouds, *P. syringa*e could eventually fall out with rain or snow to a variety of environments. Nevertheless, reports of this bacterium on wild plants have focused on weed or non-host plants in agricultural regions. *P. syringae* reported on thistle, oak, black locust (Lindemann *et al.*, 1984), *Arabidopsis thaliana* (Jakob *et al.*, 2002) and wild cherry (Vincente *et al.*, 2004) were from plants in agricultural fields, woodland plantations or nurseries. Although we suspect that *P. syringae* is widely spread in the environment, the extent to which it is present in niches outside of agricultural contexts has not been established clearly.

In this work, we sought to find *P. syringae* in pristine habitats, which it might attain primarily via fall-out with rain and snow. Substrates investigated from these habitats included lake and stream water at altitudes above cultivated zones, snow pack or firn snow melting into these lakes and streams, and the nearby flora. We also examined rain from semirural zones and freshly fallen snow from diverse locations as indirect evidence of *P. syringae* falling out with precipitation. To avoid collecting *P. syringae* scrubbed from aerosols originating from nearby plant canopies, we sampled under conditions where population sizes of local air-borne *P. syringae* were likely to be low, such as in mid or late winter or above cement or gravelled areas. We also took sample plants in forests or prairies at the border of national parks, in private properties and other areas that had never been under cultivation.

Here, we present data on the densities of populations of *P. syringae* in a range of non-agricultural substrates and evidence confirming that these strains are *bona fide P. syringae* based on genotypic and phenotypic properties, including pathogenicity. Strains isolated from snow, irrigation water and a diseased crop that belonged to the same clonal lineage were identified in this work, thereby providing evidence for a link between agricultural and non-agricultural habitats. These results led us to propose an expanded life cycle or life history for this bacterium to encompass its dissemination via the water cycle and subsequent colonization of both agricultural and non-agricultural habitats. These findings are novel for a plant pathogen and are significant from the point of view of disease epidemiology, breeding for disease resistance and disease management practices such as quarantine and chemical and biological control.

Materials and methods

Isolation of putative P. syringae *from environmental sources*

The geographic origins and substrates from which *P. syringae* was isolated are presented in Table 1. All samples were collected in sterilized or disinfested containers, were kept in a cooler during transport and were stored in a refrigerator prior to processing. The methods used to process the different substrates depended on the nature of the substrate. In all cases, aliquots of the processed material were plated on a selective medium, as described below. Except for some of the wild-plant samples collected in the early part of this study, methods were quantitative, and also the number of *P. syringae*-like bacteria per gram of tissue, per leaf or per milliliter of water was determined.

For plants, several leaves from the same plant or from the same stand of plants were pooled, weighed and macerated in a stomacher (BagMixer, Interscience, St Nom-La-Breteche, France) for 1.5–3 min in 0.1 M phosphate buffer (8.75 g K_2HPO_4 and 6.75 g KH_2PO_4 per liter, pH 6.8). The volume of buffer used depended on the mass of tissue processed. All plants collected were apparently healthy.

Snow was collected where it had accumulated to at least several centimeters depth (to avoid snow that was in contact with the ground or with plants) and where there were no animal or human tracks. Some of the snow had fallen only a few hours before sampling. The top 1 cm was brushed away and the underlying snow was collected with sterile flour scoops and put into clean plastic bags. Enough snow was collected for each sample to render at least 1.5 l of melt water. The melt water was mixed thoroughly and 1 l was filtered across a sterile nitrous cellulose filter (pore diameter 0.22 μm). The filter was recovered and agitated with a sterile stir bar for about 3 min in 10 ml of the melt water filtrate. The 100-fold concentrated melt water was then transferred to a sterile plastic container. An aliquot of the crude melt water was also conserved.

Water from lakes and streams was collected in bottles at several centimetres below the surface. Volumes of 1.5–3 l were collected at each site. The water was mixed well and then filter-concentrated in the same manner as that for snow-melt water. The 100-fold concentrated samples and aliquots of the crude water were transferred to sterile plastic containers.

At the onset of storms, rainwater was collected in large plastic buckets (volume of 30 l or greater) lined with clean plastic bags. The buckets were placed in open areas to avoid run-off or splashing from plants

Table 1 Origin of substrates from which strains of *P. syringae* able to induce a hypersensitive reaction in tobacco were isolated

Substrate	*Sample code*	*Country*	*Place name and region or state*	*Site description*	*Sampling date (month-year)*	*Population size*[a]
Wild plants						
Primula officinalis						
	P-01	France	Vaour, Tarn-et-Garonne	Domaniale-de-la-Grésigne forest	05-2003	ND
	P-02		Mézel, Alpes-de-Haute-Provence	Yard of a private home	03-2004	ND
	P-03		Chartreuse de Valbonne, Gard	Forest around a monastery	04-2004	ND
	P-04		Talant, Côte d'Ore	Roadside	04-2004	ND
	P-05		Vars, Hautes-Alpes	Vars Pass, 2100 m altitude	06-2006	8.1×10^4 CFU g^{-1}
P. grandiflora						
	P-06	France	Buoux, Vaucluse	Forest	03-2004	ND
	P-07		Mézel, Alpes-de-Haute-Provence	Yard of a private home	03-2004	ND
	P-08	Italy	Opi, Abruzzo	Along riverside in campground	04-2004	ND
P. elatior						
	P-09	France	Mézel, Alpes-de-Haute-Provence	Yard of a private home	04-2004	ND
Cultivated *Primula* spp.						
	P-10	France	Passenans, Jura	10-year-old stand, private garden	04-2004	ND
Dodecantheon pulchellum						
	P-11	USA	Gardiner, Park Co., MT	Border of Yellowstone National Park	05-2005	600 CFU per leaf
	P-12		Missoula, Missoula Co., MT	Mount Sentinel, 2000 m altitude	05-2005	3300 CFU per leaf
	P-13		Georgetown Lake, Anaconda-Deerlodge, Co., MT	On hills above the lake	06-2005	6700 CFU per leaf
Silene acaulis						
	P-14	Italy	Longet Pass, Piemonte	2600 m altitude	06-2006	7.3×10^4 CFU g^{-1}
Hutchinsia alpina						
	P-15	France	Vieux Pass, Hautes-Alpes	2600 m altitude	06-2006	4.8×10^4 CFU g^{-1}
Geranium sylvaticum						
	P-16	Italy	Chianale, Piemonte	Forest	06-2006	5.4×10^5 CFU g^{-1}
Onobrychis montana						
	P-17	Italy	Chianale, Piemonte	2000 m altitude	06-2006	7.4×10^4 CFU g^{-1}
Snow						
	S-01	France	Villard de Lans, Isère	Freshly fallen snow, 1200 m altitude	12-2005	4000 CFU l^{-1} meltwater
	S-02				02-2006	150 CFU l^{-1} meltwater
	S-03		La Clusaz, Haute Savoie	Freshly fallen snow, 1200 m altitude	02-2006	1.3×10^5 CFU l^{-1} meltwater
	S-04		Vieux Pass, Hautes-Alpes	Firn snow, 2600 m altitude	06-2006	4.5×10^3 CFU l^{-1} meltwater
	S-05		Longet Pass, Hautes-Alpes	Firn snow, 2700 m altitude	06-2006	500 CFU l^{-1} meltwater
	S-06	Austria	Obergurgl, Ötzal valley	Firn snow, 2880 m altitude	08-2006	100 CFU l^{-1} meltwater
Lake and stream water						
	W-01	France	Vieux Pass, Hautes-Alpes	Foréant Lake near the pass at 2600 m altitude	06-2006	1.4×10^3 CFU l^{-1}
	W-02		Longet Pass, Hautes-Alpes	Longet Lake, 2600 m altitude	06-2006	130 CFU l^{-1}
	W-03		Vars, Hautes-Alpes	Mouth of Riou Mounal stream, 2100 m altitude	06-2006	1.5×10^4 CFU l^{-1}
	W-04	Italy	Longet Pass, Piemonte	Bes Lake, 2600 m altitude	06-2006	2.2×10^3 CFU l^{-1}
	W-05	USA	Bozeman, Montana	Hyalite Lake in the Hyalite canyon	10-2006	10^4 CFU l^{-1}
Rain						
	R-01	France	St Saturnin-les-Avignon, Vaucluse	Rainfall in residential zone	05-2006	10^4 CFU l^{-1}
	R-02				11-2006	100 CFU l^{-1}

Table 1 Continued

Substrate	*Sample code*	*Country*	*Place name and region or state*	*Site description*	*Sampling date (month-year)*	*Population size*[a]
Epilithic biofilms						
	E-01	France	Le Vernet, Ardèche	On rocks in waterfall along roadside	11-2005	50 CFU g^{-1} wet weight
	E-02		Vieux Pass, Hautes-Alpes	Stream, 2600 m altitude	06-2006	4×10^4 CFU g^{-1} wet weight
	E-03[3]		Auzon River, Vaucluse	Stream along roadside	01-2005	272 CFU g^{-1} wet weight
	E-04[3]	USA	Palisade Falls, Gallatin Co, Montana	On rocks in waterfall in pristine woods	09-2004	5.7×10^3 CFU g^{-1} wet weight
	E-05[3]		Pine Creek Falls, Park Co., Montana	On rocks in waterfall in pristine woods, 2200 m altitude	09-2004	659 CFU g^{-1} wet weight
	E-06[3]		Mill Creek, Salt Lake City, Utah	On rocks in stream on hillside	04-2005	333 CFU g^{-1} wet weight

Abbreviation: ND, not determined.
The names of strains characterized from each substrate and their properties are indicated in Table 2.
[a]Estimated size of the *P. syringae* population in the sample. For some plant samples collected early in the study, this was not determined.
Strains were previously described by Morris *et al.* (2007).

and were placed on concrete surfaces or at sufficient heights (1.5–2 m) to avoid splashing from the ground. The rainwater was transferred to a sterile container and then filter-concentrated as described above. When at least 500 ml of rainwater was collected, the water was concentrated by a factor of 100. Otherwise, the water was concentrated by a factor of 10. Aliquots of the concentrated and the crude rainwater were stored into sterile plastic containers.

Methods for the collection and processing of epilithic biofilms are described by Morris *et al.* (2007). Homogenates of these biofilms were transferred to sterile plastic containers.

Processed samples were dilution-plated on KBC medium (Mohan and Schaad, 1987), a modification of King's medium B (King *et al.*, 1954) (KB) supplemented with cephalexin and boric acid. We have previously used this medium for the detection of low densities of *P. syringae* in irrigation water retention basins (Riffaud and Morris, 2002). Some samples were also plated on 10% tryptic soy agar (per liter: 3 g tryptic soy broth (Difco, Detroit, MI, USA), 15 g agar) to estimate the total background mesophilic bacterial flora. Plates were incubated for up to 5 days at 22–25 °C. Production of fluorescent pigment on KBC was checked at 2 days after incubation, whereas definitive counts of fluorescent and total colonies were realized after 5 days of incubation. Fluorescent colonies were transferred to KB medium and the absence of cytochrome *c* oxidase was verified by streaking some of the 48 h culture with a sterile toothpick on filter paper imbibed with a fresh solution of 1% tetramethyl-ρ-phenylenediamine dihydrochloride (Gerhardt *et al.*, 1981). Colonies lacking the cytochrome oxidase were purified and tested for their capacity to induce a hypersensitive reaction in tobacco (described below). Strains inducing this reaction were stored in 0.1 M phosphate buffer at 4 °C and in 40% glycerol at −80 °C for further characterization. A total of 140 strains were maintained for this study (Table 2). An additional 18 strains from epilithic biofilms previously described (Morris *et al.*, 2007) were also included (strains originating from samples E-03 to E-06; Table 2).

Other bacterial strains

For comparison of phenotypes and genotypes of strains isolated in this study with other strains, we used the reference strains of *P. syringae* for which the genome has been sequenced (B728a pv. *syringae*, DC3000 pv. *tomato* and 1448A pv. *phaseolicola*) (*Pseudomonas syringae* Genome Resources: http://pseudomonas-syringae.org/home.html). Other strains of *P. syringae* from agricultural sources included the following strains: CC0001, CC0023, CC0024, CC0037, CC0094, CC0125, CC0206, CC0301, CC0354 and CC0440 from diseased cantaloupe in France and Morocco (Morris *et al.*, 2000); CC0393, CC0403, CC0406 and CC0412 from water retention basins used for irrigation in southwestern France (Riffaud and Morris, 2002); and reference strains from the *Collection Française de Bactéries Phytopathogènes* (INRA-Angers, France) (*P. syringae* pv. *syringae*-type strain CFBP 1392, *P. syringae* pv. *aptata* strain CFBP 1906 and *P. syringae* pv. *atrofaciens* strain CFBP 2256). *P. viridiflava* strain PV612 isolated from diseased cantaloupe in Crete (Goumas and Chatzaki, 1998) was kindly provided by D Goumas. Other strains that were used simply to test the efficiency of the isolation medium are presented in Table 3.

Evaluation of host range and factors related to pathogenicity

The capacity of strains to induce a hypersensitive response was determined in tobacco by infiltrating fully developed leaves of plants of *Nicotiana*

tobacum L. cv. Samsun at the 10-leaf stage (bacterial suspensions of 48 h cultures at approximately 1×10^8 CFU ml^{-1}). Strains that induced the hypersensitive response within 48 h of incubation were further characterized for biochemical properties, production of syringomycin and virulence on three plant species.

Virulence of strains was determined on sugar beet (*Beta vulgaris* var. *rapa* L. cv. Sucrière), lettuce (*Lactuca sativa* L. cv. Mantila) and cantaloupe (*Cucumis melo* var. *cantalupensis* Naud. cv. Védrantais). For each strain, five plants of each species were inoculated at the two-leaf stage with a 50 µl aliquot of inoculum. The inoculum consisted of aqueous suspensions of 48-h bacterial cultures from KB plates adjusted to an A_{580} of 0.06 ($\sim 3 \times 10^8$ CFU ml^{-1}). The inoculum was infiltrated into the leaf blade of the oldest leaf near the junction with the petiole. Plants were incubated for 7 days in the greenhouse at ambient conditions of 17–25 °C. Strain CC0094 of *P. syringae*, pathogenic to all three plant species (Morris *et al.*, 2000), was used as a positive control for all inoculation series. Sterile distilled water was used as a negative control. Symptoms were observed daily, and the reaction of the plant was scored at 7 days after inoculation as 0 (no obvious symptoms), 1 (hypersensitive-like reaction restricted to the point of inoculation), 2 (slight expansion of a necrotic zone of tissue away from the point of inoculation and/or necrosis and breaking of the petiole) or 3 (expansion of a necrotic zone of tissue away from the point of inoculation, leading to

Table 2 Phenotype of *P. syringae*-like strains isolated from nonagricultural habitats

Origin[b]	strain	Ice nucleation activity[c]	Toxin production[d]	Virulence[a]		
				Cantaloupe	Sugar beet	Lettuce.
P-01	CC0627	- 5°C	7			
P-01	CC0628	-	0			
P-01	CC0629	-	7			
P-01	CC0630	- 4°C	4			
P-01	CC0631	- 6°C	1			
P-01	CC0632	- 4°C	0			
P-01	CC0633	-	0			
P-01	CC0634	- 4°C	3			
P-01	CC0635	- 3°C	2			
P-01	CC0636	- 3°C	2			
P-01	CC0637	- 4°C	1			
P-01	CC0638	- 3°C	0			
P-01	CC0639	- 4°C	1			
P-01	CC0640	- 4°C	0			
P-01	CC0641	- 4°C	0			
P-01	CC0642	- 4°C	7			
P-01	CC0643	- 5°C	0			
P-01	CC0644	- 3°C	4			
P-02	CC0654	- 4°C	7			
P-02	CC0655	-	0			
P-02	CC0656	-	0			
P-03	CC0661	-	5			
P-03	CC0664	-	0			
P-04	CC0690	- 3°C	2			
P-04	CC0691	- 4°C	5			
P-04	CC0692	- 4°C	1			
P-04	CC0693	- 3°C	1			
P-05	CC1510	- 3°C	14			
P-05	CC1511	- 3°C	14			
P-06	CC0645	- 4°C	0			
P-06	CC0646	- 4°C	0			
P-06	CC0647	- 4°C	0			
P-06	CC0648	-	0			
P-06	CC0649	-	0			
P-06	CC0650	-	0			
P-06	CC0651	-	0			
P-06	CC0652	-	0			
P-06	CC0653	-	0			
P-07	CC0658	- 3°C	6			
P-07	CC0659	- 3°C	9			
P-08	CC0669	- 3°C	0			
P-08	CC0670	- 4°C	0			
P-08	CC0671	- 4°C	0			
P-08	CC0672	- 4°C	0			
P-08	CC0673	- 3°C	2			
P-08	CC0675	-	0			
P-09	CC0660	- 3°C	4			
P-10	CC0665	-	2			
P-10	CC0668	- 3°C	2			
P-11	CC1456	- 3°C	11			
P-11	CC1457	- 4°C	>20			
P-11	CC1458	- 3°C	>20			
P-12	CC1459	- 3°C	10			
P-12	CC1460	- 3°C	13			
P-12	CC1461	- 3°C	11			
P-12	CC1462	- 3°C	11			
P-12	CC1463	- 3°C	10			
P-13	CC1464	- 3°C	5			
P-13	CC1465	- 3°C	10			
P-13	CC1466	- 3°C	11			
P-13	CC1467	- 3°C	7			
P-13	CC1468	- 3°C	9			
P-13	CC1469	- 3°C	0			
P-14	CC1506	- 3°C	10			
P-14	CC1507	- 2°C	12			
P-15	CC1512	- 5°C	>20			
P-15	CC1513	- 4°C	0			
P-15	CC1514	- 4°C	0			
P-16	CC1584	- 4°C	0			
P-17	CC1515	- 2°C	5			
P-17	CC1516	- 3°C	10			
P-17	CC1517	- 4°C	0			
S-01	CC1475	- 3°C	7			
S-01	CC1476	- 3°C	3			
S-01	CC1477	- 4°C	0			
S-01	CC1478	- 3°C	10			
S-01	CC1479	- 4°C	7			
S-01	CC1480	- 4°C	0			
S-01	CC1481	- 3°C	4			
S-01	CC1482	- 3°C	9			
S-02	CC1497	- 3°C	6			
S-02	CC1498	- 3°C	3			

Table 2 Continued

Origin[b]	strain	Ice nucleation activity[c]	Toxin production[d]	Virulence[a] Cantaloupe	Sugar beet	Lettuce.	Origin	strain	Ice nucleation activity	Toxin production	Virulence Cantaloupe	Sugar beet	Lettuce.
S-03	CC1484	- 3˚C	9				W-01	CC1544	- 5˚C	0			
S-03	CC1485	- 3˚C	8				W-02	CC1537	- 3˚C	11			
S-03	CC1486	- 4˚C	0				W-02	CC1539	- 3˚C	0			
S-03	CC1487	- 4˚C	13				W-03	CC1526	- 3˚C	3			
S-03	CC1488	- 3˚C	9				W-03	CC1529	- 4˚C	0			
S-03	CC1489	- 3˚C	8				W-03	CC1530	- 6˚C	0			
S-03	CC1490	- 4˚C	0				W-04	CC1531	-	0			
S-03	CC1491	- 3˚C	8				W-04	CC1533	- 3˚C	12			
S-03	CC1492	- 4˚C	5				W-04	CC1535	- 5˚C	2			
S-03	CC1493	- 3˚C	6				W-04	CC1536	- 3˚C	6			
S-03	CC1494	- 3˚C	5				W-05	CC1586	- 3˚C	0			
S-03	CC1495	- 4˚C	0				W-05	CC1587	- 4˚C	2			
S-03	CC1496	- 5˚C	0				R-01	CC1499	- 3˚C	2			
S-04	CC1555	- 3˚C	4				R-02	CC1594	- 5˚C	0			
S-04	CC1556	- 5˚C	0				E-01	CC1470	- 3˚C	10			
S-04	CC1557	- 2˚C	6				E-01	CC1471	- 3˚C	0			
S-04	CC1558	- 4˚C	0				E-01	CC1472	- 3˚C	0			
S-04	CC1559	- 5˚C	0				E-01	CC1473	- 3˚C	0			
S-04	CC1560	- 3˚C	6				E-01	CC1474	- 3˚C	11			
S-04	CC1562	- 5˚C	0				E-02	CC1583	- 4˚C	0			
S-04	CC1563	- 5˚C	0				E-03	CC1435	-	1			
S-04	CC1564	- 2˚C	6				E-03	CC1448	-	4			
S-04	CC1565	- 2˚C	5				E-03	CC1449	-	2			
S-04	CC1566	- 4˚C	0				E-03	CC1450	-	4			
S-04	CC1567	- 3˚C	6				E-04	CC1416	- 3˚C	0			
S-04	CC1568	- 6˚C	0				E-04	CC1417	-	0			
S-05	CC1570	- 3˚C	6				E-04	CC1420	-	0			
S-05	CC1571	- 3˚C	6				E-04	CC1421	-	0			
S-05	CC1572	- 2˚C	5				E-04	CC1424	-	0			
S-05	CC1573	- 3˚C	7				E-04	CC1425	-	0			
S-05	CC1574	- 3˚C	7				E-04	CC1426	- 2.5˚C	0			
S-05	CC1576	- 3˚C	7				E-05	CC1427	- 3˚C	0			
S-05	CC1577	- 3˚C	6				E-05	CC1428	- 3˚C	0			
S-05	CC1578	- 3˚C	7				E-05	CC1429	- 3.5˚C	0			
S-05	CC1580	- 3˚C	9				E-05	CC1430	- 3.5˚C	0			
S-05	CC1581	- 3˚C	6				E-05	CC1431	- 5˚C	0			
S-06	CC1585	- 3˚C	0				E-05	CC1432	- 4˚C	0			
W-01	CC1543	- 5˚C	8				E-06	CC1455	- 3˚C	13			

All strains below were fluorescent on KBC medium, did not have cytochrome oxidase or arginine dihydrolase, they hydrolyzed esculin and induced a hypersensitive reaction in tobacco.
[a]Virulence on plants was considered positive (black squares) if more than half of the inoculated plants gave compatible reactions (score 2 or 3). Some strains caused compatible reactions on some, but fewer than half of the plants tested (grey squares) and some strains never caused compatible reactions (white squares).
[b]The origin of strains is indicated by the sample code. These codes are indicated in Table 1.
[c]The warmest temperature of activity among 10^7 cells tested per strain is indicated. (−) indicates that no activity was detected at −6 °C or warmer.
[d]The width (mm) of the zone of inhibition against *Geotrichum candidum* is indicated.

wilting or death of the entire leaf or the entire plant). Bacteria were re-isolated from selected plants to confirm that symptoms were caused by the inoculum.

Production of syringomycin was evaluated on SRM medium (Gross, 1985), according to the bioassay based on the sensitivity of *Geotrichum candidum* (Gross and DeVay, 1977). The distance between the border of the bacterial colony and the edge of the fungal lawn (zone of inhibition) was measured after 3 and 6 days of incubation.

Characterization of biochemical traits and ice-nucleation activity of strains

The presence of arginine dihydrolase, gelatinase and levan sucrase, the reduction of nitrate and the hydrolysis of esculin were tested as previously

Table 3 Recovery of reference strains of *Pseudomonas syringae* on KBC medium

Strain	*Pathovar*	*Genomic group*[a]	*% recovery*[b]
DC 3000[c]	pv. *tomato*	1	10
CFBP[d] 70	pv. *lachrymans*	1	13
CFBP 1657	pv. *maculicola*	1	44
CFBP 1740	pv. *maculicola*	1	0.3
CFBP 1779	pv. *syringae*	1	40
CFBP 2104	pv. *lachrymans* **T**	1	0.0003
CFBP 2212	pv. *tomato* **T**	1	6
CFBP 2353	pv. *theae* **T**	1	65
B728a[c]	pv. *syringae*	2	83
CFBP 1392	pv. *syringae* **T**	2	5
CFBP 1608	pv. *syringae*	2	63
CFBP 1617	pv. *aptata* **T**	2	55
CFBP 1620	pv. *antirrhini*	2	8
CFBP 1906	pv. *aptata*	2	93
CFBP 1957	pv. *syringae*	2	0.0005
CFBP 2105	pv. *pisi* **T**	2	79
CFBP 2339	pv. *aceris* **T**	2	70
CFBP 2896	pv. *japonica*	2	17
CFBP 4139	pv. *pisi*	2	88
1448 A[c]	pv. *phaseolicola*	3	12
CFBP 1642	pv. *mori*	3	0.00002
CFBP 1647	pv. *morsprunorum*	3	32
CFBP 1656	pv. *mori*	3	12
CFBP 2106	pv. *tabaci*	3	72
CFBP 2214	pv. *glycinea* **T**	3	109
CFBP 2343	pv. *eriobotryae*	3	0.001
CFBP 2351	pv. *morsprunorum* **T**	3	0.00003
CFBP 2897	pv. *myricae*	3	0.00003
CFBP 3999	pv. *tabaci*	3	52
CFBP 4089	pv. *myricae*	3	0.00003
CFBP 4100	pv. *eriobotryae*	3	15
CFBP 4217	pv. *castaneae*	3	49

[a]Genomic groups according to Sawada *et al*. (1999).
[b]Percent of the population of cells of each strain growing on KB medium that were able to grow on KBC.
[c]These are reference strains described on the site *P. syringae* Genome Resources: http://pseudomonas-syringae.org/home.html.
[d]Strains labeled CFPB were provided by the French National Collection of Phytopathogenic Bacteria (*Collection Française de Bactérie Phytopathogène*) at INRA-Angers, M. Le Saux curator (http://www.angers.inra.fr/cfbp/index_e.html).

described by Lelliot *et al*. (1966). Ice-nucleation activity at −2 to −6 °C was determined for drops of aqueous bacterial suspensions containing a total of 10^7 cells as described previously (Morris *et al*., 2007).

Genotypic characterization of strains

Sequences of four genes of the core genome, *rpoD*, *gyrB*, *cts* and *gapA* were compared among strains isolated here and with well-described type strains of *P. syringae* pathovars. PCR amplifications were performed on aqueous suspensions of whole cells adjusted to 2×10^8 CFU ml^{-1} ($OD_{580} = 0.12$), from 48-h cultures on KB medium with the primers described by Yamamoto *et al*. (2000) for *rpoD* and *gyrB* and by Sarkar and Guttman (2004) for *cts* and *gapA*, and with reagents from the Qiagen multiplex PCR kit (Qiagen, Courtaboeuf, France) in DNA-Engine PTC200 thermal cyclers (MJ Research, Waltham, MA, USA). For each strain, 4 µl of bacterial suspension were added to 50 µl of reaction mixtures containing 25 µl of the kit's master mix, 160 pg of each of the forward and reverse primers (final concentration of 0.2 µM of each primer), 5 µl of the kit's Q-solution and sterile distilled water. For *rpoD*, 40 cycles of amplification were performed with template denaturation at 94 °C for 30 s, followed by annealing for 90 s at 63 °C for *rpoD* and *gyrB* and at 62 °C for *cts* and *gapA*, and extension at 72 °C for 1 min with the final extension lasting for 10 min. For *gyrB*, initial 20 cycles of amplification were performed with template denaturation at 94 °C for 30 s, while annealing started at 53 °C for 1 min and increased by 0.5 °C with every cycle for a final annealing temperature at 63 °C and extension at 72 °C for 2 min. This was followed by 25 additional cycles of amplification with denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s and extension at 72 °C for 2 min with the final extension lasting for 7 min. For amplification of both genes, the initial cycle was preceded by 15 min of activation of the *Taq* polymerase at 96 °C.

Amplified products were electrophoresed on 2% agarose for 25 min at 80 V and purified by using the QIAquick kit (Qiagen), as per the manufacturer's instructions. Forward and reverse sequencing was conducted by Génome Express (Meylan, France) with the sequencing primers described previously (Yamamoto *et al*., 2000; Sarkar and Guttman, 2004).

Phylogenetic analysis

The DNA sequences obtained from the PCR products corresponding to the *rpoD*, *gyrB*, *cts* and *gapA* gene fragments of our strains were aligned and cut to the same size as that of the corresponding gene sequences deposited by Sarkar and Guttman (2004) in GenBank for strains 601, 301765, YM7902, N6801, FTRS _U7805, H5E1, K93001, KOZ8101, 301702, KN203, 301020, 6606, 302941, KN221, I_6 and 36_1. These strains represent the four genomic groups identified within *P. syringae* by Sarkar and Guttman (2004) based on an MLST analysis of seven gene fragments. Sequences for strains B728a, DC3000 and 1448A of *P. syringae*, for PAO-1 of *P. aeruginosa* and for Pf05 and Pf01 of *P. fluorescens* were obtained from GenBank and also cut to the same size. Seqman and Megalign (Lasergene, DNASTAR, Madison, WI, USA) were used for this step. The four fragments were concatenated and strains with identical sequences were identified using the nonredundant database program at http://pubmlst.org/cgi-bin/mlstanalyse/mlstanalyse.pl?site = pubmlst&page = nrdb&referer = usmirror1.pubmlst.org. The nonredundant alleles were then used to construct a Bayesian tree in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), which uses the Markov chain Monte-Carlo (MCMC) method. The program was run

for 2 600 000 generations with sample frequency of 10 and the s.d. of split frequencies was stabilized below 0.006. When summarizing the substitution model parameters and trees, 65 000 samples, representing the first 25% of all samples, were used for burn-in, that is, they were discarded to achieve an accurate estimate of the posterior probability distribution in the summary tree. All potential scale reduction factor (PSRF) values were close to 1.0. Two independent runs converged on the same tree.

Evaluation of selectivity and efficiency of the isolation medium

The relative efficiency of the isolation medium KBC for strains of *P. syringae* from the three different genomic groups proposed by Sawada *et al*. (1999) was compared to that of KB. Aqueous suspensions from 48-h cultures (25 °C on KB medium) of strains listed in Table 3 were dilution plated on both KB and KBC in three replicates per dilution. Total colonies were counted for up to 4 days of incubation at 25 °C.

Results

The strains isolated from non-agricultural substrates have properties consistent with those of pathogenic *P. syringae* from agricultural sources. All strains described here from non-agricultural substrates were fluorescent on KBC medium, they neither have cytochrome oxidase nor arginine dihydrolase, they hydrolyzed esculin and induced a hypersensitive reaction in tobacco. Furthermore, phylogenetic analyses based on sequences of gene fragments of the conserved housekeeping genes *rpoD*, *gyrB*, *cts and gapA* for a subset of the non-agricultural strains and known reference strains strongly support that strains from non-agricultural habitats are *bona fide P. syringae* (Figure 1). Hence, in the presentation of the results below, the non-agricultural strains will be referred to as *P. syringae*.

P. syringae *is abundant in non-agricultural habitats*

P. syringae was isolated from all substrates listed in Table 1. These included rain, snow, mountain lakes and streams, apparently healthy wild plants and epilithic biofilms. Many of these substrates were obtained in pristine areas above 2000 m altitude that could only be accessed on foot and were well above zones of agricultural production. In rain, in snowmelt water and in lakes and streams, *P. syringae* was present at concentrations generally between hundreds to several thousands bacteria per liter. However, in some cases, concentrations were as high as 10^4–10^5 bacteria per liter. Likewise, in association with wild plants, population sizes of *P. syringae* were several hundreds to 10^5 bacteria per gram or per leaf. *P. syringae* was not always found in the substrates analyzed. Nevertheless, with the exception of fresh snow, it was detected in over half of the samples of each type of substrate analyzed (Table 4).

P. syringae *strains from non-agricultural substrates are pathogenic and ice-nucleation active; the frequency of these traits varies with substrate*

A large proportion of the strains of *P. syringae* from non-agricultural sources was virulent on one or more of the hosts tested here (Table 2, Figure 2). Over 45% consistently induced compatible reactions on at least one of the three plant species tested, whereas 13% were virulent on all three species. Half of the strains also produced toxins (considered to be syringomycin-like) that inhibited *G. candidum*. The majority of strains (85%) were also ice-nucleation active. The proportion of strains with any particular phenotype, particularly ice-nucleation activity and toxin production, varied among the different substrates. For substrates from which at least 10 strains were characterized (wild plants, snow, water and epilithic biofilms), pair-wise, two-tailed Fisher's exact tests were used to determine whether the differences in proportions were significant. No significant differences ($P \leqslant 0.05$) were observed in the frequency of strains virulent on at least one of the host plants tested among the different substrates (Figure 2). There were marked differences among substrates in the frequency of strains virulent on all three plant species tested (15% from wild plants, 6% from snow, 23% from water and 8% from epilithon), but these differences were not significant at $P \leqslant 0.05$. However, for $P \leqslant 0.1$, the frequency of strains virulent for all three species was significantly lower for snow strains than for water strains. Epilithic strains produced syringomycin less often than strains from snow ($P = 0.0004$) and from wild plants ($P = 0.02$). All strains from snow were ice-nucleation active, and this was significantly more frequent in strains from wild plants ($P = 0.0004$) and from epilithon ($P = 0.0000$) (Figure 2).

P. syringae *strains from non-agricultural habitats are genetically very similar and in some cases indistinguishable from strains isolated from disease epidemics*

Phylogenetic analyses were performed on the concatenated sequences of gene fragments from four housekeeping genes: *rpoD* (coding for sigma factor 70); *gyrB* (coding for DNA gyrase B); *cts* (also called *gltA*, coding for citrate synthase); and *gapA* (coding for glyceraldehyde-3-phosphate dehydrogenase). These four gene fragments (total length 1850 bp) were found to give reliable evolutionary relationships within the *P. syringae* species when compared with the results obtained with seven gene fragments (Hwang *et al*., 2005).

Bayesain inference was used to build a phylogenetic tree (Figure 1). The tree was rooted on

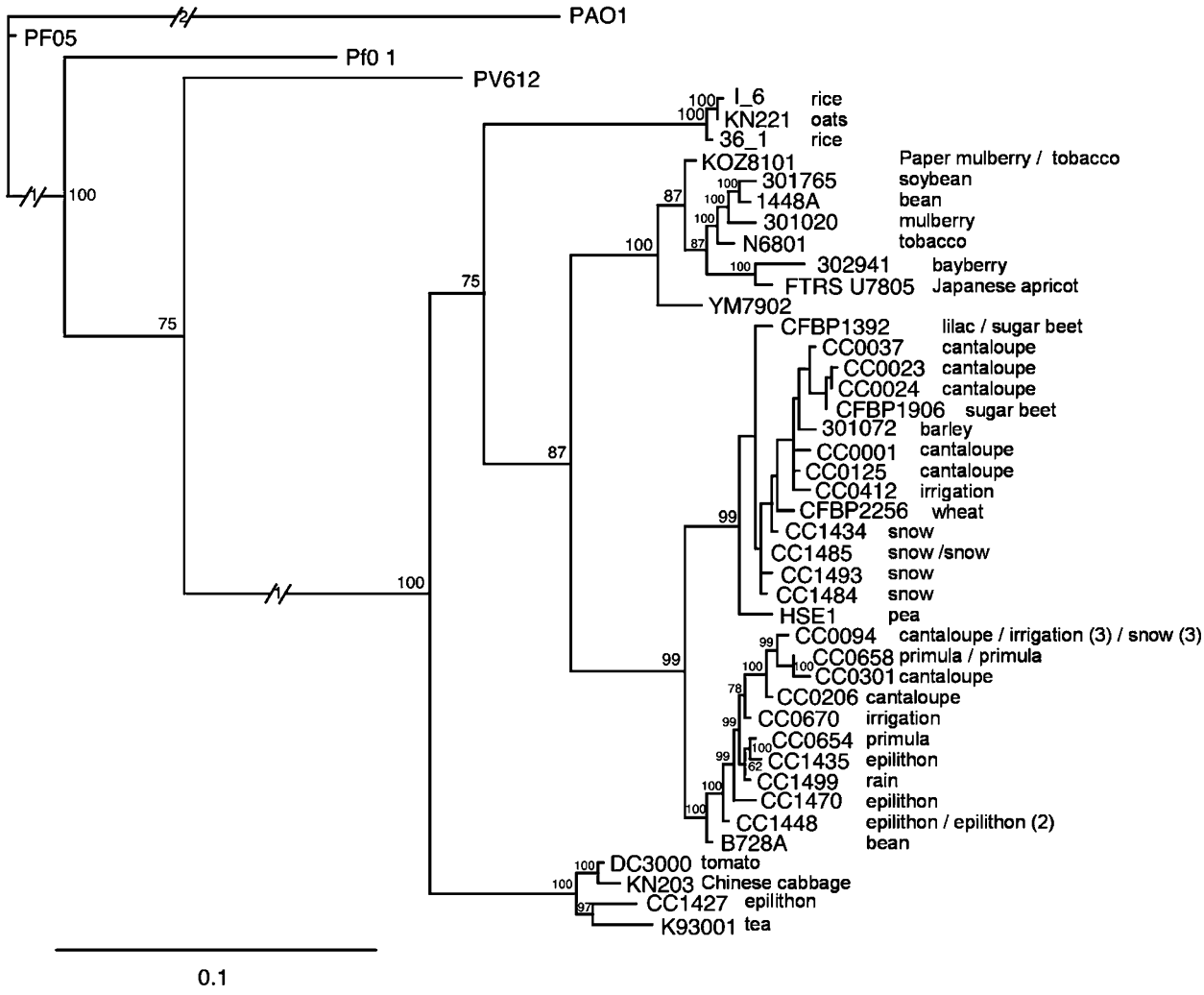

Figure 1 Phylogenetic tree constructed by Bayesian inference on the basis of the concatenated sequence of the four housekeeping gene fragments *gapA*, *cts*, *gyrB* and *rpoD*. The tree was rooted on *P. aeruginosa* PAO1. Branches that were shortened to fit the figure are indicated with /1/ and /2/. Posterior probabilities are indicated as percentages. The substrate of isolation for all *P. syringae* strains is indicated. In the case of strains with identical sequences, the source of the strain present in the tree is indicated followed by the source of any other identical strains (and by an indication of the number of identical strains from the same origin if there are more than one). There were six groups of strains with identical sequences for all four genes: (1) CFBP 1392 and 601; (2) KOZ8101 and 6606; (3) CC0037, CC0354, CC0440; (4) CC0094, CC0393, CC0403, CC0406, CC1475, CC1497 and CC1498; (5) CC0658 and CC0659 and (6) CC1448, CC1449, CC1450.

P. aeruginosa PAO1, which has a DNA identity of only approximately 65% with our strains and thus represents a sufficiently divergent outgroup. Several *P. syringae* strains representing the diversity within the species, a strain of *P. viridiflava* (the species most closely related to *P. syringae*) and two *P. fluorescens* strains were also included in the tree to precisely determine the taxonomic position of our isolates.

The Bayesian tree reveals that all but one of our isolates fall into the same branch that also includes the type strain of *P. syringae* (CFBP1392) and the sequenced *P. syringae* pv. *syringae* strain B728a. This branch has a posterior probability of 99%, which indicates a very strong statistical support for this branch. DNA identity between isolates on this branch is at least 97% for any of the gene fragments, indicating that all isolates on this branch are very closely related (see also Table 5). Moreover, this branch is part of a larger cluster with 100% support that includes exclusively *P. syringae* reference strains. The isolate CC1427 is part of this larger cluster. More precisely, CC1427 is located on a branch with a posterior probability of 100%, together with the sequenced DC3000 strain isolated from tomato and three other pathogenic *P. syringae* strains isolated from crops. The closest relative of *P. syringae*, *P. viridiflava* 612, is located outside of the *P. syringae* cluster. Therefore, phylogenetic analysis using Bayesian inference very strongly supports the conclusion that all of our isolates are members of the *P. syringae* species.

Even if our isolates cluster with pathogenic *P. syringae* strains isolated from crops, they could still form their own separate subclusters composed of strains that adapted to life in non-agricultural habitats. However, most of our strains do not cluster away from crop isolates. Most revealing is the cluster containing CC0094, CC0658 and CC0301 (Figure 1). The strains CC0658 and CC0659 (with identical sequence) were isolated from a wild primula plant and CC0301 was isolated from cultivated cantaloupe, while the strains identical in all four gene fragments to the cantaloupe strain CC0094 were isolated from irrigation water and from freshly fallen snow, which strongly suggests that CC0094-like strains cycle through all three substrates.

Table 4 Frequency of samples containing detectable populations of *P. syringae* among the total samples analyzed

Substrate	*Number of samples analyzed*	*Number of samples harboring P. syringae*[a]
Primulaceae	21	14
Other wild plants	5	4
Snow (firn)	5	3
Snow (fresh)	9	3
Lake and stream waters	8	5
Rain	4	2
Epilithic biofilms[b]	16	7

[a]Number of samples with detectable levels of strains corresponding to the three initial selection criteria: fluorescent on KBC medium, lacking cytochrome oxidase and causing a hypersensitive reaction in tobacco. Several samples contained only strains that did not induce a hypersensitive reaction. These strains will be described elsewhere.
[b]Values for epilithic biofilms include samples reported in a previous study (Morris *et al*., 2007).

The isolation medium permits isolation of strains representing all genomic groups of P. syringae but does not allow recovery of all strains at the same rate of efficiency

To evaluate the potential bias of the isolation medium for the types of *P. syringae* strains recovered from non-agricultural habitats, we determined the plating efficiency of the selective isolation medium KBC compared with KB medium on which all strains tested here can grow. KBC had a varying degree of selectivity for reference strains compared to KB. Percent recovery on KBC of the reference strains varied between just above 0% to over 100% relative to KB medium (Table 3). All of the three genomic groups identified by Sawada *et al*. (1999)

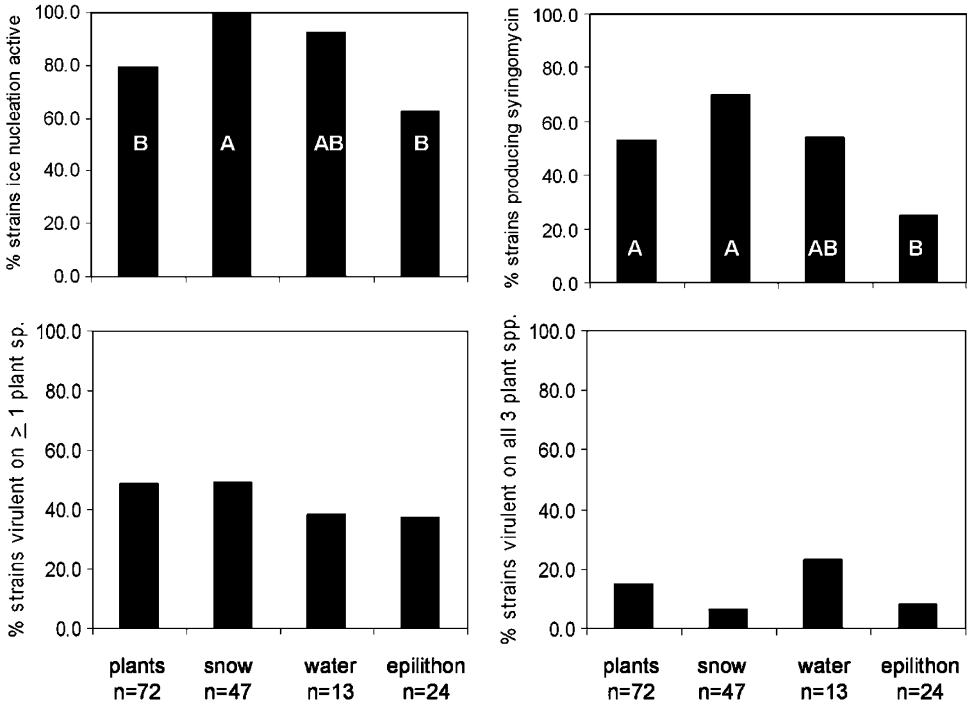

Figure 2 Frequency of ice-nucleation activity, of syringomycin-like toxin production and virulence in at least one or to all three host plants tested among strains from four non-agricultural substrates. Frequencies were calculated from results presented in Table 2. For syringomycin production, frequencies presented here concern strains with zones of inhibition $\geqslant 2$ mm. For virulence, frequencies were calculated in terms of strains producing compatible reactions on at least half of the plants tested per species (black squares in Table 2). The total number of strains characterized for each substrate is listed in parentheses below the substrate name. For each trait, the values represented by bars marked with a same letter are not significantly different ($P \leqslant 0.05$) according to a two-tailed Fisher's exact test. The Fisher's test was conducted for all pair-wise comparisons of substrates. For virulence, there were no significant differences.

Table 5 Similarity of strains of *P. syringae* from nonagricultural substrates to reference strains

Reference strain	*Non-agricultural strain*	*% difference in sequence*[a]				*Identical strains*[b]
		rpoD	*gyr*	*cts*	*gapA*	
CFBP 1392	CC1434	0.2	1.9	0.0	1.0	
	CC1485	0.2	1.7	0.0	0.0	CC1488, CC1494
	CC1493	0.2	1.7	0.0	0.8	
	CC1484	0.2	2.1	0.0	0.2	
B728A	CC1475	0.0	2.8	0.9	0.6	CC0094, CC0393, CC0403, CC0406, CC1497, CC1498
	CC0658	0.4	3.0	0.2	0.0	CC0659
	CC654	0.4	2.8	0.2	0.6	
	CC1435	0.4	2.3	0.7	0.6	
	CC1499	0.0	2.8	0.9	0.6	
	CC1470	1.4	2.8	1.2	0.0	
	CC1448	0.0	2.6	0.0	0.0	CC1449, CC1450
DC3000	CC1427	1.6	2.6	1.9	1.4	

[a]Similarity is expressed as the percent difference in sequence for partial sequences of 449 bases for *rpoD*, 480 bases for *gyrB*, 427 bases for *cts* and 497 bases for *gapA*.
[b]Strains with 100% sequence similarity for the partial sequences of all four genes.

had strains that could grow at recovery rates of over 50% of the total number of cells introduced onto the medium. On the other hand, numerous strains in each group were recovered at much lower rates. Only about 10% of the cells of DC3000 and 1448A that could grow on KB were recovered on KBC, whereas about 80% of the cells of B728a that grew on KB were recovered on KBC.

Discussion

Our results clearly demonstrate that plant pathogenic strains of *P. syringae* are ubiquitous in a wide range of non-agricultural substrates. Furthermore, we have also identified strains belonging to a same clonal lineage from both agricultural and non-agricultural substrates. Many of the non-agricultural substrates are non-plant substrates associated with alpine habitats or with the water cycle including snow, mountain lakes and streams, epilithic biofilms and rain. All species of wild plants sampled in this study also harbored pathogenic strains of *P. syringae*. The plant species examined here are all found in alpine and sub alpine zones. The Primulaceae, in particular, are also typically found in humid soils. Many of the samples collected here bordered lakes and streams. *Silene acaulis* forms dense mats on rocks without direct contact with the soil. Nevertheless, because of the altitude at which this perennial plant grows, it is likely to be repeatedly exposed to snow during its life.

Coupled to the current understanding of the ecology of *P. syringae* in agricultural systems and reports of its dissemination, our results lead us to suggest the life history or life cycle for *P. syringae* described in detail in Figure 3. This cycle was based on an initial proposition of Sands *et al.* (1982), suggested 25 years ago, for which we now provide significant corroborative evidence. We suggest that the water cycle is an important driver for dissemination of *P. syringae*, transporting it to a variety of permissive ecological niches, in addition to plants, in which it can survive. The high frequency (100%) of ice-nucleation active strains in snow samples relative to samples from other substrates corroborates the suggestion of Sands *et al.* (1982) that the ice-nucleation activity of *P. syringae* plays a role in this cycle. Nevertheless, this question will need to be further evaluated by an interdisciplinary approach combining microbiology, meteorology and atmosphere physics.

Water, in particular flowing water, has clearly been shown to harbor several species of plant pathogenic bacteria. For example, *Ralstonia solanacearum* can survive in aquatic habitats in association with plant roots (Elphinstone *et al.*, 1998; Wenneker *et al.*, 1999). Analysis of DNA from lake sediments in copper-mining regions also revealed the presence of *R. solanacearum*-like bacteria (Konstantinidis *et al.*, 2003). *Dickeya chrysanthemi* (formerly *Erwinia chrysanthemi)* is thought to be a part of the indigenous microorganisms on weeds and in sediments in alpine streams in Australia (Cother and Gilbert, 1990). Numerous studies illustrated that *Pectobacterium carotovorum* (formerly *E. carotovora*) is present in a wide range of surface and underground waters including those used in irrigation (McCarter-Zorner *et al.*, 1984; Harrison *et al.*, 1987). Franc (1988) further revealed that this bacterium was present in at least 80% of samples of ocean water and rainwater collected on the west coast of the United States and in samples of aerosols along the coast. It was also present in about 5% of snow samples collected in the Rocky Mountains where precipitation occurs from air masses originating over the Pacific west coast of the United States. This led him to propose that *P. carotovorum*

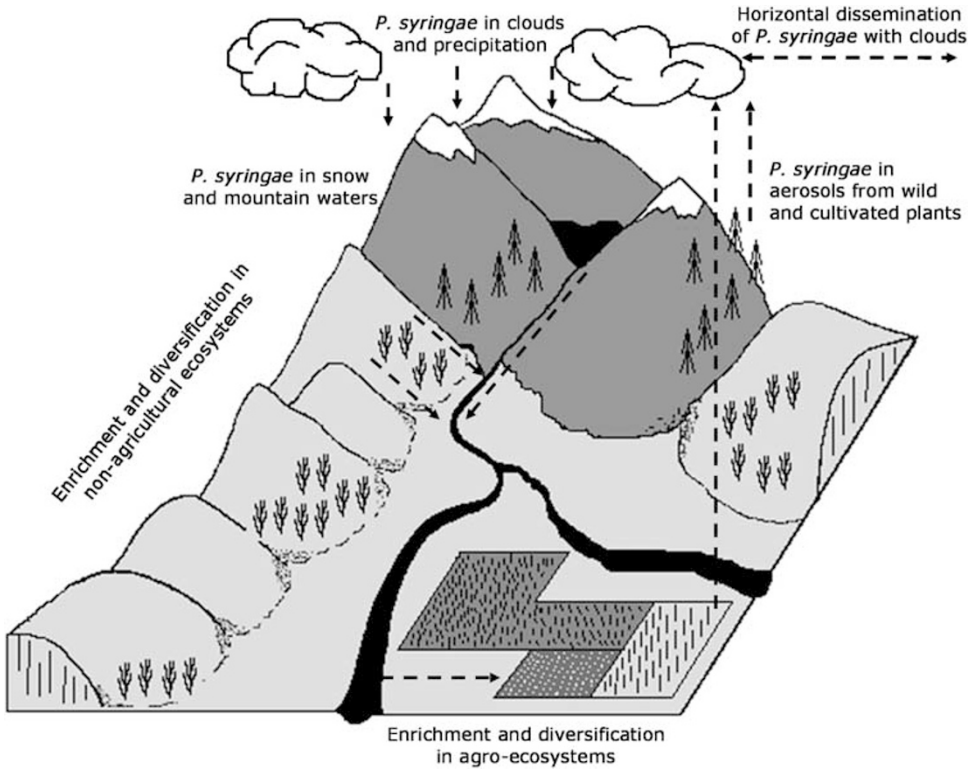

Figure 3 Hypothetical life cycle of *P. syringae*. Cells of this bacterium in clouds (Sands *et al*., 1982; Amato *et al*., 2007) and those scrubbed from the atmosphere are deposited with rain and snow. *P. syringae* is stocked in snow, but the duration of survival likely depends on environmental conditions and on the biotype of the bacterium. Rain and melting snow containing *P. syringae* feed into lakes and streams. These waters might be habitats for proliferation in the bulk water and/or in epilithic biofilms (Morris *et al*., 2007); streams are also vectors of dissemination. Water run-off from melting snow, rain, and streams and lakes transport *P. syringae* to wild plants and also wash bacteria from these plants and disseminate them elsewhere. These various niches harboring *P. syringae* (wild plants, snow, water and epilithic biofilms) each offer different selective pressures that likely have differential effects on the various biotypes of the *P. syringae* population. Some of these niches might contribute to the diminution of certain biotypes of *P. syringae*, whereas others might foster multiplication. Percolation of water underground as part of its trajectory down mountains will expose *P. syringae* to environmental conditions possibly differing in chemical concentrations, oxygen tension and other physical–chemical parameters compared to surface waters, thereby adding on additional selective pressures. Use of river waters for irrigation transports *P. syringae* to cultivated plants and to agricultural equipment. *P. syringae* will also come into contact with cultivated plants via rain or via irrigation systems based on collection of rain water (Riffaud and Morris, 2002) via aerial dissemination and via the other vectors previously described in studies of this bacterium in agricultural contexts. Certain biotypes of *P. syringae* might be better adapted than others to the stresses encountered in agricultural systems. *P. syringae* in the epiphytic phase on plants (wild or cultivated) are taken up by aerosols and transported to clouds in which they could be transported horizontally.

in ocean waters is taken up by aerosols, is transported inland by clouds and falls out inland with precipitation. This cycle also included the proposition that *P. carotovorum* enhanced cloud formation thereby enhancing rainfall due to the activity of its cells as cloud condensation nuclei (Franc and DeMott, 1998). In spite of the well-supported proposition of Franc and the other hypotheses concerning the role of upward flux in aerosols, of precipitation and of water flow in the long-distance dispersal of bacterial plant pathogens, plant pathology has remained relatively agrocentric in its consideration of the life cycles of pathogens. As far as we are aware, none of the life cycles of plant pathogenic bacteria presented in plant pathology textbooks and reviews takes into account the potential non-agricultural reservoirs to any significant extent. Here, we present a clearly illustrated hypothetical life cycle with the intent to promote further studies to validate and enrich our hypothesis for *P. syringae*, as well as to encourage new hypotheses for other plant pathogens.

The overall abundance of the different genotypes and phenotypes of *P. syringae* described in this study was likely influenced by the isolation medium used. Strains similar to B728a were the most abundant in the collection of non-agricultural strains examined here. This might be because bacteria related to B728a were more efficiently isolated on KBC medium than were those related to DC3000 and 1448A or because B728-like strains are in fact more abundant in non-agricultural substrates than DC3000-like and 1448A-like strains. Super-selectivity is a classic dilemma associated with the use of culture media in studies of population structure. Nevertheless, without this selective medium, which leads to a reduction of a factor of up to 10^{-4} of the background microflora

(Riffaud and Morris, 2002), it would have been impossible to isolate *P. syringae* from certain substrates. Furthermore, the bias of KBC medium is likely identical for all substrates. Hence, although the absolute population structure of each substrate is likely biased, we feel that our observations of comparative trends in population structure are not significantly influenced by the isolation medium. The relative abundance of 1448A-like and DC3000-like strains compared to B728-like strains in the analyzed substrates will need to be determined in future studies possibly taking advantage of culture-independent metagenomic approaches (Committee on Metagenomics, 2007).

The substrates in which *P. syringae* was detected might simply offer means of survival and accumulation of bacteria immigrating with water flow. However, our data suggest that selection pressures associated with the different substrates lead to different biotype frequencies. This may be the result of differential growth and survival in these substrates or due to the need for certain phenotypes to attain access to the substrate. For example, *P. syringae* might need to be active as an ice nucleus to become incorporated into snow (Morris *et al.*, 2004). This could explain why all strains from snow were ice-nucleation active, whereas significantly fewer strains from other sources were active as ice nuclei. Likewise, epilithic biofilms might offer a protective niche for strains that are not armed with syringomycin or related toxins, thereby allowing for a greater abundance of these deficient strains relative to other substrates. The different substrates also harbored varying frequencies of strains of *P. syringae*-like bacteria that did not induce HR in tobacco (data not presented here). The trends observed here should be confirmed with more extensive and completely randomized sampling of these substrates. Nevertheless, they suggest that non-plant substrates can play a role in structuring populations of *P. syringae* and therefore might be veritable habitats.

Our observations for *P. syringae* and those of Franc and co-workers concerning *P. carotovorum* evoke questions regarding sources and sinks in the flow of pathogens with the water cycle. Both of these plant pathogens can develop large populations when associated with plants, especially when plants become diseased. Based on current understanding of their abundance and population dynamics in agro-ecosystems, it is reasonable to propose that strains in non-agricultural habitats originate from plants, enter the water cycle via aerosol formation and then return to agricultural systems via rainfall or the use of irrigation waters containing these bacteria. It is currently unknown whether these pathogens proliferate to any significant extent in non-agricultural habitats, non-plant substrates in particular, and whether they can, in fact, be the sources of pathogens for epidemics. These features of the life cycle of these plant pathogens merit investigation.

Due to the current agrocentric view of the life cycles of plant pathogens, interest in the forces structuring populations of pathogens such as *P. syringae* has focused on cultivated plants (Hirano and Upper, 2000; Sarkar and Guttman, 2004; Guttman *et al.*, 2006). Our results open the door to studies of the comparative roles of a range of niches in the evolution of *P. syringae*, in the emergence of various phenotypes and in the organization of its genome. Such studies will likely lead to re-evaluation of the importance of coevolution of *P. syringae* with plants and the impact of plants on the emergence of pathotypes. The abundance of this bacterium in diverse substrates provides the opportunity to formulate hypotheses on the role of these substrates in driving the evolution of *P. syringae*—the body of alternative hypotheses needed for a rigorous evaluation of the notion of coevolution. The life history that we propose for *P. syringae* is also a rich context for testing more recent hypotheses concerning the emergence of pathotypes. In particular, Arnold *et al.* (2007) have suggested that adaptation of bacteria to an ensemble of multiple stresses leads to the acquisition of virulence factors and to the emergence of pathogenic variants. Mechanisms of adaptation to varied niches or habitats might shed light on the enigma of broad host range. The development of cultural practices and plant breeding strategies for effective disease management critically relies on an understanding of the environmental factors to which plant pathogens adapt and on an overall comprehension of their ecology both within and outside of agro-ecosystems.

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