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Selective isolation of indigenous *Pseudomonas syringae* strains with ice nucleation activity properties from a ski resort

Alexander L. SAVVIDES[1], Christos P. ANDRIOPOULOS[1], Konstantinos K. KORMAS[2], Dimitris G. HATZINIKOLAOU[1], Efstathios A. KATSIFAS[1] and Amalia D. KARAGOUNI[1*]

[1] *National and Kapodistrian University of Athens, Faculty of Biology, Department of Botany, Microbiology Group, 15781 Athens, Greece*

[2] *University of Thessaly, Faculty of Agricultural Sciences, Department of Ichthyology and Aquatic Environment, 38446 Nea Ionia, Greece*

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Four sampling sites were selected in the area of Velouhi mountain, Greece in order to screen for *Pseudomonas syringae* isolates with high ice nucleation activity from a ski resort environment. Bacterial isolates (n = 147) were obtained from soil and phyllosphere samples. Seven isolates exhibited morphological, biochemical and physiological profile similar to *P. syringae*. Phylogenetic relationships of the seven isolates were determined by 16S rRNA gene sequencing. Two isolates were phylogenetically affiliated to *P. syringae*, three to *P. viridiflava*, one to *P. avellanae*, and one *Pseudomonas* strain could not be assigned to a known species. The seven isolates were examined for their ice-nucleation activity properties. Three out of the seven studied isolates exhibited ice nucleation activity from –4.67 to –4.35 ice nuclei per cell, values similar to those obtained from a known ice-nucleation protein producer *P. syringae* strain and therefore could be used for the production of artificial snow in ski resort areas with short snow periods.

Key words: Greece, ice nucleation, *Pseudomonas syringae*, 16S rRNA, BOX-PCR, ski resort.

INTRODUCTION

Pseudomonas syringae participates in phyllosphere bacterial communities as a pathogen, ice nucleator, and epiphyte. Less common habitats are soil, rainwater and air where it seems to play an important role in the water cycle (Morris *et al*., 2004; 2008). Among the diversity of microbes that colonize leaves, none has received wider attention than *P. syringae*, as it is the first recombinant organism (*ice*$^{-}$ *P. syringae*) to be deliberately introduced into the environment (Gurian-Sherman & Lindow, 1993). More than 80 plant species growing in many different parts of the world are listed as hosts for strains of *P. syringae* and approximately 50 pathogenic varieties of this bacterial strain were described in the past (Hirano & Upper, 1995; 2000).

* Corresponding author: tel.: +30 210 7274704, fax: +30 210 7274901, e-mail: akar@biol.uoa.gr

The ice nucleation protein, that facilitates the formation of ice crystals at temperatures above –10˚C, is responsible for a high amount of crop losses each year. *Pseudomonas syringae* is not only the most widely distributed Ice Nucleation Active (INA) bacterium in nature but also among the most active. The discovery of biological ice nucleation proteins on the leaf surface opened biotechnological topics such as snow making and food processing. The largest volume commercial use of *P. syringae* is found in the spray-ice application area of artificial snowmaking, which has been shown to offer significant advantages over non-biologically nucleated snowmaking systems (Cochet & Widehem, 2000).

Aiming to isolate naturally occurring *P. syringae* strains with high ice nucleation activity, we selected sampling sites from a ski resort environment with very short snow period and a mean value of 24.5 days snow presence per year for the last 42 years. The ski resort was at the area of Velouhi Mountain, Karpe-

nisi, Greece. Soil and phyllosphere samples from the most common plants were collected from various sites. BOX-PCR analysis was performed for the differentiation of *Pseudomonas* isolates. Furthermore *P. syringae* isolates were identified by means of 16S rRNA gene sequence. Phylogenetic relationships of six type strains, two reference strains and the seven *P. syringae* isolates (based on their 16S rRNA sequences) were demonstrated. Three out of the seven isolates presented quite high ice nucleation activity, close to that of the reference strain *P. syringae* pv. *syringae* (CP000075).

MATERIALS AND METHODS

Bacterial strains, media and sampling

Pseudomonas syringae pv. *syringae* B728a (CP000075) and *P. syringae* pv. *tomato* DC3000 (AE016853) were used as reference strains throughout the phenotypical analysis, BOX PCR analysis and ice nucleation assay. Media used for isolation and cultivation of the reference strains and the isolates were Nutrient Agar (NA) (Atlas, 2000), King's B (KMB) medium (King *et al.*, 1954) and *P. syringae* Selective (PSSM) medium (Atlas, 2000). Media used for the phenotypic characterization were Sucrose Nutrient Agar (SNA, Nutrient Agar containing 50 g l^{-1} sucrose) and Thornley's 2A medium (Atlas, 2000). Incubation temperature for all cultures was 25°C. The isolates were recovered from the area of Velouhi mountain (Karpenisi, Greece 38°55′ N, 21°49′ E), nine km from Karpenisi in the Evrytania Prefecture. This ski resort was chosen because it is one of the most popular ski resorts in Greece and no commercial snow making bacteria or bacterial products have ever been used in the sampling area. Four sampling sites were chosen based on criteria of altitude and average winter temperature (Table 1). All samples were collected in November 2006. Approximately 100 g of soil was collected from 10 cm depth using a sterile 8 cm diameter plastic core. The sampling procedure followed a two dimensional matrix. The total sampling area was 20 m^2 and five soil samples were collected from each sampling site. Each soil sample was 2 to 10 m apart from each other and in order to avoid the effect of the proximity of plants, each soil sample was at least one meter far away from plant roots (Lipson & Schmidt, 2004). Each sample was placed separately in new Ziploc plastic bag, transported to the laboratory, and stored at 4°C until processing. The soil samples were passed through a 2-mm sieve before soil analysis, to remove rocks. Afterwards, ten g of each individual sample of every sampling site were mixed to provide a representative sample (50 g) for each sampling site. The standard time of leaves sampling was 08.00 to 10.00 to minimize the possible effect of the short-term fluctuations in *P. syringae* population. (Lindemann & Upper, 1985; Hirano & Upper, 1995). Portions of leaves of approximately 20 g were cut into pieces of ~5 cm^2. Each leaves sample was placed in a new Ziploc plastic bag, transported to the laboratory, and stored at 4°C until processing.

Isolation and phenotypical characterization of P. syringae isolates

Soil samples (50 g) were placed in sterile Erlenmeyer flasks, mixed with 500 ml sterile Ringer's solution

TABLE 1. Detailed description and characteristics of sampling sites. (a), (b) and (c) are different members of the family

	Sampling site 1 (ski resort slope)	Sampling site 2 (ski resort slope)	Sampling site 3 (off ski resort slopes)	Sampling site 4 (18 km south of ski resort)
Soil pH	6.8 ± 0.2	6.7 ± 0.2	6.8 ± 0.2	6.7 ± 0.2
Average winter temperature (°C)	7 ± 1	9 ± 1	12 ± 2	15 ± 2
Elevation (m)	1956	1890	1740	980
Plant genus and families*	*Cerastium*	Compositae	*Abies* (a)	*Cercis*
	Compositae (a)	*Daphne*	*Abies* (b)	Gramineae
	Compositae (b)	*Euphorbia*	Gramineae (a)	*Junglans*
	Daphne	Gramineae	Gramineae (b)	Leguminosae
	Gramineae (a)	Labiatae	*Prunus* (a)	*Platanus* (a)
	Gramineae (b)	*Phlomis*	*Prunus* (b)	*Platanus* (b)
	Labiatae	Scrophulariaceae	*Prunus* (c)	*Prunus*
	Phlomis	*Urtica*	*Rosa*	*Quercus*

*Plant genus and families from where phyllosphere samples derived

(0.25 strength) and shaken on an orbital shaker at maximum speed (500 rev min^{-1}) for 2 hrs. Leaf samples were placed in sterile Erlenmeyer flasks, mixed with 200 ml sterile Ringer's solution enriched with peptone (1 g l^{-1}) and shaken on an orbital shaker at maximum speed (500 rev min^{-1}) for 2 hrs (Lindow *et al.*, 1978). Mixtures were allowed to settle before making serial dilutions (up to 10^{-8}) of the supernatant fluids and plating on agar media (NA, KMB and PSSM) in triplicate. After incubation, each morphologically different bacterial colony was picked up and purified by further culture. All isolates were preserved in 30% (w/v) glycerol solution as a protective agent at –80°C.

Gram staining was performed using the Bactident Aminopeptidase kit (Merck, Germany). The KMB medium was used for detection of fluorescein. After 24-48 hrs of growth in the dark, colonies were examined for fluorescence under UV light (King *et al.*, 1954). Each isolate which was phenotypically characterized as *Pseudomonas* was further characterized according to the LOPAT test (Goszczynska *et al.*, 2000).

BOX-PCR analysis

BOX sequences are part of one out of three groups of repeated sequences that have been described for the prokaryotic genome. These sequences contain palindromic parts 35-40 bp in size. The BOX-PCR method is using specific primers for high conserved and repeated DNA sequences that exist in multiple copies in specific intergenic areas at the genome of the most Gram positive and negative bacteria. The BOX element (BOX1A) was amplified using the BOXA1R primer 5′- CTACGGCAAGGCGACGC TGACG-3′ (Rademaker & de Bruijn, 1997). The PCR amplification procedure was as described by Rademaker & Bruijn (1997). The BOX products were electrophorized in a 1.5 % (w/v) agarose gel for 16 hrs at 1.9 V cm^{-1} in 1×TAE. The PCR reaction for each one of the seven *P. syringae* isolates as well as for the two reference strains was repeated three times for reproducibility.

Taxonomy and phylogenetic analysis of P. syringae isolates

The selected *P. syringae* isolates were harvested from 1 ml overnight culture in Nutrient Broth at 30°C and 200 rev min^{-1}. DNA extraction was performed following the protocol of Haught *et al.* (1994). DNA concentration was determined with a spectrofluorimeter and adjusted to 40 ng μl^{-1}. A region of 16S rRNA was amplified by PCR using F27 and R1492 primers (Lane, 1991). Thereafter, 16S rRNA PCR products were extracted from agarose gel with Nucleospin Extract® PCR kit (Macherey-Nagel GmbH, Germany) following the manufacturer's protocol. Sequence data were obtained by capillary electrophoresis (Macrogen Inc., Korea). Each product was sequenced in one direction. The size of each sequence was approximately 900 bp. Similarity searches of the GenBank database were performed with BLAST (www.ncbi.nih.gov). Sequence data were compiled using the ARB software (Ludwig *et al*., 2004) and aligned with sequences obtained from the ARB and GenBank databases. Phylogenetic analyses was performed using the neighbour-joining method as determined by distance Jukes-Cantor analysis, implemented in PAUP* (Swofford, 2003). Heuristic searches under minimum evolution criteria used 1000 random-addition replicates, followed by tree bisection-reconnection topological rearrangements. Nodal support for 16S rRNA datasets was evaluated through 1000 bootstrapped pseudoreplicates. The GenBank accession numbers of sequences retrieved for compiling the final dataset were EU045445, EU045446, EU045447, EU045448, EU117208, EU117209 and EU117210.

Ice nucleation assay

The ice nucleation activity was quantified by the droplet-freezing technique described by Arvanitis *et al*. (1995). Each strain was cultivated in an overnight culture in Nutrient Broth at 24°C. The whole culture of each strain was serially (10-fold) diluted, and twenty droplets (10 μl each) from each dilution were placed on the surface of an aluminium foil sheet (spray coated with a 2% solution of paraffin in xylem and heat dried to remove the solvent) floating on an ethanol bath set at –9°C for 5 minutes. Ice nucleation activity was calculated by the equation of Vali (1971) using the "Ice" nucleation software program, version 1993, which was kindly provided by Professor C. Drainas, and was expressed as the logarithm of ice nuclei per CFU [log(ice nuclei/cell)] of the whole culture.

RESULTS

The total population of bacteria of the different sampling sites ranged from 1.1×10^2 to 4.7×10^6 CFU per g of sample. Differences among the three media were not statistically significant. Of all isolates, 47% were recovered from soil and 53% from phyllosphere.

TABLE 2. Characterization of the 7 selected isolates

Isolate Designation	GenBank accession number	Sequence alignment No of nucleotides*	Sequence alignment % identity**	Closest phylogenetic relative (GenBank accession number)
Pseudomonas sp. 1069	EU045445	927	99.24	*Pseudomonas* sp. (AY689083)
Pseudomonas sp. 1102	EU045446	883	99.31	*P. syringae* pv. *coryli* (AJ889841)
Pseudomonas sp. 1131	EU045447	911	98.20	*P. viridiflava* (AM182934)
Pseudomonas sp. 1133	EU045448	918	98.37	*P. viridiflava* (AM182934)
Pseudomonas sp. 1138	EU117208	925	98.59	*P. viridiflava* (AM182934)
Pseudomonas sp. 2048	EU117209	922	95.56	*P. avellanae* (AJ889839)
Pseudomonas sp. 4032	EU117210	913	99.99	*P. syringae* pv. *coryli* (AJ889841)

* The number of 16S rRNA nucleotides used for the alignment

** The percentage identity with the 16S rRNA sequence of the closest phylogenetic relative

From all sampling sites, soil or phyllosphere bacterial isolates were obtained, except for the soil sampling site 3. Approximately half of the 147 morphologically different bacterial colonies were collected from sampling site 1, with 41 isolates derived from soil and 30 from phyllosphere. Thirty-five isolates were obtained from sampling site 2 (13 of from soil and 22 from phyllosphere). Thirty-one isolates were obtained from sampling site 4, 15 of them were from soil and 16 from phyllosphere. Less isolates were obtained from sampling site 3. Nearly all isolates (144 out of 147) were characterised as Gram negative. The Gram negative isolates were examined with the LOPAT test and formation of fluorescent pigments was tested on KMB. Eighty-nine out of 144 isolates which were negative in the Kovacs' oxidase test were grown on KMB for the detection of fluorescent pigments under UV light. Only 31 of those proved to be positive and 23 out of 31 isolates did not exhibit arginine dihydrolase activity. Seven of these did not show pectolytic activity and were positive in the Tobacco hypersensitivity reaction. According to the above phenotypic characteristics those seven isolates had a profile similar to *P. syringae*.

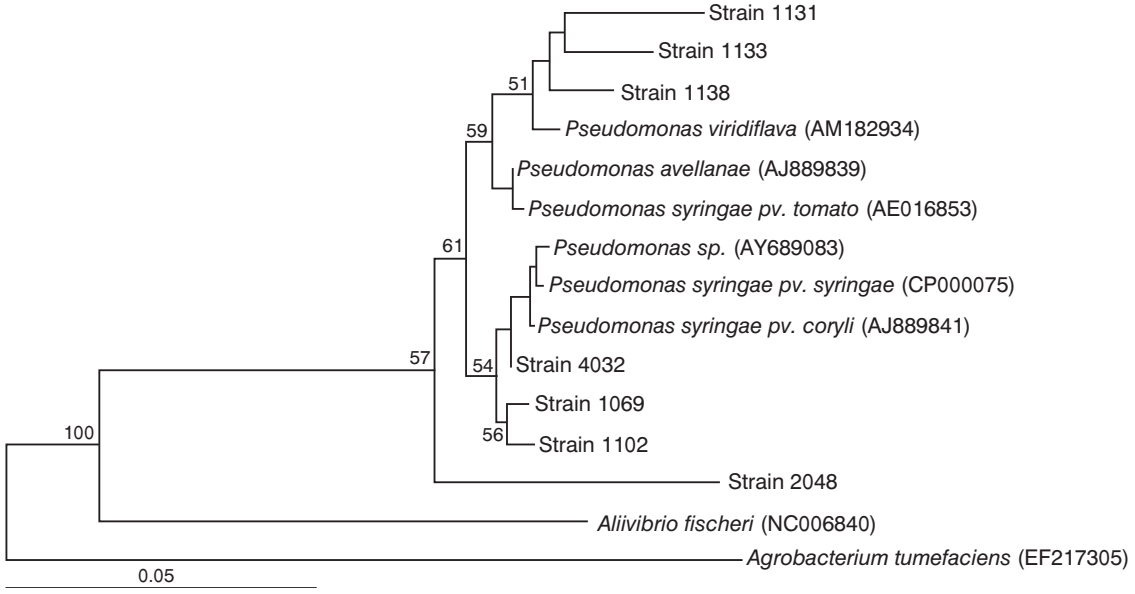

FIG. 1. Neighbor-Joining tree (Jukes-Cantor distance) based on 16S rRNA of the type strains, reference strains and *Pseudomonas* sp. isolates. One thousand bootstrap analyses were conducted, and bootstrap values greater than 50% are indicated at the nodes. The tree was rooted with *Agrobacterium tumefaciens*. Scale bar represents 5% estimated distance.

TABLE 3. Ice nucleation activity of isolated strains grown in Nutrient Broth at 24˚C

Isolate	log (ice nuclei per cell)
P. syringae pv. *syringae* B728a (CP000075)*	–4.08
Pseudomonas sp. 1102	–4.35
Pseudomonas sp. 2048	–4.97
Pseudomonas sp. 4032	–4.67
P. syringae pv. *tomato* DC3000 (AE016853)**	NF***

* Positive Control
** Negative Control
*** No freezing effects were recorded after the first 5 minutes on a –9˚C plate

Using BOX-PCR analysis (300-3000 bp) the two *P. syringae* reference strains were successfully discriminated, producing different band profile. In terms of the above seven *P. syringae* isolates, 5 unique fingerprints were derived, defining the isolates as 5 different strains. Isolates 1131, 1133 and 1138 shared the same BOX-PCR profile. These isolates were isolated from sampling site 1 and they had high sequence homology with *P. viridiflava* (AM182934) (Table 2).

The partial 16S rRNA gene sequences of the seven selected isolates were aligned with sequences from GenBank databases. The results presented in Table 2 indicated that *Pseudomonas* sp. isolate 1069 exhibited sequence homology with strain *Pseudomonas* sp. (AY689083); isolate *Pseudomonas* sp. 1102 exhibited high sequence similarity with our isolate *Pseudomonas* sp. 4032 (EU117210) which had 99.99% sequence similarity with strain *P. syringae* pv. *coryli* (AJ889841) and finally *Pseudomonas* sp. isolate 2048 had high sequence similarity with *P. avellanae* (AJ889839). Phylogenetic relationships among the studied *Pseudomonas* isolates on the basis of comparison of 16S rRNA sequences were further inferred by the neighbour-joining method (Fig. 1). The phylogram clustered the strains into sequence similarity groups. Isolates 1131, 1133 and 1138 are grouped in one cluster and are closely related with *P. viridiflava* (AM182934). Isolate 4032 is grouped in the same cluster with *Pseudomonas* sp. (AY689083), *P. syringae* pv. *syringae* (CP000075) and *P. syringae* pv. *coryli* (AJ889841). Isolates 1069 and 1102 are grouped to the cluster which is closely related to isolate 4032 as described above.

Only three *Pseudomonas* isolates, namely 1102, 2048 and 4032, showed ice nucleation activity (Table 3). Comparing the ice nucleation activity of the above isolates with the type strain of *P. syringae* pv. *syringae* (CP000075), isolate 1102, isolated from sampling site 1, exhibited the highest activity, close to that of the type strain [–4.35 log (ice nuclei per cell)].

DISCUSSION

Aiming to isolate indigenous *P. syringae* strains with high ice nucleation protein production, we have chosen a ski resort environment with a very short snow period (Cochet & Widehem, 2000). In this study, only seven isolates out of 147 bacterial isolates were characterized as *P. syringae* according to their biochemical profile. Only three out of these seven strains showed ice nucleation activity. As it was expected, the seven strains were isolated from phyllosphere which is a common habitat for *Pseudomonas* strains (Hirano & Upper, 2000). Five out of seven isolates were obtained from sampling site 1. The plant species diversity in sampling site 1 was quite different from the other three sampling sites (Table 1), which possibly explains why most of *Pseudomonas* strains were recovered from that site. It is known that plant species, among other factors such as daily cycles, temperature, radiation, humidity, wind velocity and leaf wetness, affect the *Pseudomonas* population size (Hirano & Upper, 1995).

BOX-PCR analysis was performed for the discrimination of the *P. syringae* isolates at the strain level. An adequate number of bands was obtained according to Rademaker & de Bruijn (1997), indicating that BOX-PCR can be applied for molecular typing within the genus *Pseudomonas*. BOX-PCR is generally used to distinguish related strains of the same species in several bacterial groups (Meintanis *et al.*, 2008). The isolates 1131, 1133 and 1138 that exhibit the same BOX-PCR profile, were derived from the same sampling site and shared the same biochemical characteristics with *P. viridiflava* (AM182934). The 16S rRNA sequence analysis showed that with the exception of isolate 1069 all seven isolates could be assigned to the *group* of *P. syringae* according to Anzai *et al.* (2000). Isolate 1069 could also be assigned to the same group based on our phylogenetic analysis (Fig. 1).

Hirano & Upper (1995, 2000) suggested that strains with ice nucleation activity are of a high biotechnological interest. Although it is not clear from the commercial products (like SNOWMAX®) whether they contain genetically modified bacteria, it is safer, under the considerations of EU regulations, to use indigenous isolates with biotechnological properties for two reasons: firstly they are well adapted to the local environment and secondly the application of such bacteria does not interfere with the existing biodiversity. The above suggestion supports the potential use of isolate 1102 in production of artificial snow for areas like the Karpenisi Ski Resort where the extension of the snow period is essential. Nevertheless the results for bacterial ice nuclei production were not satisfactory enough and further work is now in progress to improve productivity by cultivating the isolate 1102 in continuous flow culture. The results so far indicate that the ice nuclei production from isolate 1102 is above –2.8 log (ice nuclei per cell). Additionally it is in our plans to perform the ice nucleation spectra to estimate the temperature that the above isolate will initiate freezing. Depending on the outcome of the results, field experiments will be carried out in Karpenisi area.

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