Microb Ecol (2008) 56:283–291
DOI 10.1007/s00248-007-9346-7

ORIGINAL ARTICLE

62-kb Plasmids Harboring *rulAB* Homologues Confer UV-tolerance and Epiphytic Fitness to *Pseudomonas syringae* pv. *syringae* Mango Isolates

F. M. Cazorla · J. C. Codina · C. Abad · E. Arrebola · J. A. Torés · J. Murillo · A. Pérez-García · A. de Vicente

Received: 27 July 2007 / Accepted: 8 November 2007 / Published online: 6 December 2007

Abstract The presence of genetic determinants homologous to *rulAB* genes for ultraviolet (UV) radiation resistance was determined in a collection of *Pseudomonas syringae* pv. *syringae* strains isolated from mango. The potential role of these plasmids in UV tolerance and ecological fitness in the mango phyllosphere was also evaluated. Nearly all of the 62-kb plasmids present in the *P. syringae* pv. *syringae* strains hybridized with a *rulAB* probe, but these 62-kb plasmids showed differences in restriction patterns. *In vitro* assays of tolerance to UV radiation of *P. syringae* pv. *syringae* strains showed a higher survival of the strains harboring the 62-kb plasmids compared to strains lacking plasmids when exposed to UVC or UVA+B fractions. Similar results were observed when transconjugants harboring the 62-kb plasmid were tested. Survival assays were carried out under field conditions, and a higher survival of *P. syringae* pv. *syringae* strains harboring 62-kb plasmids under direct solar radiation on the adaxial surface of leaves was also observed. When the assays were carried out in shady areas or on the abaxial surface of leaves, survival time was comparable for all the assayed strains, whether or not they contained a 62-kb plasmid hybridizing to *rulAB*. Our results indicate that *P. syringae* pv. *syringae* strains harboring 62-kb plasmids show an increase in ecological fitness when colonizing the mango phyllosphere.

F. M. Cazorla · J. C. Codina · C. Abad · E. Arrebola · A. Pérez-García · A. de Vicente (✉)
Grupo de Microbiología y Patología Vegetal-Unidad Asociada CSIC, Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga,
Campus de Teatinos,
29071 Málaga, Spain
e-mail: adevicente@uma.es

J. A. Torés
Estación Experimental "La Mayora", C.S.I.C. Algarrobo-Costa, 29750 Málaga, Spain

J. Murillo
Laboratorio de Patología Vegetal, E.T.S. de Ingenieros Agrónomos, Universidad Pública de Navarra,
31006 Pamplona, Spain

Introduction

Mango (*Mangifera indica* L.) is one of the most important tropical and subtropical fruit crops in the world [22]. In Europe, most mango production is located in Southern Spain and Portugal. However, the commercial viability of this crop has been frequently threatened by the occurrence of bacterial apical necrosis, a disease elicited by *Pseudomonas syringae* pv. *syringae* [5, 6].

P. syringae pv. *syringae* is not only a phytopathogenic bacterium but also is an epiphyte associated with plant surfaces [13]. *P. syringae* pv. *syringae* exhibits an epiphytic phase achieving and maintaining large populations on healthy plants, where they are exposed to stressful conditions. Epiphytic *P. syringae* populations serve as inocula that can subsequently invade their hosts and initiate disease [6, 14]. The plant leaf surface (phyllosphere) is a changing environment subject to rapid and extreme fluctuations in parameters such as temperature, relative humidity, and solar radiation [33]. During the summer in Southern Spain, the phyllosphere is a difficult place for bacteria to survive because plants are exposed to heavy doses of solar radiation (including ultraviolet wavelengths) [9].

Approximately 3.2% of the total energy of solar radiation is in the ultraviolet (UV) range [8], which is categorized by photobiologists into three classes by

wavelength. UVC wavelengths (<290 nm) do not reach the earth's surface, as they are completely screened by ozone and oxygen. Ultraviolet wavelengths reaching the earth's surface are classified as UVA (320–400 nm) and UVB (290–320 nm) [16]. Lethality due to UVA exposure is attributed to the broad-spectrum effects of the intracellular generation of reactive oxygen species, including hydrogen peroxide, superoxide anion, and singlet oxygen, and not to direct DNA damage [26]. In contrast, the UVB component of solar ultraviolet radiation, as well as UVC, causes direct DNA damage; such lesions can be lethal in the absence of DNA repair mechanisms [10, 15, 25].

Ultraviolet radiation affects phyllosphere bacterial communities by playing an important role in the increased fitness of bacterial inhabitants. A number of autoecological studies have addressed the ability of phyllosphere bacterial strains to overcome environmental stresses, including ultraviolet radiation, and many of these studies have quite naturally focused on fitness characteristics of plant pathogenic strains (reviewed in [2]).

Phyllosphere bacterial communities are diverse and are composed of organisms displaying a range of tolerance to DNA-damaging ultraviolet radiation [15, 33]. Mechanisms to avoid UVA-induced oxidative stress in bacteria include carotenoid pigmentation and catalase and superoxide dismutase activities [15]. Furthermore, the production of an extracellular polysaccharide layer capable of absorbing ultraviolet radiation has also been implicated [21], although DNA repair mechanisms appear to be of higher importance for UV protection.

In *P. syringae*, tolerance to ultraviolet radiation is primarily conferred by the plasmid-encoded *rulAB* DNA-repair operon [32]. The *rulAB* genes are homologous to DNA repair operon *umuDC* of *Escherichia coli*, which is regulated as part of the SOS cellular system [10]. In the case of *P. syringae*, *rulAB* requires a functional *recA* gene for its expression, suggesting that *rulAB* may also be regulated by a SOS-like cellular system [32]. Expression of the *rulAB* operon is induced by DNA damage and typically results in a 10- to 30-fold increase in cell survival [19]. Likewise, the *rulAB* genes have been associated with the tolerance to UV radiations in *P. syringae* [32, 34] and also with the bacterial survival on phyllosphere [15, 33]. The importance of epiphytic growth on leaf surfaces to the epidemiology of most *P. syringae*–host interactions may explain the wide distribution of the *rulAB* determinant.

The aims of this study were to demonstrate the relationship between indigenous 62-kb plasmids harboring genetic determinants homologous to the *rulAB* genes and the tolerance to UV radiation in *P. syringae* pv. *syringae* isolated from mango and, furthermore, to determine its relevance to bacterial fitness in a plant phyllosphere exposed to heavy doses of solar radiation.

Materials and Methods

Microorganisms and Growth Conditions

Most of the *P. syringae* pv. *syringae* strains used in this study were isolated and identified from previous studies [5, 6, 7] and are listed with their principal characteristics in Table 1. Bacterial strains were routinely cultured on King's B medium (KBM) at 25°C. *P. syringae* pv. *syringae* FF5 is a copper-sensitive and plasmidless strain with a high efficiency of transformation by electroporation [31]; it was used to construct a stable kanamycin-resistant mutant (FF5-km) [7]. The FF5-km strain (Cu^s and km^r) was used as a receptor strain to perform conjugation experiments.

Biparental matings with *P. syringae* pv. *syringae* UMAF0081 harboring a 62-kb plasmid (Cu^r) and *P. syringae* pv. *syringae* FF5-km (km^r) were carried out as previously described [7] to obtain 62-kb plasmid transconjugants (Cu^r-km^r). Briefly, donor and recipient strains were grown overnight in Luria–Bertani broth (LB) and mixed in a 1:1 ratio, and the mixture was incubated overnight in LB at 27°C. Transconjugants were selected on mannitol-glutamate-yeast extract medium (MGY) [3] supplemented with 1 mM copper sulfate and 30 μg/ml of kanamycin. Two selected transconjugant strains (UMAFCB and UMAFCK, Table 1) were used to study the role of this 62-kb plasmid harboring the copper resistance genes and the *rulAB* homologs in UV radiation tolerance.

Chromosomal and Plasmid DNA Isolation and Characterization

Chromosomal DNA was extracted following a previously described method [27] with minor modifications. Briefly, bacterial cell pellets were frozen at −20°C for 15 min and then treated with a solution containing Tris, EDTA, sucrose, and lysosyme at 4°C. The suspension was treated with an alkaline solution with sodium dodecyl sulfate and stabilized with sodium acetate. The DNA pellet was obtained and washed with phenol/chloroform/isoamilic alcohol and precipitated. Chromosomal DNA was separated by electrophoresis in 0.8% agarose gels.

Plasmid DNA was isolated according to a modified alkaline lysis method [7, 38] and separated by electrophoresis on 0.6% agarose gels [24]. Plasmid size was estimated by comparison with plasmids from *Pseudomonas syringae* pv. *tomato* PT23. Purified plasmid DNA digested with *Eco*RI was separated by electrophoresis with 1% agarose gels.

Southern Blot Analyses

Plasmid or chromosomal DNA obtained as described above was transferred to nylon membranes and hybridized to

Table 1 Native plasmid content and presence of genetic determinants hybridizing to *rulAB* and *copABCD* probes in plasmid or chromosomal DNA, from *Pseudomonas syringae* pv. *syringae* (Pss) strains isolated from mango

Strain	Isolated from	Plasmid content (Kb)	*rulAB* hybridization of			*copABCD* hybridization
			Plasmid DNA	*Eco*RI-digested plasmid DNA	Chromosomal DNA	
Pss UMAF0114	Málaga, Spain	120	–	NT	–	+(120)[e]
Pss UMAF0167	Málaga, Spain	120	–	–	–	+(120)
Pss UMAF0001	Málaga, Spain	88, 62	+(62)[e]	+(1.2)[e]	NT	+(62)
Pss UMAF0049	Málaga, Spain	88, 62	+(62)	+(1.2)	NT	+(62)
Pss UMAF0119	Málaga, Spain	88, 62	+(62)	+(1.2)	NT	+(62)
Pss UMAF0123	Málaga, Spain	88, 62	+(62)	NT	NT	+(62)
Pss UMAF0139	Málaga, Spain	88, 62	+(62)	+(1.2)	NT	+(62)
Pss UMAF0048	Málaga, Spain	62	+(62)	+(1.2)	NT	+(62)
Pss UMAF0069	Málaga, Spain	62	+(62)	NT	NT	+(62)
Pss UMAF0081	Málaga, Spain	62	+(62)	+(1.2)	–	+(62)
Pss UMAF0128	Málaga, Spain	62	+(62)	+(1.2)	NT	+(62)
Pss UMAF0158	Málaga, Spain	62	+(62)	NT	NT	–
Pss UMAF0170	Málaga, Spain	62	+(62)	+(1.2)	NT	+(62)
Pss UMAF0176	Málaga, Spain	62	+(62)	+(13)	–	–
Pss UMAF0181	Málaga, Spain	62	+(62)	+(13)	NT	–
Pss UMAF1029	Huelva, Spain	62	+(62)	NT	–	–
Pss UMAF1065	Huelva, Spain	62	+(62)	NT	–	+(62)
Pss UMAF1094	Huelva, Spain	62	+(62)	NT	–	–
Pss UMAF1110	Huelva, Spain	62	+(62)	NT	–	+(62)
Pss UMAF2012	Faro, Portugal	62	+(62)	NT	–	NT
Pss UMAF2016	Faro, Portugal	62	+(62)	NT	–	+(62)
Pss UMAF6582[a]	Málaga, Spain	62	+(62)	NT	NT	NT
Pss Ps35	Israel	62	–	NT	–	NT
Pss UMAF1128	Huelva, Spain	62, 45	+(62)	NT	–	+(45)
Pss UMAF2026	Faro, Portugal	45	–	NT	NT	+(45)
Pss UMAF0005	Málaga, Spain	Plasmidless	–		–	NT
Pss UMAF0118	Málaga, Spain	Plasmidless	–		–	–
Pss UMAF0138	Málaga, Spain	Plasmidless	–		–	NT
Pss UMAF0174	Málaga, Spain	Plasmidless	–		–	–
Pss UMAF0509	Málaga, Spain	Plasmidless	–		–	NT
Pss UMAF2010	Mesines, Portugal	Plasmidless	–		–	–
Pss UMAF2025	Faro, Portugal	Plasmidless	–		–	+(chr)
Pss UMAF2802	Canarias, Spain	Plasmidless	–		–	NT
Pss UMAF2901	Granada, Spain	Plasmidless	–		–	NT
Pss Ps10	Israel	Plasmidless	–		–	NT
Pss UMAF6003[b]	Málaga, Spain	Plasmidless	–		–	NT
Pss UMAF0049c4[c]	Cazorla et al. [7]	62	+(62)	+(1.2)	NT	NT
Pss UMAF0119c5[c]	Cazorla et al. [7]	62	+(62)	+(1.2)	NT	NT
Pss FF5-km	Cazorla et al. [7]	Plasmidless	–	–	–	–
Pss UMAFCB[d]	This study	62	+(62)	+(1.2)	NT	+(62)
Pss UMAFCK[d]	This study	62	+(62)	+(1.2)	NT	+(62)
Ps pv. *tomato* PT23	Bender and Cooksey [3]	100,83,65,35	+(100, 35)	NT	–	+(83, 35)

– Hybridization has not been observed; *NT* not tested

[a] Isolated from a peach tree

[b] Isolated from a wild plant (*Crocus* sp.)

[c] These strains are derivatives from UMAF0049 and UMAF0119 respectively, cured of the 88-kb plasmid but harboring the 62-kb plasmid.

[d] Transconjugant strains (Cu^r, km^r) selected from biparental matings of Pss FF5-km (km^r) × Pss UMAF0081 (Cu^r).

[e] In parentheses, the size of plasmids and *Eco*RI restriction fragments of 62-kb plasmid or chromosome hybridizing with *rulAB* or *copABCD* probes

radioactive probes following standard procedures [28]. Additionally, approximately 5 μg of plasmid DNA was digested with *Eco*RI, separated by electrophoresis in 1% agarose gel and likewise, also transferred to nylon membranes, and hybridized to different probes. Hybridization probes were a 4.4-kb *Pst*I fragment from pCOP2, containing the *copABCD* genes from *P. syringae* pv. *tomato* PT23 [4], a 0.7-kb *Pst*I-*Bam*HI fragment from pAKC, containing part of the *rulAB* genes from *P. syringae* pv. *tomato* PT23 [29, 32], and a 0.8-kb *Eco*RI fragment from pAKC, containing the *oriV* gene from *P. syringae* pv. *tomato* PT23 [23]. The probes were labeled with [α-^{32}P] dCTP using the random primer method [28]. Hybridization signals were detected after a scan in a FUJI BAS 1500 using a FUJIX BAS IP-CST 2040 cassette and analyzed with the MacBas software (Fuji Photo Film, Tokyo, Japan). All experiments were performed at least twice.

Resistance of *P. syringae* pv. *syringae* Strains to UV Radiation

To evaluate tolerance to UVC radiation, bacterial suspensions (10^8–10^9 CFU ml^{-1}) of selected *P. syringae* pv. *syringae* strains were irradiated in a chamber, under UV-lamps (Sylvania G1578, Barcelona, Spain) with a maximum emission at 253.4 nm; UVC irradiance in these experiments was 0.033 W m^{-2}. After 10, 20, 30, and 60 s of irradiation, serial dilutions were performed and plated on KBM, incubated at 28°C for 48 h [6], and bacterial counts were determined.

Similar assays were performed to evaluate the effect of simultaneous exposure to UVA and UVB radiation, but in this case, experiments were carried out at two different irradiance levels. The various studies were carried out under one lamp TL-12 (Philips, Eindhoven, The Netherlands), one lamp QP-351, and two lamps QP-340 (Q-Panel Lab Products, Bolton, UK) to cover the UV spectra (250–400 nm), and using a Folex-295 filter (Folex GmbH, Dreieich, Germany) to avoid the UVC radiations below 295 nm. The irradiance levels were monitored by measurement with a LICOM Li-1800 radiometer (UW Li-Cor Radiation Sensors, Lincoln, USA). In the first set of experiments, bacterial suspensions were simultaneously exposed to high intensities of UVA (55.2 W m^{-2}) and UVB (4.11 W m^{-2}) radiation, and bacterial counts were determined after 30, 60, 75, and 90 min of exposure as described above. In a second set of experiments, the applied irradiance levels were 22 W m^{-2} of UVA and 1.4 W m^{-2} of UVB, which approximate the conditions of a very sunny summer day in Southern Spain [9]. Bacterial counts were determined every hour during the 4-h experiment. All of these experiments were repeated at least three times.

Survival of *P. syringae* pv. *syringae* on Mango Leaf Surfaces Under Sunlight

Survival curves of *P. syringae* pv. *syringae* cells exposed to direct sunlight on mango leaves were performed in two groups of 2-year-old mango trees grown in pots, during July at the "La Mayora" Experimental Station (Málaga, Spain). One group of 15 trees was located in a sunny area directly exposed to sunlight, whereas the other group was maintained in a shady area. Eight drops of 20 μl of bacterial suspensions (10^8–10^9 CFU ml^{-1}) were placed on the upper (adaxial) or on the lower (abaxial) surface of mango leaves, under direct sunlight. Three leaf samples from different trees containing bacterial cells of each assayed strain were taken every hour (from 10:00 to 13:00 hours) and the *P. syringae* levels determined on KBM after incubation at 28°C for 48 h, as previously described [6]. The UVA and UVB radiation intensities at leaf surfaces during the experiments (Table 2) were determined with a Macam UV203 radiometer (Macam Photometrics, Livingston, UK).

Statistical Analysis

Data were statistically analyzed using analysis of variance followed by Fisher's least significant difference test (p=0.05) using Statistical Package for the Social Sciences (SPSS) software (SPSS, Chicago, USA). All the experiments were performed at least three times.

Results

Detection of Homology to *rulAB* Probes Associated with 62-kb Plasmids

Plasmid occurrence was examined by agarose gel electrophoresis of DNA from 37 *P. syringae* pv. *syringae* strains, which were mostly isolated from mango (Table 1), and to illustrate the observed plasmid profiles, a picture of a gel obtained with some representative strains is presented in

Table 2 UVA and UVB radiation intensities at mango leaf surfaces at Estación Experimental "La Mayora" during the field experiments, shown in Fig. 5

Conditions	UV-A radiation (W m^{-2})	UV-B radiation (W m^{-2})
Adaxial leaf/sunny	20.8–49.9	0.21–3.10
Abaxial leaf/sunny	2.4–6.2	0.02–0.54
Adaxial leaf/shady	2.1–3.5	0.00–0.20
Abaxial leaf/shady	0.1–0.9	0.00–0.00

Fig. 1a. Results showed the frequent presence of a 62-kb plasmid (62.2% of tested strains), either alone or in addition to either an 88- or 45-kb plasmid. Two strains showed a 120-kb plasmid (Table 1).

Detection of genetic determinants homologous to *rulAB* was performed by Southern blot experiments, showing hybridization signals in nearly all 62-kb plasmids (95.5%), with the unique exception of strain *Ps*35 (Table 1). In contrast, genetic sequences homologous to *rulAB* were not detected in either strains lacking the 62-kb plasmid or possessing other plasmids (120-, 88-, and 45-kb). To illustrate these *rulAB* hybridization experiments, a representative picture is shown in Fig. 1b.

All 62-kb plasmids were determined to belong to the family of pPT23A-like plasmids, having a replication origin homologous to *oriV* (data not shown); however, they are not identical and show a degree of diversity. Only 68% of the 62-kb plasmids hybridized with a DNA probe specific for genes *copABCD*, conferring resistance to copper (Table 1). The 62-kb plasmids could be classified in at least two different groups with respect to the *Eco*RI pattern of hybridization to *rulAB* (1.2- and 13-kb; Table 1, Fig. 1c).

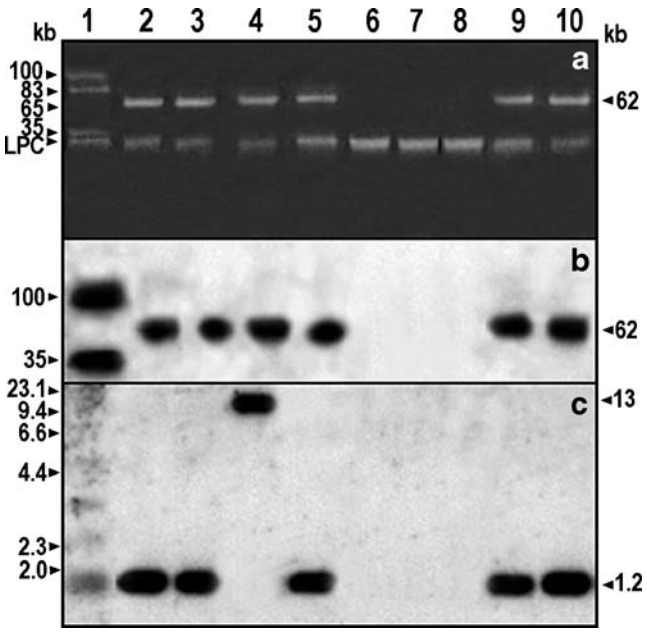

Figure 1 Detection of indigenous plasmids and homologous sequences to *rulAB* genes in some representative *P. syringae* pv. *syringae* strains isolated from mango. **a** Native plasmids of *P. syringae* pv. *syringae* strains separated by electrophoresis on a 0.6% agarose gel. **b** Southern blot analysis of plasmid DNA from the gel shown in A using the *rulAB* probe. **c** Southern blot analysis of plasmid DNA with the *rulAB* probe, after *Eco*RI digestion. *Lane 1*: *P. syringae* pv. *tomato* PT23 (used as molecular size marker and hybridization control). *P. syringae* pv. *syringae* isolates from mango are in *lanes 2*, UMAF0081; *3* UMAF0048; *4* UMAF0176; *5* UMAF0170; *7* UMAF6003; *8* UMAF0167. In *lanes 9 and 10*, *P. syringae* UMAFCB and UMAFCK, transconjugant strains of *P. syringae* pv. *syringae* FF5-km (*lane 6*) and *P. syringae* pv. *syringae* UMAF0081 (*lane 2*). *LPC* Linear plasmid and/or chromosome

Relationship Between UV Tolerance and Occurrence of 62-kb Plasmids

To study the role of the 62-kb plasmids harboring *rulAB* homologs in the resistance to UV radiation, survival experiments after UV light exposure were performed. Firstly, we employed UVC radiation because the higher energy of UVC wavelengths more readily distinguishes differences in the UV sensitivity of individual strains [35]. Strains harboring 62-kb plasmids showed a high level of tolerance to the UVC radiation doses assayed (Fig. 2), exhibiting reductions of approximately four orders of magnitude after 60 s of exposure. In contrast, strains lacking plasmids or plasmid-encoded *rulAB* homologues genes (UMAF0167) showed a lower tolerance, exhibiting reductions in the population levels of six orders of magnitude (Fig. 2). To confirm the association between the 62-kb plasmid and UV tolerance, survival experiments after UVC light exposure were carried out with two transconjugants with a 62-kb *rulAB*+ plasmid and their parental strains (*P. syringae* pv. *syringae* UMAF0081 and *P. syringae* pv. *syringae* FF5-km). We observed a significant higher UV-tolerance by transconjugant strains compared to the parental plasmidless strain FF5-km (Fig. 2).

We next tested the relationship between tolerance of UVA+B and the occurrence of 62-kb plasmids carried by some of the *P. syringae* strains. Firstly, as a comparison to other published works, we tested bacterial tolerance at high intensity levels of UVA [16, 33] and UVB [12] (Fig. 3). When high doses of UVA and UVB radiation were used, the strains harboring the 62-kb plasmid showed a higher tolerance to UVA+B exposure, in comparison to the plasmidless strains, with differences in the surviving bacterial population of approximately three orders of magnitude (Fig. 3). A second set of experiments was carried out to simulate natural conditions; UVA+B light effect on *P. syringae* pv. *syringae* strains was studied by the exposure of bacterial suspensions to intensities of UVA and UVB radiation similar to those that can be measured on the Mediterranean basin in summer (Fig. 4) and that are two to three times lower than those used in the previous experiments (Fig. 3). A higher survival of strains harboring the 62-kb plasmid with respect to the plasmidless strains was also observed. Bacterial population levels of strains lacking 62-kb plasmids were up to four orders of magnitude lower than the most resistant strain. However, the population levels of strains harboring the 62-kb plasmid were only reduced by one order of magnitude (Fig. 4). When transconjugant strains were used in both experiments, similar

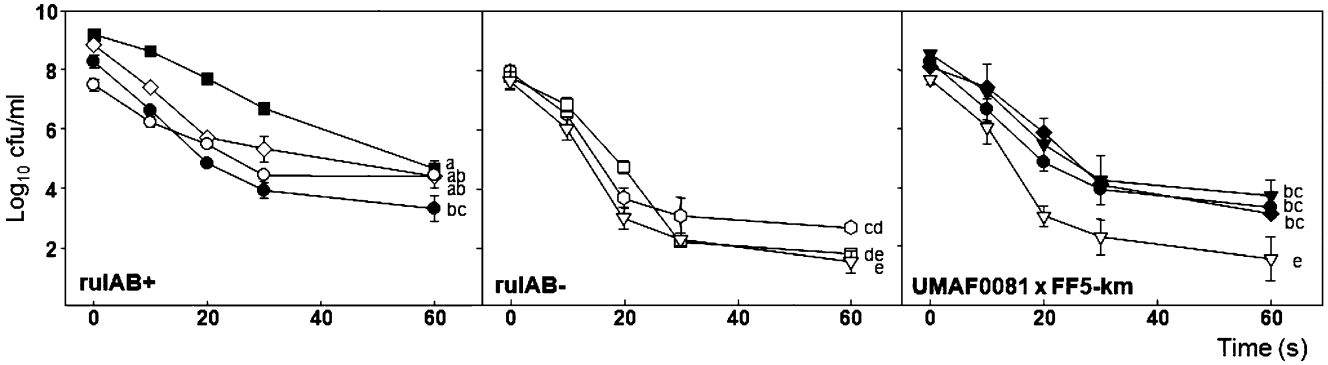

Figure 2 Survival of *P. syringae* pv. *syringae* strains under UVC radiation with an irradiance maximum of 0.033 W m^{-2} at 253.4 nm. The following strains were studied: UMAF0048 (*open circle*), UMAF0081 (*filled circle*), UMAF0167 (*open hexagon*), UMAF0170 (*filled square*), UMAF0176 (*open diamond*), UMAF6003 (*open square*), FF5-km (*open inverted triangle*), UMAFCB (*filled diamond*), UMAFCK (*filled inverted triangle*). *Different letters* denote that mean values at the final point of the assay (60 s) were significantly different, according to least significant difference test (p=0.05). *Bars* represent the standard deviation from three independent experiments

results to those shown by wild strains harboring the native 62-kb plasmid were observed (Figs. 3 and 4).

62-kb Plasmids Confer Enhanced Epiphytic Fitness on Mango Phyllosphere Under Sunlight

To verify if 62-kb plasmids could play an important role in the persistence of *P. syringae* pv. *syringae* in the phyllosphere of mango, bacterial survival was evaluated in field assays on mango leaves, both in their adaxial and abaxial surfaces and in two groups of trees, one situated in a sunny area and the other in a shady place (Table 2). *P. syringae* pv. *syringae* strains harboring 62-kb plasmids showed significantly higher survival ability on mango phyllosphere directly exposed to sunlight in sunny summer days than plasmidless strains. Strains harboring the plasmid with *rulAB* homologs showed a decrease in bacterial population levels between two and three orders of magnitude after 4 h of exposure to direct sunlight, whereas plasmidless strains showed a higher decrease in bacterial populations of nearly five orders of magnitude (Fig. 5a). In contrast, there were no significant differences when parallel assays were performed in shady areas (Fig. 5c, d) or when bacteria were placed on the abaxial surfaces of leaves directly exposed to solar radiation (Fig. 5b).

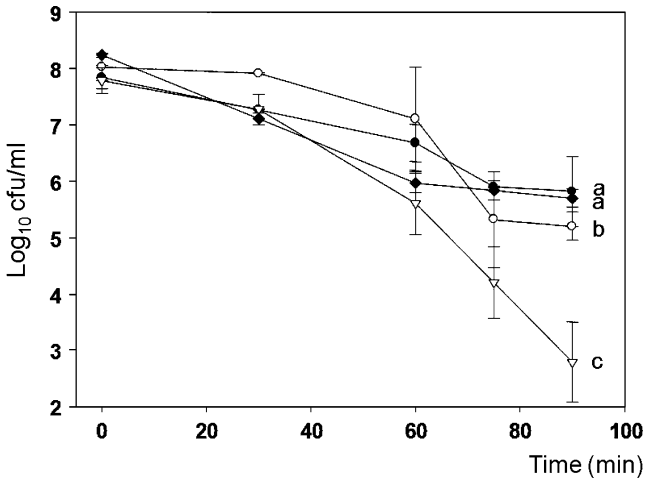

Figure 3 Survival of *P. syringae* pv. *syringae* strains after different exposures to high intensities of UVA + B radiation (55.2 W m^{-2} of UVA and 4.11 W m^{-2} of UVB). The following strains were studied: UMAF0048 (*open circle*), UMAF0081 (*filled circle*), FF5-km (*open inverted triangle*), UMAFCB (*filled diamond*). *Different letters* denote that mean values at the final point of the assay (90 min) were significantly different, according to least significant difference test (p= 0.05). *Bars* represent the standard deviation from three independent experiments

Discussion

P. syringae pv. *syringae* is regularly exposed to natural UV radiation in its phyllosphere habitat [34], which can cause extensive DNA damage and ultimately in cell death. However, most strains of this and other pathovars of *P. syringae* contain a copy of the *rulAB* genes that were first described as associated to a 68-kb plasmid in *P. syringae* pv. *syringae* and that confer variable levels of resistance to this damaging radiation [32, 34, 35]. *P. syringae* pv. *syringae* strains causing apical necrosis of mango are present in high number in buds of infected trees, which likely offer an effective shield against phytosanitary treatments [7] and UV radiation, although their populations drop significantly during the hot, dry, and bright days of summer in Southern Spain [6]. In this study, the genetic analysis of *P. syringae* pv. *syringae* isolates from the mango phyllosphere has revealed the frequent presence of genetic sequences, homologous to the *rulAB* operon, which were exclusively associated with 62-kb plasmids. Hybridization with a DNA probe specific for the replication protein of plasmid pPT23A indicates that all the 62-kb plasmids could be included in the pPT23A-like family [7, 11, 29]. It is noteworthy that the 62-kb plasmid band corresponds to a

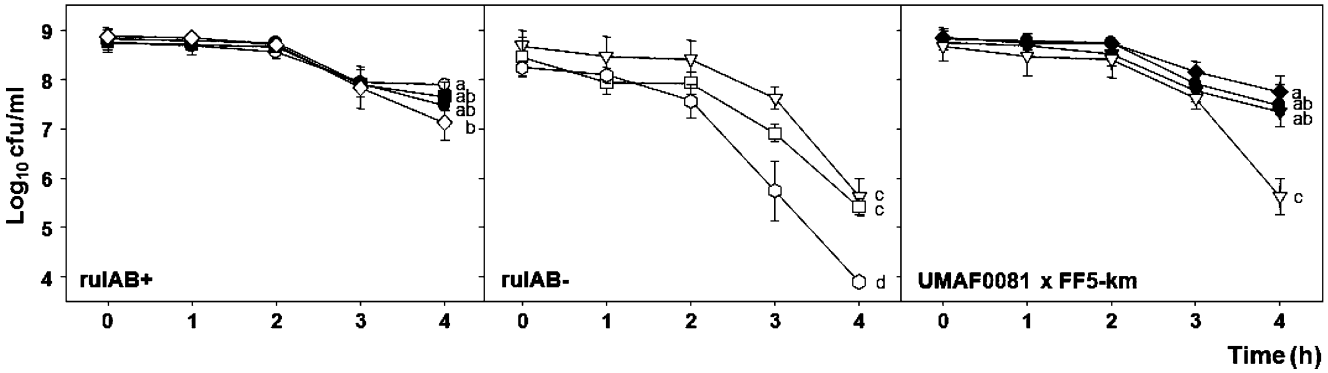

Figure 4 Survival of *P. syringae* pv. *syringae* strains under UVA + B radiation. UVA radiation and UVB radiation were simultaneously applied at irradiance doses of 22 W m^{-2} and 1.4 W m^{-2}, respectively, close to normal summer values in Málaga (Spain). The following strains were studied: UMAF0048 (*open circle*), UMAF0081 (*filled circle*), UMAF0167 (*open hexagon*), UMAF0170 (*filled square*), UMAF0176 (*open diamond*), UMAF6003 (*open square*), FF5-km (*open inverted triangle*), UMAFCB (*filled diamond*), UMAFCK (*filled inverted triangle*). *Different letters* denote that mean values at the final point of the assay (4 h) were significantly different, according to least significant difference test (p=0.05). *Bars* represent the standard deviation from three independent experiments

population of plasmids that shows fundamental differences in gene content and organization. Furthermore, the 62-kb plasmids showed an asymmetrical gene content; therefore, using specific probes for the *rulAB* genes and the *copABCD* genes, conferring resistance to copper, we detected 62-kb plasmids that hybridized to only the *rulAB* probe or to both, with the exception of strain Ps35, which did not hybridize with any of these probes (Table 1). In addition, the size of the *Eco*RI fragments hybridizing to the *rulAB* probe was

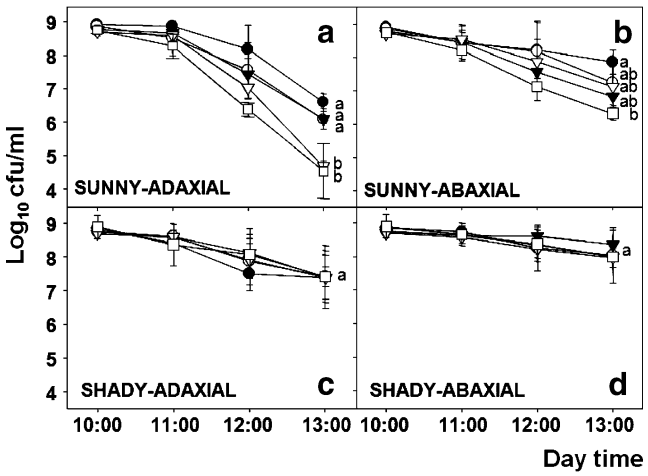

Figure 5 Survival of *P. syringae* pv. *syringae* strains on mango leaves exposed to natural solar radiation (see Table 2). **a** *P. syringae* pv. *syringae* strains were placed on the adaxial leaf surface under direct solar radiation on a sunny day. **b** The same environmental conditions in **a** but *P. syringae* pv. *syringae* strains were placed on the abaxial leaf surface. **c** *P. syringae* pv. *syringae* strains were placed on the adaxial leaf surface under indirect solar radiation in a shady area. **d** The same environmental conditions in **c** but *P. syringae* pv. *syringae* strains were placed on the abaxial leaf surface. The following strains were studied: UMAF0048 (*open circle*), UMAF0081 (*filled circle*), UMAF6003 (*open square*), FF5-km (*open inverted triangle*), UMAFCK (*filled inverted triangle*). *Different letters* denote that mean values at the final point of the assay were significantly different, according to least significant difference test (p=0.05). *Bars* represent the standard deviation from three independent experiments

either 1.2 or 13 kb for different plasmids, which indicates the existence of further genetic differences among the 62-kb *rulAB*+ plasmids. The analysis of restriction profiles of individual 62-kb plasmids [7] suggest that the *rulAB* and the *copABCD* homologues are situated in a single 62 kb plasmid and not in two different 62-kb plasmids coexisting in the same strain. Hybridization data with DNA digested with restriction enzymes also suggest that each strain contains only a single type of the 62-kb plasmids harboring the *rulAB* homologues (Table 1) or the copper resistance genes [7]. Additionally, the sum of the fragments generated by restriction digest of the 62-kb plasmid band [7] also indicate that there is only one species of 62-kb plasmids in those strains that did not contain homologues of *rulAB* or *copABCD*.

In spite of its importance, there are few studies about the UV radiation effects on the microbiota present on exposed plant surfaces (e.g. [18]) and, particularly, of bacterial plant pathogens that survive in the phyllosphere [14]. In this study, we evaluated the ecological role of the 62-kb plasmids harboring *rulAB* homologues in the resistance of *P. syringae* pv. *syringae* to UV radiation. The *rulAB* determinants are homologous to DNA repair systems such as the chromosomal *umuDC* genes, regulated as part of the cellular SOS system in *E. coli* [10], and to other plasmid-encoded determinants such as *mucAB* and *samAB*, from *Salmonella typhimurium* [30, 37]. In addition, *rulAB*, as occurs with *umuDC*, could be implicated in the repair of DNA damage produced by UVB or UVC radiation [19, 20] and, for this reason, in the bacterial resistance to UV [12, 15, 32]. Our results clearly show increased survival of *P. syringae* strains harboring 62-kb plasmids, compared to plasmidless strains, when exposed to different types of UV radiation, including the UVC fraction; similar results have been described by other authors for bacterial strains harboring the *rulAB* operon [12, 32, 33]. UVC exposure

assays were important to readily distinguish differences in the UV sensitivity of individual strains because the UVC fraction includes the most bactericidal wavelength, 260 nm [1]. However, the higher UVC tolerance of *P. syringae* strains containing 62-kb plasmids is probably of little ecological significance due to the complete screening of the UVC wavelengths by the stratospheric ozone layer, which prevents this radiation from reaching the earth's surface [19].

P. syringae pv. *syringae* strains harboring 62-kb plasmids also showed an increased tolerance to high doses of UVA+B radiation (Fig. 3) that was comparable to that observed by other authors in strains containing the *rulAB* genes [19, 32, 34]. More importantly, these strains also showed significantly higher survival (two to three orders of magnitude after 4 h) than strains lacking *rulAB* homologs when artificially exposed to intensities of UVA+B radiation similar to those reaching the earth's surface in our latitude during summer (Fig. 4). There were substantial differences in the bacterial response to these different radiation intensities; at high irradiance levels (Fig. 3), *rulAB*+ bacteria were more rapidly inactivated than at the normal irradiance levels (two to three times lower, Fig. 4). The slight reduction in populations of *P. syringae* carrying a 62-kb plasmid could be explained by an increasing dryness and a high temperature towards the end of the experiment that, as it has been stated by some authors, could stress this bacterium [33, 36]; indeed, we observed a similar decline in bacterial populations on shady leaf surfaces 3 h after inoculation (Fig. 5c). In all these experiments, a derivative of the plasmidless strain *P. syringae* pv. *syringae* FF5 containing a 62-kb plasmid showed a significant increase in its resistance to UV radiation, which was comparable to wild-type *P. syringae* pv. *syringae* strains containing *rulAB* homologues. Altogether, our data strongly support the idea that the *rulAB* homologues associated to 62-kb plasmids in *P. syringae* pv. *syringae* could be responsible for the increased resistance to UV light, probably by eliminating DNA damage under UV radiation [17, 34].

Interestingly, the strain UMAF0167, which harbors a 120-kb plasmid, shows significantly higher resistance to UVC than plasmidless strains FF5-km and UMAF6003 (Fig. 2) and statistically significant lower resistance to UVA +B than FF5-km and UMAF6003 (Fig. 4). This could suggest that the 120-kb plasmid of UMAF0167 encodes a mechanism that enhances UVC resistance, but it is not *rulAB* homolog and perhaps shows that *rulAB* homologues in 62-kb plasmids would be more relevant for UVA+B resistance.

The higher tolerance to UV radiation conferred by the 62-kb plasmids was further confirmed in field assays by monitoring bacterial populations on artificially inoculated mango leaves (Fig. 5). Again, we observed a significantly higher survival of *P. syringae* pv. *syringae* strains harboring 62-kb plasmids with respect to the plasmidless strains. This difference was only apparent on the adaxial side of leaves exposed to direct sunlight, whereas shaded areas and on the abaxial side of leaves the populations of bacteria containing or lacking *rulAB* homologues were comparable. Bacteria inoculated on the abaxial surface of leaves, regardless of their possession of 62-kb plasmids, showed longer survival periods, which could reduce the ecological advantage of the *rulAB* determinants because the *P. syringae* pv. *syringae* strains infecting mango spend a large part of their life cycle protected inside buds [6]. In spite of this, however, the majority of the strains examined in this work harbored 62-kb *rulAB*+ plasmids and showed increased tolerance to UV radiation. This could be in part explained because these *rulAB*+ strains could colonize areas of the mango plant and other plant hosts that are under direct solar radiation, which would facilitate their dispersal, as has been previously shown [16, 33, 34]. Additionally, it is possible that the 62-kb *rulAB*+ plasmids carry other determinants that increase the fitness of the bacterium and that the *rulAB* homologs are selected for in part by genetic hitchhiking.

In conclusion, the presence of genetic determinants homologous to *rulAB* on 62-kb plasmids has been determined in many *P. syringae* pv. *syringae* strains isolated from mango in the Mediterranean area. The strains harboring these plasmids showed a higher tolerance to UV radiation and also demonstrated an increased survival on mango phyllosphere exposed to intense solar radiation.

Acknowledgements This work was partially supported by grants from Junta de Andalucía (PAI, Grupo AGR-169 and Project AGR-2471) and from Plan Nacional de I + D + I del Ministerio de Educación y Ciencia of Spain (AGL2001-1837-CO2–01, AGL2004-03143 and AGL2005-06347-C03–01). We want to acknowledge D. del Pino for his technical assistance in some experiments. We also wish to thank F. López-Figueroa, J. Aguilera, and E. Pérez-Rodríguez for light measurements and work facilities at Laboratorio de Fotobiología, Univ. de Málaga. We also acknowledge J.M. Farré and J.M. Hermoso for helpful suggestions and work facilities at E.E. La Mayora (CSIC, Spain). We thank G.W. Sundin for kindly providing us with some of the bacterial strains and DNA probes used in this study and critical review of the manuscript.

References

1. Atlas RM, Bartha R (1998) Microbial ecology: fundamentals and applications. Benjamin-Cummings, Menlo Park (USA)
2. Beattie G, Lindow SE (1995) The secret life of foliar bacterial pathogens on leaves. Ann Rev Phytopathol 33:145–172
3. Bender CL, Cooksey D (1986) Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: Conjugative transfer and role in copper resistance. J Bacteriol 165:534–541
4. Bender CL, Cooksey D (1987) Molecular cloning of copper resistance genes from *Pseudomonas syringae* pv. *tomato*. J Bacteriol 169:470–474

5. Cazorla FM, Olalla L, Torés JA, Codina JC, Pérez-García A, de Vicente A (1997) *Pseudomonas syringae* pv. *syringae* as microorganism involved in apical necrosis of mango: characterization of some virulence factors. In: Rudolph K, Burr TJ, Mansfield JW, Stead D, Vivian A, von Kietsell (eds) *Pseudomonas syringae* Pathovars and Related Pathogens. Kluwer, Dordrecht, The Netherlands, pp 82–87
6. Cazorla FM, Torés JA, Olalla L, Pérez-García A, Farré JM, de Vicente A (1998) Bacterial apical necrosis of mango in Southern Spain: a disease caused by *Pseudomonas syringae* pv. *syringae*. Phytopathology 88:614–620
7. Cazorla FM, Arrebola E, Sesma A, Pérez-García A, Codina JC, Murillo J, de Vicente A (2002) Copper resistance in *Pseudomonas syringae* strains isolated from mango is encoded mainly by plasmids. Phytopathology 92:909–916
8. Cutchis P (1974) Stratospheric ozone depletion and solar ultraviolet radiation on earth. Science 184:13–19
9. Figueroa FL, Jiménez C, Viñegla B, Pérez-Rodríguez E, Aguilera J, Flores-Moya A, Altamirano M, Lebert M, Häder DP (2002) Effects of solar UV radiation on photosynthesis of the marine angiosperm *Posidonia oceanica* from southern Spain. Mar Ecol Prog Ser 230:59–70
10. Friedberg EC, Walker GC, Siede W (1995) DNA repair and mutagenesis. American Society for Microbiology, Washington, DC, USA
11. Gibbon MJ, Sesma A, Canal A, Wood JR, Hidalgo E, Brown J, Vivian A, Murillo J (1999) Replication regions from plant-pathogenic *Pseudomonas syringae* plasmids are similar to ColE2-related replicons. Microbiology 145:325–334
12. Gunasekera TS, Sundin GW (2006) Role of nucleotide excision repair and photoreactivation in the solar UVB radiation survival of *Pseudomonas syringae* pv. *syringae* B728a. J Appl Microbiol 100:1073–1083
13. Hirano SS, Upper CD (1990) Population biology and epidemiology of *Pseudomonas syringae*. Annu Rev Phytopathol 28:155–177
14. Hirano SS, Rouse DI, Clayton MK, Upper CD (1995) *Pseudomonas syringae* pv. *syringae* and bacterial brown spot of snap bean: a study of epiphytic phytopathogenic bacteria and associated diseases. Plant Dis 79:1085–1093
15. Jacobs JL, Sundin GW (2001) Effect of solar UV-B radiation on a phyllosphere bacterial community. Appl Environ Microbiol 67:5488–5496
16. Jacobs JL, Carroll TL, Sundin GW (2005) The role of pigmentation, ultraviolet radiation tolerance, and leaf colonization strategies in the epiphytic survival of phyllosphere bacteria. Microb Ecol 49:104–113
17. Joux F, Jeffrey WH, Lebaron P, Mitchell DL (1999) Marine bacterial isolates display diverse responses to UV-B radiation. Appl Environ Microbiol 65:3820–3827
18. Kadivar H, Stapleton AE (2003) Ultraviolet radiation alters maize phyllosphere bacterial diversity. Microb Ecol 45:353–361
19. Kim JJ, Sundin GW (2000) Regulation of the *rul*AB mutagenic DNA repair operon of *Pseudomonas syringae* by UV-B (290 to 320 nanometers) radiation and analysis of *rul*AB-mediated mutability *in vitro* and *in planta*. J Bacteriol 182:6137–6144
20. Koch WH, Fernández de Henestrosa AR, Woodgate R (2000) Identification of *muc*AB-like homologues on two IncT plasmids, R394 and Rts-1. Mut Res 457:1–13
21. Leach JG, Lilly VG, Wilson HA, Purvis MR Jr (1957) Bacterial polysaccharides: the nature and function of the exudate produced by *Xanthomonas phaseoli*. Phytopathology 47:113–120
22. Litz RE (1997) The Mango: Botany. Production and Uses. CAB International, Wallingford, UK
23. Murillo J, Keen NT (1994) Two native plasmids of *Pseudomonas syringae* pathovar *tomato* strain PT23 share a large amount of repeated DNA including replication sequences. Mol Microbiol 12:941–950
24. Murillo J, Shen H, Gerhold D, Sharma AK, Cooksey D, Keen NT (1994) Characterization of pPT23B, the plasmid involved in syringolide production by *Pseudomonas syringae* pv. *tomato*. Plasmid 31:275–287
25. Pfeifer GP (1997) Formation and processing of UV photoproducts: effects of DNA sequence and chromatin environment. Photochem Photobiol 65:270–283
26. Pourzand C, Tyrell RM (1999) Apoptosis, the role of oxidative stress and the example of solar UV radiation. Photochem Photobiol 70:380–390
27. Ramos-González MI, Ramón MA, Ramos JL (1991) Conjugational transfer of recombinant DNA in cultures and in soils: host range of *Pseudomonas putida* TOL plasmid. Appl Environ Microbiol 57:3020–3027
28. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Spring Harbor, NY, USA
29. Sesma A, Sundin GW, Murillo J (1998) Closely related plasmid replicons coexisting in the phytopathogen *Pseudomonas syringae* show a mosaic organization of the replication region and altered incompatibility behaviour. Appl Environ Microbiol 64:3948–3953
30. Smith BT, Walker GC (1998) Mutagenesis and more: *umu*DC and the *Escherichia coli* SOS response. Genetics 148:1599–1610
31. Sundin GW, Bender CL (1993) Ecological and genetic analysis of copper and streptomycin resistance in *Pseudomonas syringae* pv. *syringae*. Appl Environ Microbiol 59:1018–1024
32. Sundin GW, Kidambi SP, Ulrich M, Bender CL (1996) Resistance to ultraviolet light in *Pseudomonas syringae*: sequence and functional analysis of the plasmid-encoded *rul*AB genes. Gene 177:77–81
33. Sundin GW, Jacobs JL (1999) Ultraviolet radiation (UVR) sensitivity analysis and UVR survival strategies of a bacterial community from the phyllosphere of field-grown peanut (*Arachis hypogeae* L.). Microb Ecol 38:27–38
34. Sundin GW, Murillo J (1999) Functional analysis of the *Pseudomonas syringae rul*AB determinant in tolerance to ultraviolet B (290–320 nm) radiation and distribution of *rul*AB among *P. syringae* pathovars. Environ Microbiol 1:75–87
35. Sundin GW, Jacobs JL, Murillo J (2000) Sequence diversity of *rul*A among natural isolates of *Pseudomonas syringae* and effect on function of *rul*AB-mediated UV radiation tolerance. Appl Environ Microbiol 66:5167–5173
36. Wilson M, Hirano SS, Lindow SE (1999) Location and survival of leaf-associated bacteria in relation to pathogenicity and potential growth within the leaf. Appl Environ Microbiol 65:1435–1443
37. Woodgate R, Sedgwick SG (1992) Mutagenesis induced by bacterial *umu*DC proteins and their plasmid homologues. Mol Microbiol 6:2213–2218
38. Zhou C, Yang Y, Jong AY (1990) Miniprep in ten minutes. BioTechniques 8:172–173