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Regulation of the *rulAB* Mutagenic DNA Repair Operon of *Pseudomonas syringae* by UV-B (290 to 320 Nanometers) Radiation and Analysis of *rulAB*-Mediated Mutability In Vitro and In Planta

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The effects of the *rulAB* operon of *Pseudomonas syringae* on mutagenic DNA repair and the transcriptional regulation of *rulAB* following irradiation with UV-B wavelengths were determined. For a *rulB*::Km insertional mutant constructed in *P. syringae* pv. syringae B86-17, sensitivity to UV-B irradiation increased and UV mutability decreased by 12- to 14-fold. *rulAB*-induced UV mutability was also tracked in phyllosphere populations of B86-17 for up to 5 days following plant inoculation. UV mutability to rifampin resistance (Rif^r) was detected at all sampling points at levels which were significantly greater than in nonirradiated controls. In *P. aeruginosa* PAO1, the cloned *rulAB* determinant on pJJK17 conferred a 30-fold increase in survival and a 200-fold increase in mutability following a UV-B dose of 1,900 J m^{-2}. In comparative studies using defined genetic constructs, we determined that *rulAB* restored mutability to the *Escherichia coli umuDC* deletion mutant RW120 at a level between those of its homologs *mucAB* and *umuDC*. Analyses using a *rulAB*::*inaZ* transcriptional fusion in *Pseudomonas fluorescens* Pf5 showed that *rulAB* was rapidly induced after UV-B irradiation, with expression levels peaking at 4 h. At the highest UV-B dose administered, transcriptional activity of the *rulAB* promoter was elevated as much as 261-fold compared to that of a nonirradiated control. The importance of *rulAB* for survival of *P. syringae* in its phyllosphere habitat, coupled with its wide distribution among a broad range of *P. syringae* genotypes, suggests that this determinant would be appropriate for continued investigations into the ecological ramifications of mutagenic DNA repair.

The involvement of bacterial plasmids in increasing the survival of their hosts following irradiation with UV wavelengths was first reported in 1965 by Howarth (13), who was working with the ColIb-P9 plasmid in *Salmonella enterica* serovar Typhimurium LT2. Howarth also noted that the frequency of mutants in irradiated cultures of serovar Typhimurium LT2 (ColIb-P9) was increased (14). These two initial observations have been followed by the discovery that a large number of bacterial plasmids from many incompatibility groups confer phenotypes of increased UV survival and mutability (43, 52). Genes conferring the UV mutability phenotype are also chromosomally located in some cases, an important example being the *umuDC* operon of *Escherichia coli*.

The *umuDC* operon is one component of the SOS regulon of *E. coli*, a set of approximately 20 unlinked genes which are coordinately regulated in response to DNA damage (42). The *umuDC* operon is regulated by the *recA* and *lexA* gene products, with the UmuDC and RecA proteins alone required for UV mutability (42). LexA functions as a repressor through binding to a conserved DNA sequence (SOS box) located within the promoter region of SOS regulon genes (3, 25). The irradiation of *E. coli* cells with UV wavelengths results in the occurrence of DNA lesions of which the cyclobutane pyrimidine dimer and the pyrimidine(6-4)pyrimidinone photoproduct are the most typical (8); these lesions can result in a blockage of DNA polymerase activity, leading to a stalling of replication. Cellular perception of DNA damage is thought to occur through the binding of RecA to single-stranded DNA immediately downstream of a DNA lesion; during this process, RecA is converted to an activated form (RecA*) (42). RecA* then mediates a self-cleavage reaction of LexA, resulting in the removal of LexA and allowing the expression of the SOS response genes (24).

Following the expression of *umuDC*, a posttranslational self-modification of UmuD (removal of the first 24 amino acids of UmuD) mediated by RecA* is required to generate the active UmuD′ protein (5, 40). UmuD′ forms a homodimer (UmuD′$_2$) which complexes with UmuC, resulting in the mutagenically active UmuD′$_2$C complex (4). The function of the UmuD′$_2$C complex in mutagenic DNA repair (MDR) occurs as a result of translesion DNA synthesis (42, 50). The model for replicative lesion bypass includes DNA pol III holoenzyme complex, the UmuD′$_2$C complex, and RecA* (48). However, recent evidence has shown that the UmuD′$_2$C complex itself may be sufficient in translesion synthesis with the possibility of either the UmuD′$_2$C complex or UmuC itself functioning as a DNA polymerase (36, 49).

To date, five distinct plasmid-carried *umuDC* homologs have been characterized at the sequence level. These include *impCAB*, *mucAB*, *rulAB*, *rumAB*$_{R391}$, and *samAB* (19, 26, 31, 33, 46). Each of these sequences contains a consensus LexA-binding site within the respective promoter regions and a conserved internal cleavage site within the *umuD* homolog. With the exception of *rulAB*, the other known MDR systems were isolated from enterobacteria and have not been well characterized in terms of their contribution to the ecological fitness of their hosts. The *rulAB* operon was originally cloned from

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pPSR1, an indigenous plasmid from *Pseudomonas syringae* pv. syringae A2, and was initially characterized for its role in UV radiation (UVR) tolerance (46). Although not absolutely required for survival, the UVR tolerance phenotype conferred by *rulAB* was subsequently shown to increase *P. syringae* populations by 10- to 30-fold in its leaf surface (phyllosphere) habitat (47). *rulAB* is the most distantly related to *umuDC* of the other plasmid-carried *umuDC* homologs, as *rulA* and *rulB* share only 30.9% and 41.5% amino acid similarity to *umuD* and *umuC*, respectively (42). However, *rulAB* does share the important features of this group, including its function in UVR tolerance and its lack of expression and activity in a *P. syringae recA* background (46). Recent evidence has also shown that *rulAB* is widely distributed among strains within and among pathovars of *P. syringae* (39, 47), suggesting the importance of this determinant to a wide range of genotypes.

Our laboratory is interested in further elucidating the role of the *rulAB* system in the population biology of *P. syringae*. To that end, we are fully characterizing the functional roles of *rulAB* (UVR tolerance and MDR), along with analyzing the regulation of this determinant so that we can ultimately address the biological significance of the *rulAB* system to *P. syringae* in its natural environment. Our studies reported here utilize UV-B (290 to 320 nm) wavelengths, which is in contrast to most analyses of DNA repair and UVR-induced mutagenesis in microorganisms, where higher-energy UV-C (254 nm) wavelengths are used. In nature, UV-C wavelengths are screened by the stratospheric ozone layer and do not reach the earth's surface; thus, the use of UV-B wavelengths is relevant from an ecological standpoint. The most important types of UV-B-induced DNA lesions appear to be cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidinone photoproducts (30). Ecological studies have shown that solar UV-B radiation has profound deleterious effects on microorganisms dwelling in locations with high sunlight exposure, including surface aquatic habitats and the phyllosphere (11, 15, 45).

In this study, we report an analysis of the MDR capacity of *rulAB* and an analysis of mutability in a *P. syringae* strain containing a stable insertional mutation within *rulAB*. We also examined the MDR potential of *rulAB*-containing *P. syringae* in planta and show that UVR-induced mutability occurs in leaf surface populations at rates similar to those observed in vitro. We further demonstrate that *rulAB* complements an *E. coli umuDC* mutant and compare the MDR activity of *rulAB* with that of *mucAB* and *umuDC* in *E. coli* and *P. aeruginosa* by using defined genetic constructs. Finally, we analyzed the regulation of *rulAB* in response to increasing doses of UV-B (290 to 320 nm) radiation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains, plasmids, and specific oligonucleotides utilized which were relevant to this study are listed in Table 1. All bacterial strains were grown in Luria-Bertani (LB) medium (Difco) or King's medium B (KB) (17); *E. coli* strains and *Pseudomonas aeruginosa* PAO1 were grown at 37°C, and *Pseudomonas fluorescens* Pf5 and *P. syringae* pv. syringae B86-17 were grown at 28°C. Plasmid transfer from *E. coli* to *Pseudomonas* strains was accomplished by triparental mating using the helper plasmid pRK2013. The exconjugants were selected on MG medium (16) or *Pseudomonas* isolation agar (Difco) supplemented with appropriate antibiotics. Antibiotics were added to the media in the following concentrations: ampicillin, 75 μg ml^{-1}; carbenicillin, 150 μg ml^{-1}; cycloheximide, 100 μg ml^{-1}; gentamicin (Gm), 20 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; rifampin, 100 μg ml^{-1}; and spectinomycin, 40 μg ml^{-1}.

Molecular biology techniques. Plasmid isolation from *P. syringae* was accomplished using the technique of Crosa and Falkow (6). Restriction digestions, isolation of DNA fragments from agarose gels, PCR amplifications, ligations, and Southern transfer to nylon membranes were performed using standard techniques (38). DNA fragments used as probes were labeled with digoxigenin-11-dUTP (Genius kit; Boehringer Mannheim, Indianapolis, Ind.) following the instructions of the manufacturer. Hybridizations at 65°C followed by high-stringency washes were performed as described previously (44).

Construction of *P. syringae* pv. syringae GWS242 (B86-17 *rulB*::Km). The *rulAB* gene disruption on the native plasmid pB8617A of strain B86-17 was created via gene replacement using a homologous recombination strategy. The intact *rulAB* sequence on pB8617A was initially cloned into pBluescript II SK(+) as a 6.4-kb *Bam*HI fragment (pJJK1) and subcloned as a partial 3.8-kb *Eco*RI fragment into pGem 7zf−, creating pJJK12. This plasmid was then digested with *Bss*HII, resulting in the removal of a 0.6-kb segment from within *rulB*. Following *Bss*HII digestion, the ends were made blunt by incubation with the Klenow fragment of DNA polymerase (Life Technologies), and the 1.2-kb kanamycin resistance (Km^r) gene cassette (released from pBSL86 as a *Hin*cII fragment) was ligated in. The entire DNA region was excised as a 4.4-kb *Xba*I-*Bam*HI fragment and ligated into the suicide gene replacement vector pJQ200SK, creating pJJK16. pJJK16 contained approximately 1.0 kb of flanking DNA sequences on each side of the *rulAB* (*rulB*::Km) sequence. Following transfer of pJJK16 into *P. syringae* pv. syringae B86-17, those *P. syringae* cells in which a plasmid integration event had occurred were selected on MG supplemented with Gm. Several isolated Gm^r colonies were then cultured in LB broth containing Km. After 2 days of incubation, 0.1-ml aliquots were plated onto LB containing Km and 5% sucrose to counterselect against the *sacB* gene encoded on pJQ200SK. The sucrose-resistant (Suc^r) colonies recovered were subsequently tested for sensitivity to Gm as a phenotypic assay for loss of the vector sequences. Since there are no *Bam*HI sites within *rulAB* or the Km^r cassette, the increased size of pB8617A *rulB*::Km was visualized by comparing the sizes of the *Bam*HI fragment from pB8617A and pB8617A *rulB*::Km, which hybridized to an internal *rulAB* gene probe consisting of the 0.7-kb *Hin*dIII-*Pst*I fragment from pGWS140. Further confirmatory hybridizations were done using the Km^r cassette from pBSL86 as a probe both to the *Bam*HI plasmid digests and to *Bam*HI digests of total genomic DNA from *P. syringae* pv. syringae B86-17 and GWS242 to ensure that there was only one insertion of the Km^r cassette within the GWS242 genome.

UV-B irradiation and MDR assays. Bacterial strains were grown to late log phase in LB medium containing the appropriate antibiotics; 1 ml of the cultures was pelleted, washed with an equal volume of sterile saline (0.85% NaCl) solution, and resuspended in an equal volume of saline. The cell suspension was then diluted with an additional 9 ml of saline and placed in a sterile glass petri dish. Cell suspensions were irradiated with UV-B (peak, 302 nm) wavelengths using an XX-15M model UV-B lamp (UVP Products, San Gabriel, Calif.) filtered through cellulose diacetate (Kodacel; Eastman Kodak, Rochester, N.Y.) to eliminate any UV-C wavelengths (<290 nm) given off. The UV-B lamp was turned on 15 min prior to use to allow for stabilization of the UVR output. The energy output of the lamp was monitored with a UV-X radiometer (UVP Products) and determined to be 2.8 J m^{-2} s^{-1}. Cell suspensions were mixed continuously while receiving the UV-B dose to eliminate survival as a result of shading. After irradiation, surviving cells were enumerated by dilution plating conducted under conditions that minimized photoreactivation. For the MDR assays, 0.1 ml of untreated cells and cells from all UV-B treatments were diluted in 0.9 ml of sterile saline and added to 1 ml of 2× LB broth. Following overnight incubation under dark conditions, appropriate dilutions of cell suspensions were plated on LB alone and LB containing rifampin. The frequency of mutation to rifampin resistance (Rif^r) was calculated as the number of Rif^r mutants per 10^8 cells.

Analysis of MDR in phyllosphere populations of *P. syringae* pv. syringae B86-17. *P. syringae* pv. syringae B86-17 was grown on LB agar medium for 18 h before inoculation. The cells were washed and then resuspended in 0.1 M potassium phosphate buffer (pH 7.0), and cell suspensions were adjusted turbidimetrically to approximately 5×10^9 cells ml^{-1}. Bush bean plants (*Phaseolus vulgaris* cv. "Blue Lake 214") were grown under controlled conditions in a growth chamber (24°C, 80% relative humidity, 240-μmol m^{-2} s^{-1} light intensity, and 12-h photoperiod) with care taken during watering to prevent nonspecific leaf surface colonization. Experiments consisted of spraying inoculum of strain B86-17 onto the primary bean leaves using an air brush (Badger model 350; Badger Co., Franklin Park, Ill.) until the leaf surfaces were uniformly coated with the cell suspension. Immediately and at 24, 72, and 120 h after inoculation, four of the leaves were excised and individually irradiated with a 500-J m^{-2} dose of UV-B by using the XX-15M lamp as described above. An additional four leaves were also removed, and these leaves were not irradiated as a control. The leaves were placed onto sterile, moist paper towels in a covered sterile Bio-Assay dish (Nunc Products) and incubated under dark conditions for 12 h at 25°C. After incubation, each leaf was sonicated for 7 min in 20 ml of sterile washing buffer (0.1 M potassium phosphate [pH 7.0] and 0.1% peptone). The combined washing buffer from four leaves per treatment was reduced to 1 ml by centrifugation, and appropriate serial dilutions of the leaf washings were plated onto KB agar medium amended with cycloheximide alone (KBc) and KBc containing rifampin. A total of six independent experiments were conducted. The data were normalized based on total leaf surface populations and reported as the number of Rif^r cells per 10^8 total cells. A one-way analysis of variance based on UV-B irradiation was done to compare the mean number of Rif^r cells per 10^8 total cells at each sampling time; means were differentiated using a *t* test.

Phenotypic comparison of *rulAB* with *mucAB* and *umuDC*. The individual coding sequences of the *mucAB*, *rulAB*, and *umuDC* MDR operons were amplified via PCR using the following oligonucleotide primers and source DNA: (i) *mucAB Nde* 5′, *mucAB Bam* 3′, and pRW144 for *mucAB*; (ii) *rulAB Nde* 5′, 3′

TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers utilized in this study and their relevant characteristics

Strain, plasmid, or oligonucleotide primer	Relevant characteristics[a] or nucleotide sequence[b]	Source or reference
Bacterial strains		
E. coli		
DH10B	Plasmid-free strain used for cloning	9
RW120	*lexA*$^+$ *recA*$^+$ Δ(*umuDC*)*595*::*cat*	12
P. aeruginosa		
PAO1	UV^s, no detectable plasmids	A. M. Chakrabarty
P. fluorescens		
Pf5	UV^s, *ina*-negative	27
P. syringae pv. syringae		
B86-17	UV^r, contains *rulAB* on pB8617A	21
GWS242	As B86-17 but also *rulB*::Km	This study
Plasmids		
pBluescript SK(+)	Ap^r, cloning vector	Stratagene
pBSL86	Source of Km^r cassette	1
pCR2.1	Ap^r Km^r, direct cloning vector for PCR products	Invitrogen
pET-5a	Ap^r, source of Shine-Dalgarno sequence	Promega
pGem7zf−	Ap^r, cloning vector	Promega
pJB321	Cb^r, broad-host-range cloning vector	2
pJQ200SK	Gm^r *sacB*, suicide gene replacement vector	34
pPROBE KI′	Km^r, broad-host-range, *inaZ* reporter vector	S. E. Lindow
pRK2013	Helper plasmid for triparental matings	7
pRW144	Sp^r, 2.4-kb *mucAB* as *Bam*HI in pGB2	12
pRW154	Sp^r, 2.8-kb *umuDC* as *Eco*RI in pGB2	12
pB8617A	*rulAB*$^+$, native plasmid from *P. syringae* pv. syringae B86-17	This study
pGWS140	0.7-kb *Hin*dIII-*Pst*I from pSM1 in pBluescript SK(+)	47
pJJK1	6.4-kb *rulAB* as *Bam*HI from pB8617A in pBluescript SK(+)	This study
pJJK5	1.7-kb *mucAB* as *Nde*I-*Bam*HI from pRW144 in pET-5a	This study
pJJK12	3.8-kb *rulAB* as partial *Eco*RI from pJJK1 in pGem7zf−	This study
pJJK15	1.2-kb Km^r cassette as *Hin*cII from pBSL86 in pJJK12 at blunt-ended *Bss*HII	This study
pJJK16	4.4-kb *rulA*, *rulB*::Km as *Xba*I-*Bam*HI in pJQ200SK	This study
pJJK17	3.8-kb *rulAB* as *Xba*I-*Bam*HI from pJJK12 in pJB321	This study
pJJK20	0.75-kb *umuDC* promoter as *Sph*I-*Xba*I in pET-5a	This study
pJJK21	1.7-kb *rulAB* as *Nde*I-*Bam*HI from pJJK12 in pJJK20	This study
pJJK22	0.75-kb *umuDC* promoter as *Sph*I-*Xba*I in pJJK5	This study
pJJK23	1.7-kb *umuDC* as *Nde*I-*Eco*RI from pRW154 in pET-5a	This study
pJJK24	0.75-kb *umuDC* promoter as *Sph*I-*Xba*I in pJJK23	This study
pJJK25	2.45-kb *umuDC* promoter + *rulAB* as *Sal*I-*Bam*HI in pJB321	This study
pJJK26	2.45-kb *umuDC* promoter + *mucAB* as *Sph*I-*Bam*HI in pJB321	This study
pJJK27	2.45-kb *umuDC* promoter + *umuDC* as *Sph*I-*Eco*RI in pJB321	This study
pJJK40	1.1-kb *rulAB* promoter region in pCR2.1	This study
pJJK41	0.9-kb *rulAB* promoter as *Hin*dIII-*Eco*RI from pJJK40 in pPROBE KI′	This study
Oligonucleotide primers		
mucAB Nde 5′	5′-GGAATTCCATATGAAGGTCGATATTTTTG-3′	This study
mucAB Bam 3′	5′-GATCGGATCCTTATTTGATGGTGGCTATTGG-3′	This study
rulAB Nde 5′	5′-GGAATTCCATATGAACGTCAAAATACTCGGGCGG-3′	This study
3′ *rulB* TAA *Bam*HI	5′-GATCGGATCCTTACTTTACAACCCACAGCTG-3′	This study
rul PX	5′-GGATTGGGAAAACCGGCAGGG-3′	This study
umuDC Nde 5′	5′-GATCCATATGTTGTTTATCAAGCCTGCGG-3′	This study
umuDC Eco 3′	5′-GATCGAATTCTTATTTGACCCTCAGTAAATCAG-3′	This study
umu Pro 5′ *Sph*I	5′-GATCGCATGCGAGCAATTGCGTCGC-3′	This study
umu Pro 3′	5′-GTACTCTAGACTGCCTGAAGTTATACTG-3′	This study

[a] Abbreviations: Ap, ampicillin; Cb, carbenicillin; Gm, gentamicin; Km, kanamycin; Sp, spectinomycin.
[b] Restriction sites incorporated in primers are underlined. GGATCC, *Bam*HI; GAATTC, *Eco*RI; CATATG, *Nde*I; GAGCTC, *Sac*I; GCATGC, *Sph*I; TCTAGA, *Xba*I.

rulB TAA *Bam*HI, and pJJK12 for *rulAB*; and (iii) *umuDC Nde* 5′, *umuDC Eco* 3′, and pRW154 for *umuDC*. The amplified product from pRW144 was confirmed as having the correct size on an agarose gel, digested with the appropriate restriction enzymes, and ligated into pET-5a, creating pJJK5. A 0.75-kb region upstream of *umuDC* on the *E. coli* chromosome was next amplified from pRW154 using the primers *umu* Pro *Sph*I 5′ and *umu* Pro 3′, digested, and ligated upstream of the *mucAB* coding sequence in pJJK5, creating pJJK22. The other genetic constructs were made by first ligating the *umuDC* promoter into pET-5a, creating pJJK20, and then ligating the respective *rulAB* or *umuDC* amplified coding sequence into pJJK20. In each case, the translational start site of the MDR coding sequences was the ATG sequence within the *Nde*I sequence of pET-5a, allowing optimal spacing from a Shine-Dalgarno sequence present on the pET-5a vector. The final constructs, including the *umuDC* promoter sequences, Shine-Dalgarno sequence from pET-5a, and the respective MDR coding sequences were ligated into pJB321, creating pJJK25, pJJK26, and pJJK27. Each of these plasmids was transferred into *E. coli* RW120 and *P. aeruginosa* PAO1 and utilized in MDR assays as described above.

Determination of *rulAB* promoter activity using the *inaZ* reporter gene. The intergenic sequences between *rulAB* and *repA* on pB8617A were fused to the promoterless ice nucleation gene (*inaZ*) in pPROBE KI′ in order to quantify *rulAB* expression following UV-B irradiation. The primers T7 (Promega) and *rul* PX were used to amplify a 1.1-kb fragment from pJJK12; the PCR product was directly ligated into pCR2.1, creating pJJK40. A 0.9-kb *Hin*dIII-*Eco*RI fragment was subsequently excised from pJJK40 and ligated into pPROBE KI′, resulting

in a transcriptional fusion of the *rulAB* promoter region with *inaZ* in the final construct, pJJK41. Ice nucleation activity (INA) was chosen as a reporter because of the sensitivity with which INA can be detected and the large range of INAs which can be quantified (28). INA is also easily quantified in situ, and our future experiments would be geared toward *rulAB* expression analysis in the phyllosphere. Unfortunately, most strains of *P. syringae* produce the ice nucleation protein naturally; however, we felt that the sensitivity of the reporter would enable us to detect differences in expression resulting after small differences in the UV-B dose and that it justified the use of *inaZ* even though a different *Pseudomonas* species would be used in the assay.

The *rulAB*::*inaZ* expression analyses were performed in *P. fluorescens* Pf5, an ice nucleation-negative strain, following the confirmation of *rulAB* activity (increased UV-B survival and MDR) in strain Pf5. Cells of *P. fluorescens* Pf5 (pJJK41) were grown overnight in KB broth containing Km, pelleted by centrifugation, resuspended in 0.01 M potassium phosphate buffer (pH 7.0), and exposed to various UV-B doses using the methods described above. Twenty-five ml of UV-B-irradiated or nonirradiated cells was then cultured in 25 ml of 2× KB broth under dark conditions. Aliquots were withdrawn immediately upon culture and at designated intervals up to 18 h following irradiation and then resuspended in a small volume of 0.01 M potassium phosphate buffer for assessment of INA. The number of ice nuclei per cell was estimated by the droplet-freezing assay as described by Lindow (23), with the number of ice nuclei calculated as the fraction of droplets that froze within 5 min. Dilution plating on KB was used to count the number of viable cells so that the data could be normalized on the basis of numbers of ice nuclei per cell.

RESULTS

Construction and analysis of a *rulB* insertional mutant of *P. syringae* pv. syringae B86-17. The *rulAB* determinant was initially isolated from the indigenous plasmid pPSR1 from *P. syringae* pv. syringae A2, a pathogen of ornamental pear trees (46). In this and subsequent studies, it was of interest to work with the *rulAB* determinant of a *P. syringae* strain which was pathogenic on bean, a plant host which is more easily utilized in growth chamber studies. A bean-pathogenic strain, *P. syringae* pv. syringae B86-17, was chosen for use; this strain has a phenotype of UV tolerance and encodes *rulAB* on its large indigenous plasmid (47). For our experimental analyses, the *rulAB* determinant from pB8617A, the indigenous plasmid harbored by *P. syringae* pv. syringae B86-17, was subcloned as a 3.8-kb *Xba*I-*Bam*HI fragment in the broad-host-range low-copy-number vector pJB321, creating the plasmid pJJK17.

The function of *rulAB* in elevating UV survival and MDR was evaluated by using *P. syringae* pv. syringae GWS242 (B86-17 *rulB*::Km), a strain that was constructed by gene replacement using a sucrose-mediated counterselection system. Confirmation of the insertion of a kanamycin cassette within *rulB* was done using Southern hybridization analysis (data not shown). The UV-B sensitivity and mutability of *P. syringae* pv. syringae GWS242 were then compared with those of the wild-type B86-17 strain. The *rulB*::Km mutant GWS242 showed an increased UV-B sensitivity of approximately threefold at the highest dose level utilized (Fig. 1A). UV-B survival was restored to wild-type levels by the *rulAB*-containing plasmid pJJK17 (Fig. 1B). The increased sensitivity of the *rulB*::Km mutant GWS242 was of a lower magnitude than those observed previously by using two other *P. syringae* pv. syringae strains containing *rulAB* insertional mutations created by Campbell integration (47). This observation could be due to the insertional mutation occurring only within *rulB* of pB8617A, leaving the *rulA* sequence intact, or because *P. syringae* pv. syringae B86-17 contains additional UV-B-protective mechanisms which are not present in the strains utilized in the previous study.

An examination of UV-B mutability showed that the mutation frequency of spontaneous Rif^r in *P. syringae* pv. syringae B86-17 is elevated following UV-B irradiation (Fig. 1C). This increase in mutation frequency is not observed in cultures of strain B86-17 which are not exposed to UV-B irradiation, suggesting that the phenotype is inducible (Fig. 1C). A reduction

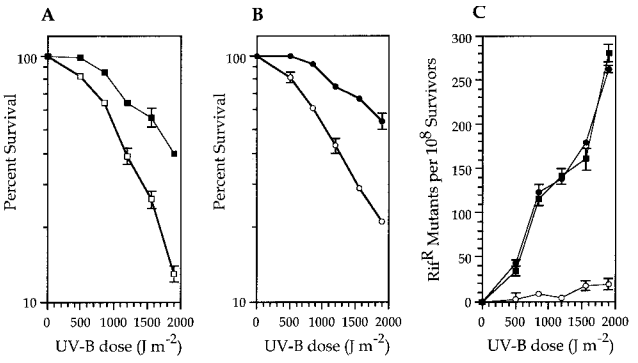

FIG. 1. (A) Survival of *P. syringae* pv. syringae B86-17 (■) and GWS242 (□); (B) survival of GWS242(pJB321) (○) and GWS242(pJJK17) (●) after UV-B irradiation. Each data point represents the mean (±the standard error of the mean) from three replicate UV sensitivity experiments. (C) Effect of the *rulB*::Km mutation in GWS242 on UV-inducible mutagenesis. Rif^s strains were irradiated with different doses of UV-B, samples were removed to initiate cultures that were incubated in LB for 18 h, and the number of Rif^r colonies was determined. The number of spontaneous mutations conferring Rif^r in the absence of UV-B irradiation has been subtracted. Each data point represents the mean (± the standard error of the mean) from three replicate experiments. Symbols: ■, *P. syringae* pv. syringae B86-17; ●, GWS242(pJJK17); ○, GWS242(pJB321).

in mutability of 12- to 14-fold at UV-B doses of ≥850 J m^{-2} was observed in the *rulB*::Km strain GWS242; mutability at wild-type rates was restored when GWS242 was complemented with *rulAB* on the plasmid pJJK17 (Fig. 1C). It should be noted that the mutability of strain GWS242 (Fig. 1C) was also increased to a small degree at each UV-B dose compared to that of nonirradiated GWS242 (≤1 Rif^r mutant per 10^8 survivors recovered at each UV-B dose). In our experiments, we typically observed between 10 and 30 Rif^r mutants per 10^8 survivors at the higher (1,500 and 1,900 J m^{-2}) UV-B doses (Fig. 1C). This observation raises the possibility that an additional source of UV-B mutability is present within the B86-17 genome.

Analyses of additional, natural *P. syringae* strains which did not contain *rulAB* also indicated a phenotype of UV-B mutability following irradiation with 1,500-J m^{-2} UV-B (J. J. Kim and G. W. Sundin, unpublished data), albeit at a significantly lower rate than that of *rulAB*-containing strains. Thus, to fully evaluate the contribution of *rulAB* to UV-B survival and mutability, we utilized *P. aeruginosa* PAO1, a naturally UV-sensitive strain which, according to previous studies, does not contain a functional MDR system within its chromosome (29, 41). We transferred either pJJK17 or the vector pJB321 into strain PAO1 by triparental mating and subsequently determined that *rulAB* conferred an increase in UV-B survival to this strain of as much as 30-fold at the highest UV-B dose (Fig. 2A). UV-B mutability was also significant (up to a 200-fold increase in Rif^r mutants following irradiation at 1,900 J m^{-2}) and clearly inducible by UV-B irradiation (Fig. 2B).

In planta UV-B mutability of *P. syringae* pv. syringae B86-17. The role of the *rulAB* determinant in enabling *P. syringae* strains to maintain and increase population size in their natural leaf surface (phyllosphere) habitat has been previously established (47). However, we have not previously assessed UV-B mutability in planta and, to our knowledge, the occurrence of MDR has not been previously shown using any bacteria in their natural habitat. Our studies were done by inoculating populations of *P. syringae* pv. syringae B86-17 onto bean leaves, excising the leaves at various sampling times after inoculation, irradiating the leaves with UV-B at 500 J m^{-2}, and subsequently incubating the excised leaves for 12 h in a sterile,

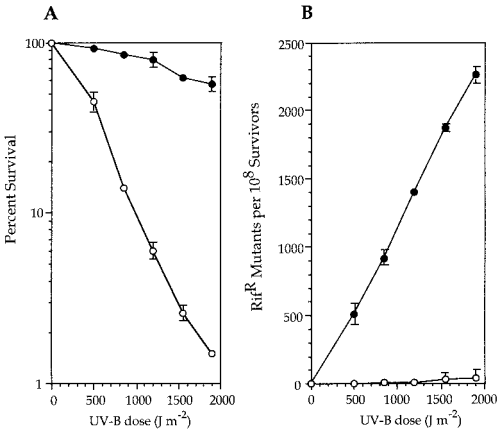

FIG. 2. (A) Survival of *P. aeruginosa* PAO1(pJB321) (○) and PAO1(pJJK17) (●) after UV-B irradiation. Each data point represents the mean (±the standard error of the mean) from three replicate UV sensitivity experiments. (B) Analysis of *rulAB*-mediated MDR in PAO1. Rif^s strains were irradiated with different doses of UV-B, samples were removed to initiate cultures that were incubated in LB for 18 h, and the number of Rif^r colonies was determined. The number of spontaneous mutations conferring Rif^r in the absence of UV-B irradiation has been subtracted. Each data point represents the mean (±the standard error of the mean) from three replicate experiments. Symbols: ○, PAO1(pJB321); ●, PAO1(pJJK17).

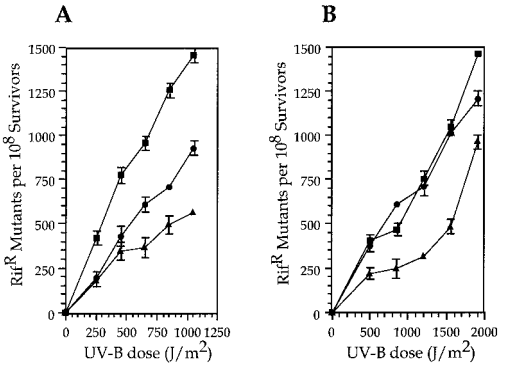

FIG. 3. Ability of defined genetic constructs encoding *rulAB*, *mucAB*, and *umuDC* to restore mutagenesis functions to an *E. coli umuDC* strain and to induce mutagenesis in *P. aeruginosa* PAO1. (A) *E. coli* strain RW120 [*lexA*$^+$ *recA*$^+$ Δ(*umuDC*)*595*::*cat*]. The number of spontaneous mutations conferring Rif^r in the absence of UV-B irradiation has been subtracted. Symbols: ●, *rulAB* (pJJK25); ■, *mucAB* (pJJK26); ▲, *umuDC* (pJJK27). (B) *P. aeruginosa* PAO1. As with *E. coli* RW120, the number of spontaneous mutations conferring Rif^r in the absence of UV-B irradiation has been subtracted. Symbols: ●, *rulAB* (pJJK25); ■, *mucAB* (pJJK26); ▲, *umuDC* (pJJK27). In panels A and B, each data point represents the mean (±the standard error of the mean) from three replicate UV sensitivity experiments.

high-humidity environment. Following the incubation, bacteria were removed from the leaves by sonication and enumerated through plating on KBc alone and KBc containing rifampin. A total of six experiments were done, with the frequency of mutation to Rif^r shown to be significantly greater from irradiated leaves than from nonirradiated control leaves at all sampling points (Table 2). Over time, the ratio of Rif^r cells recovered from irradiated leaves to those recovered from nonirradiated leaves decreased slightly (4.8- to 2.9-fold) (Table 2). The levels of increase in mutability (at 500 J m^{-2}) observed immediately and at 24 h following inoculation were similar to those observed in the previous experiments performed in vitro (Fig. 1C).

Phenotypic comparison of *rulAB* with *mucAB* and *umuDC*. Since *rulAB* was found to be distantly related to *umuDC* at the amino acid level (42), it was of interest to determine if *rulAB* was functionally similar to other well-characterized MDR operons and especially to determine if *rulAB* could restore mutability functions to an *E. coli umuDC* mutant. In order to facilitate phenotypic-analysis studies of *rulAB*, it was desirable to choose hosts in which there was minimal interfering background activity in terms of mutability. We chose two hosts for these experiments, *E. coli* RW120, in which the chromosomal source of MDR, the *umuDC* determinant, was deleted, and *P. aeruginosa* PAO1, a strain which does not encode an MDR determinant. Comparisons of *rulAB* with its homologs *mucAB* and *umuDC* were done using the defined genetic constructs pJJK25, pJJK26, and pJJK27. These constructs only differed by the inclusion of the individual MDR systems; the promoter, Shine-Dalgarno sequences, and vector were identical among them.

TABLE 2. Analysis of *rulAB*-mediated MDR in *P. syringae* pv. syringae B86-17 at each sampling point following inoculation to the phyllosphere of bean

Category of leaf	No. of Rif^r mutants per 10^8 cells recovered at each time point (in h) after inoculation:[a]			
	0 h	24 h	72 h	120 h
Nonirradiated control	3.8	3.3	3.6	4.5
Irradiated at 500 J m^{-2}	18.3***	16.3**	13.0*	13.0*

[a] Values reported are the means from six independent experiments. Significant differences between means at each sampling time are indicated (*, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$).

Restoration of UV-B mutability was first assessed in *E. coli* RW120 containing pJJK25, pJJK26, pJJK27, or the pJB321 vector. As expected, the use of the *umuDC* promoter sequences to control the expression of the MDR systems resulted in UV-B mutability being inducible. The *mucAB* determinant resulted in the largest frequency of mutation to Rif^r (Fig. 3A), confirming previous observations of mutability with *mucAB* using UV-C radiation in strain RW120 (19). Mutability of strain RW120 containing *rulAB* on pJJK25 was at an intermediate level between those of *mucAB* and *umuDC* (Fig. 3A). The additional significance of these observations is that *rulAB* complemented the *umuDC* mutation in *E. coli* RW120. When *P. aeruginosa* PAO1 was used as the host, the positions of each MDR system remained the same, with *mucAB* resulting in the highest mutation frequency (Fig. 3B). However, little difference in mutability was observed between *mucAB* and *rulAB* at four of the five UV-B levels utilized, with a small increase in *mucAB* mutability observed only at the largest UV-B dose (Fig. 3B).

Regulation of the *rulAB* promoter. We used the ice nucleation gene, *inaZ*, as a reporter in our regulation analyses because of the sensitivity of ice nucleation as a reporter (28) and the relative ease of measurement of this reporter when used for in planta studies, which will be reported elsewhere. We chose to use *P. fluorescens* Pf5 for our studies, since Pf5 does not possess INA, and also because Pf5 grows at 25°C, a temperature at which INA is readily expressed (28). Prior to the utilization of *P. fluorescens* Pf5 in our studies, we determined that the *rulAB* determinant was active in this strain, functioning in the increase of both UV-B survival and mutability (data not shown).

Since *P. fluorescens* Pf5 was more sensitive to UV-B irradiation than *P. syringae* pv. syringae B86-17, we irradiated with lower doses of UV-B in the regulation experiments. Following

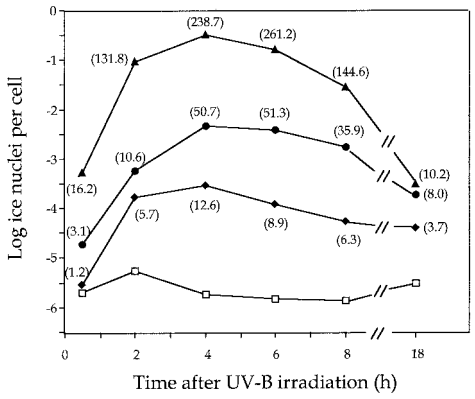

FIG. 4. Analysis of the activity of a *rulAB* promoter fusion with a promoterless *inaZ* gene in *P. fluorescens* Pf5 following UV-B irradiation. *P. fluorescens* Pf5 (pJJK41) was irradiated with different doses of UV-B, samples were removed to initiate cultures which were incubated in KB, and at the designated time points, cells were removed and analyzed for INA using the droplet-freezing assay. Each data point represents the mean from three replicate experiments. Values in parentheses indicate the fold increase in InaZ concentration at each data point relative to that of the nonirradiated control. Symbols for amounts of UV-B irradiation of Pf5(pJJK41): ◆, 75 J m^{-2}; ●, 150 J m^{-2}; ▲, 300 J m^{-2}; □, nonirradiated control.

the irradiation of Pf5 containing the *rulAB* promoter-*inaZ* fusion construct pJJK41 with UV-B at 75, 150, and 300 J m^{-2}, cultures were initiated using the irradiated cells and samples were removed at designated times for INA analysis. An increase in expression was observed within 30 min at the two higher UV-B doses and within 2 h at the 75-J m^{-2} dose (Fig. 4). The increase in *rulAB* promoter activity continued until 4 h after irradiation with the observed levels of INA ranging as high as 10^5 greater than those for the nonirradiated control (Fig. 4). There is an exponential relationship between INA and the abundance of ice nuclei, with INA increasing with the square of InaZ concentration until INA reaches 10^{-1} nuclei per cell (22). Between 10^{-1} and 10^0 ice nuclei per cell, INA increases with the third power (cube) of InaZ concentration (28). Thus, quantification of InaZ levels in our experiments reflects the transcriptional activity of the *rulAB* promoter and, when compared to InaZ levels detected in the control strain containing the pPROBE KI′ vector, indicates that *rulAB* transcription was increased up to 261-fold at 4 h after irradiation with the 300-J m^{-2} dose (Fig. 4). Because of the exponential relationship between INA and the abundance of ice nuclei, calculations of fold increases in promoter activity are sensitive to the INA measurements in the nonirradiated control treatment. Thus, the decrease in INA between 4 and 6 h after irradiation in the control treatment was large enough to result in an elevated increase in InaZ levels (Fig. 4, 150- and 300-J m^{-2} treatments) even though the INA observed at these treatments had actually decreased. Overall, a stair-step effect was consistently observed, with the lower UV-B doses resulting in lower promoter activity at all time points. INA levels expressed from the *rulAB* promoter were still elevated through 18 h after irradiation, although this observation could also be due to the stability of the InaZ protein (Fig. 4).

DISCUSSION

The *rulAB* operon, which is widely distributed among diverse strains of *P. syringae* isolated from many plant hosts, plays an important functional role in protecting strains from the DNA-damaging effects of UV-B radiation. Exposure of *P. syringae* strains to UV-B wavelengths present in solar radiation would be predicted to occur with regularity in the phyllosphere habitat of this organism. Analysis of the UV-B mutability of the *rulAB* operon in *E. coli* RW120 indicated that *rulAB* is functionally similar to *umuDC* and other previously characterized MDR operons, such as *mucAB* and $rumAB_{R391}$. Indeed, *rulAB* shares many other features with the known MDR systems, including the presence of a binding site for the LexA repressor within its putative promoter region, possession of a conserved internal cleavage site within the *rulA* sequence, and lack of function in a *recA* background (46). Each of the known *umu*-like systems varies in its ability to promote mutagenesis in different cellular backgrounds. For example, the approximately threefold increase in our experiments of mutagenesis of *mucAB* in *E. coli* RW120 as compared to that of *umuDC* has been observed by others as well and is attributed to a more efficient processing of MucA to the truncated MucA′ form in an *E. coli* host (10). It is interesting to note that the difference in mutation frequency between *rulAB* and *mucAB* was reduced in the *P. aeruginosa* PAO1 background (Fig. 3B) due to a larger decrease in MucAB-mediated MDR in *P. aeruginosa* than in *E. coli*. This is probably due to a decreased affinity of the MucAB system with *P. aeruginosa* RecA or DNA polymerase.

In the *Pseudomonas* backgrounds, *rulAB* has a large effect on UV-B survival for *P. syringae* as noted previously (46, 47) and for *P. aeruginosa* in the current study. Although the rates of survival of *P. aeruginosa* PAO1(pJJK17) and *P. syringae* pv. syringae B86-17 (*rulAB*+) were similar for UV-B doses up to 1,900 J m^{-2}, the frequency of mutation to Rif^r was approximately sevenfold higher in *P. aeruginosa*. The increased mutation frequency in *P. aeruginosa* could be due to the relative contribution of other non-MDR systems and *rulAB* to the overall repair effort. At the 1,900-J m^{-2} UV-B dose, the survival rate of *P. aeruginosa* PAO1(pJB321) is approximately 20-fold lower than that of *P. syringae* pv. syringae GWS242(pJB321), suggesting that *P. syringae* is more efficient in non-MDR than *P. aeruginosa*.

Through analysis of UV-B mutability in the *rulB*::Km strain GWS242, we demonstrated a small (10- to 20-fold) increase in mutability to Rif^r, an observation that we have also made when examining other natural *P. syringae* strains which do not contain *rulAB* (Kim and Sundin, unpublished). These observations are consistent with the occurrence of another source of UV mutability within the *P. syringae* genome. Hybridizations of *Bam*HI-digested genomic DNA of the *rulAB*-negative strain *P. syringae* pv. syringae FF5 with a *rulAB* probe under low-stringency conditions resulted in the observation of a single hybridizing band (Kim and Sundin, unpublished). We are currently attempting to clone this chromosomal sequence and determine its role in UV survival and mutability. Other examples of MDR systems cooccurring on plasmids and chromosomes in a single strain have been examined in serovar Typhimurium (31) and more recently in *Shigella flexneri* (37). In serovar Typhimurium, the chromosomally encoded $umuDC_{ST}$ determinant is active in UV-inducible mutagenesis, while the plasmid-encoded *samAB* determinant does not contribute to UV mutability (18). The situation is reversed in *S. flexneri*, as the plasmid-encoded *impCAB* determinant is required for UV mutability while the chromosomally encoded *umuDC* operon is not expressed following UV irradiation and therefore is apparently unable to promote UV-induced mutagenesis (37).

Thus, in each of these situations, only one of the MDR operons plays the important role in UV mutability, which leads to the obvious question—why are two operons present? Recently, Opperman et al. (32) showed that the *E. coli umuDC* gene products play an additional role in cell cycle control following DNA damage. These authors demonstrated individ-

ual roles for the UmuD and UmuD′ proteins and proposed a model in which the UmuD and UmuC proteins were involved in a delay in the recovery of DNA replication following DNA damage (32). RecA-mediated cleavage of UmuD and the formation of the UmuD′$_2$C complex would then be required for MDR mediated by translesion synthesis; this would be followed by the resumption of DNA replication (32, 35). Cell cycle control and a delay in replication restart following DNA damage are presumably important because they allow for non-MDR processes such as excision repair to occur, ultimately lowering the cellular mutational load. Organisms which encode two MDR systems in which one is functionally dominant in terms of actual repair may utilize the other system in cell cycle control. Alternatively, the formation of chimeric MDR complexes may also be involved in the posttranslational regulation of these systems. We are interested in examining the chromosomal source of UV mutability in *P. syringae* in order to understand the contribution of this system and *rulAB* to the overall repair process and UV survival.

The regulation analyses performed in this study indicate that *rulAB* is expressed in a UV-inducible manner with increased promoter activity in response to increasing UV-B dosage. The rapid induction of expression, maintenance of transcriptional activity at high levels for 4 to 6 h following irradiation, and overall importance of the *rulAB* determinant to UV-B survival suggest that MDR could play a major role in *P. syringae* survival in the environment. As more studies show the significance of DNA repair processes to organismal survival in habitats with high solar radiation exposure (e.g., reference 15), it would be important to determine the occurrence and role of MDR in organisms in these habitats.

It was previously shown that *rulAB*-containing *P. syringae* strains maintained significantly larger phyllosphere populations following UV-B irradiation (47). Our current results from assays involving phyllosphere populations of *P. syringae* pv. syringae B86-17 indicate that MDR occurs and is detectable during the period when inoculated cells are establishing an infection on their host. Populations of *P. syringae* pv. syringae inoculated to bean leaf surfaces typically initiate disease, which is manifested by the occurrence of leaf spots, within 4 to 6 days. In our experiments, we observed leaf spot symptoms at the 5-day sampling time. Our data indicate that UV-B mutability occurs at levels similar to those recorded in vitro when UV-B irradiation is administered immediately after inoculation. Over time, as strain B86-17 established an infection on its bean host, the frequency of UV-B mutability to Rif^r was slightly reduced (from 4.8- to 2.9-fold) but still significantly greater than in a nonirradiated control. As *P. syringae* strains become established in the phyllosphere, it is thought that a proportion of the cell population colonizes sites on leaves which are protected from external stresses (51). However, our data on UV-B mutability in the phyllosphere and previous data on *rulAB*-mediated UV-B survival in the phyllosphere (47) also clearly indicate the important contribution of the *rulAB* determinant to relative fitness during colonization and establishment of infection in planta.

The importance of *rulAB* to UV tolerance and the potential recurring necessity of this determinant for survival in the phyllosphere seem to distinguish *rulAB* from the other *umu*-like operons from an ecological standpoint. Each of the other *umu*-like operons characterized to date has been isolated from enteric organisms, whose exposure to UV radiation or chemical mutagens would probably be sporadic. One example of the consequence of a limited necessity for MDR in enteric organisms may be an attenuation of activity of the corresponding MDR determinant. For example, when located on its natural plasmid R46, the activity of *mucAB* is repressed by another gene located approximately 2 kb away (20). The role of some of the enteric-organism *umu*-like operons in UV protection is also unclear, as inactivation of either or both $umuDC_{ST}$ and *samAB* in serovar Typhimurium has no effect on UV survival (18). Thus, the distinguishing features of the *rulAB* system in terms of activity when present on its native plasmid, function in UV tolerance, and potentially daily expression in response to solar UV damage make this system appropriate for continued analysis of the ecological and evolutionary ramifications of UV-induced mutagenesis.

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