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# Sequence Diversity of *rulA* among Natural Isolates of *Pseudomonas syringae* and Effect on Function of *rulAB*-Mediated UV Radiation Tolerance

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**The *rulAB* locus confers tolerance to UV radiation and is borne on plasmids of the pPT23A family in *Pseudomonas syringae*. We sequenced 14 *rulA* alleles from *P. syringae* strains representing seven pathovars and found sequence differences of 1 to 12% within pathovar syringae, and up to 15% differences between pathovars. Since the sequence variation within *rulA* was similar to that of *P. syringae* chromosomal alleles, we hypothesized that *rulAB* has evolved over a long time period in *P. syringae*. A phylogenetic analysis of the deduced amino acid sequences of *rulA* resulted in seven clusters. Strains from the same plant host grouped together in three cases; however, strains from different pathovars grouped together in two cases. In particular, the *rulA* alleles from *P. syringae* pv. lachrymans and *P. syringae* pv. pisi were grouped but were clearly distinct from the other sequenced alleles, suggesting the possibility of a recent interpathovar transfer. We constructed chimeric *rulAB* expression clones and found that the observed sequence differences resulted in significant differences in UV (wavelength) radiation sensitivity. Our results suggest that specific amino acid changes in RulA could alter UV radiation tolerance and the competitiveness of the *P. syringae* host in the phyllosphere.**

The pPT23A plasmid family encompasses the majority of native plasmids identified in the plant-pathogenic bacterium *Pseudomonas syringae*; these plasmids share a gene (*repA*) required for plasmid replication and, in most cases, additional areas of homology (16, 30, 35, 38). pPT23A-type plasmids are diverse in size, and multiple plasmids sharing large regions of repeated sequences may be present in the same cell (3, 8, 30, 35). Plasmids in the pPT23A family can encode determinants of importance in host-pathogen interactions such as the coronatine biosynthesis locus, which increases virulence, and the avirulence genes *avrD, avrPphC*, and *avrPphF*, which affect host range (1, 21, 46). Additional sequences known to be encoded on plasmids of the pPT23A family include the *stbCBAD* locus involved in plasmid stability (18), copper resistance determinants (6) and the streptomycin resistance transposon Tn*5393* (39), and insertion sequence elements including IS*51*, IS*801*, IS*870*, and IS*1240* (1, 18, 30). A common feature of all of these determinants is that functional loci encoding these traits are typically limited in distribution to small groups of *P. syringae* pathovars.

Given the distribution of the pPT23A plasmid family throughout *P. syringae* pathovars, it is likely that these plasmids encode a "backbone" of traits of general importance to the *P. syringae* species. We are interested in the evolution of the pPT23A plasmid family in *P. syringae*, including determining the range of pathovars encompassed by particular plasmid lineages, characterizing genes encoded on these plasmids, and delineating the importance of horizontal transfer in pPT23A plasmid biology. In a previous study, we suggested that subgroups of pPT23A-like plasmids formed stable cohesive lineages as defined by Riley and Gordon (32), and further genetic evidence implied that individual plasmids had resided within their host strain for long time periods (39). Thus, to further understand the biology of the pPT23A plasmid family, it was desirable to study a locus that is widely distributed among *P. syringae* pathovars and might encode a trait of general importance.

One such locus is the *rulAB* operon, a homolog of the *umuDC* mutagenic DNA repair system first described in *Escherichia coli* (37). This operon encodes tolerance to UV radiation (UVR) and was recently cloned and characterized from a pPT23A-like plasmid from *P. syringae* pv. syringae (41). In contrast to other known pPT23A family loci, functional copies of the *rulAB* determinant are widely distributed and have recently been described in at least 14 pathovars of *P. syringae* (42). *rulAB*$^+$ *P. syringae* strains vary widely in their tolerance to UVR (42), and we wished to determine if specific sequence alterations accounted for these observations. A functional *rulAB* locus is critical for the maintenance of population size on leaf surfaces that are irradiated with UV-B radiation (42). The importance of epiphytic population growth on leaf surfaces to the epidemiology of most *P. syringae*-host interactions (19) may explain the wide distribution of the *rulAB* determinant.

Our objectives in this study were (i) to compare sequences of *rulA* both within the pathovar syringae and among six other pathovars and (ii) to determine if the observed sequence differences affect the contribution of *rulA* to *rulAB*-mediated UVR tolerance.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids utilized in this study are listed in Tables 1 and 2, respectively. *Escherichia coli* and *Pseudomonas aeruginosa* strains were grown at 37°C on Luria-Bertani medium (Difco Laboratories, Detroit, Mich.) and *Pseudomonas* isolation agar (PIA) (Difco), respectively. *P. syringae* strains were grown at 28°C on King's medium B (24) or Luria-Bertani medium. When necessary, media were supplemented with the following antibiotics at the indicated concentrations (in micrograms per milliliter): ampicillin, 75; carbenicillin, 200; gentamicin, 50; kanamy-

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TABLE 1. Bacterial strains used in this study and relevant characteristics

| Strain | Relevant characteristic(s) | Source or reference[a] |
|---|---|---|
| *E. coli* DH10B | Plasmid-free strain used for cloning | 17 |
| *P. aeruginosa* PAO1 | UV-sensitive, plasmid-free host | AMC |
| *Pseudomonas syringae* | | |
| pv. lachrymans 1188-1 | Isolated from zucchini; California | DAC |
| pv. maculicola 438 | Isolated from crucifers; California | CLB |
| pv. phaseolicola 0886-19 | Isolated from bean; location unknown | CLB |
| pv. pisi 1086-2 | Isolated from pea; location unknown | CLB |
| pv. savastanoi 0886-21 | Isolated from olive; location unknown | CLB |
| pv. syringae | | |
| 4918 | Isolated from butterfly pea; Uganda | ICMP |
| 5D425 | Isolated from apricot; California | DCG |
| 8B48 | Isolated from ornamental pear; Oklahoma | GWS |
| A2 | Isolated from ornamental pear; Oklahoma | 38 |
| B86-17 | Isolated from bean; New York | DEL |
| BBS32-5 | Isolated from bean; Colorado | DEL |
| HS191 | Isolated from millet; Australia | AKV |
| pv. tomato | | |
| OK-1 | Isolated from tomato; Oklahoma | CLB |
| PT14 | Isolated from tomato; California | CLB |

[a] AKV, A. K. Vidaver; CLB, C. L. Bender; AMC, A. M. Chakrabarty; DAC, D. A. Cooksey; DCG, D. C. Gross; DEL, D. E. Legard; GWS, G. W. Sundin; ICMP, International Collection of Microorganisms from Plants.

cin, 25; and rifampin, 100. Triparental matings, using the helper plasmid pRK2013, were done to mobilize plasmid constructs into *P. aeruginosa* PAO1.

**UV sensitivity characterization.** We used either UV-B or UV-C radiation in our UV sensitivity experiments. The UV-B sensitivity of the *P. syringae* strains following a dose of 590 J $m^{-2}$ (biologically effective dose calculated using the DNA damage action spectrum of Setlow [36]) was determined; this survival value can be compared to those of *P. syringae* strains assayed previously (42). UV-C radiation also was used because the higher-energy UV-C wavelengths more readily distinguish differences in the UV sensitivity of individual strains.

We grew cells to late log phase ($OD_{600}$=1.3) in LB medium containing carbenicillin. The cells were pelleted, washed in 0.85% NaCl, and resuspended at a concentration that was 10-fold less than that of the growth culture in 15 ml of 0.85% NaCl in a sterile glass petri dish (100 by 15 mm). The cell suspensions were exposed to UV-B radiation (maximum output at 302 nm) from an XX-15M

TABLE 2. Bacterial plasmids used in this study and relevant characteristics

| Plasmid[a] | Relevant characteristic(s) |
|---|---|
| pBluescript SK(+) | $Ap^r$; cloning vector |
| pCR2.1 | Vector for direct cloning of *Taq* PCR products |
| pET-5a | $Ap^r$; expression vector |
| pJB321 | $Ap^r$; broad-host-range cloning vector |
| pJJK20 | *E. coli umuDC* promoter amplified from pRW154 and ligated into pET-5a as *Sph*I-*Xba*I |
| pJJK21 | *rulAB* coding sequence from B86-17 ligated into pJJK20 as *Nde*I-*Bam*HI |
| pRK2013 | Helper plasmid for triparental matings |
| pRW154 | Source of the *E. coli umuDC* promoter |
| p183-1 | *rulA* PCR product from strain HS191 in pCR2.1 |
| p191-1 | *rulA* PCR product from strain 4918 in pCR2.1 |
| p201-1 | *rulA* PCR product from strain BBS32-5 in pCR2.1 |
| p202-1 | *rulA* PCR product from strain B86-17 in pCR2.1 |
| p202-2 | *rulA* coding sequence from p202-1 ligated into pJJK21 as *Nde*I-*Hin*dIII |
| p202A-202B | Cassette containing *rulA* from B86-17, *rulB* from B86-17, and UV-inducible *umuDC* promoter ligated into pJB321 as *Sal*I-*Bam*HI |
| p205-1 | *rulA* PCR product from strain 5D425 in pCR2.1 |
| p205-2 | *rulA* coding sequence from p205-1 ligated into pJJK21 as *Nde*I-*Hin*dIII |
| p205A-202B | Cassette containing *rulA* from 5D425, *rulB* from B86-17, and UV-inducible *umuDC* promoter ligated into pJB321 as *Sal*I-*Bam*HI |
| p216-1 | *rulA* PCR product from strain 1086-2 in pCR2.1 |
| p216-2 | *rulA* coding sequence from p216-1 ligated into pJJK21 as *Nde*I-*Hin*dIII |
| p216A-202B | Cassette containing *rulA* from 1086-2, *rulB* from B86-17, and UV-inducible *umuDC* promoter ligated into pJB321 as *Sal*I-*Bam*HI |
| p219-1 | *rulA* PCR product from strain 0886-19 in pCR2.1 |
| p223-1 | *rulA* PCR product from strain OK-1 in pCR2.1 |
| p240-1 | *rulA* PCR product from strain 8B48 in pCR2.1 |
| p241-1 | *rulA* PCR product from strain A2 in pCR2.1 |
| p241-2 | *rulA* coding sequence from p241-1 ligated into pJJK21 as *Nde*I-*Hin*dIII |
| p241A-202B | Cassette containing *rulA* from A2, *rulB* from B86-17, and UV-inducible *umuDC* promoter ligated into pJB321 as *Sal*I-*Bam*HI |
| p244-1 | *rulA* PCR product from strain 0886-21 in pCR2.1 |
| p244-2 | *rulA* coding sequence from p244-1 ligated into pJJK21 as *Nde*I-*Hin*dIII |
| p244A-202B | Cassette containing *rulA* from 0886-19, *rulB* from B86-17, and UV-inducible *umuDC* promoter ligated into pJB321 as *Sal*I-*Bam*HI |
| p366-1 | *rulA* PCR product from strain PT14 in pCR2.1 |
| p366-2 | *rulA* coding sequence from p366-1 ligated into pJJK21 as *Nde*I-*Hin*dIII |
| p366A-202B | Cassette containing *rulA* from PT14, *rulB* from B86-17, and UV-inducible *umuDC* promoter ligated into pJB321 as *Sal*I-*Bam*HI |
| p377-1 | *rulA* PCR product from strain 1188-1 in pCR2.1 |

[a] Sources or references for the following plasmids are as indicated: pBluescript SK(+), Stratagene, La Jolla, Calif.; pCR2.1, Invitrogen, Carlsbad, Calif.; pET-5a, Promega, Madison, Wis.; pJB321, reference 5; pRK2013, reference 12; and pRW154, reference 20. All other plasmids were generated in this study.

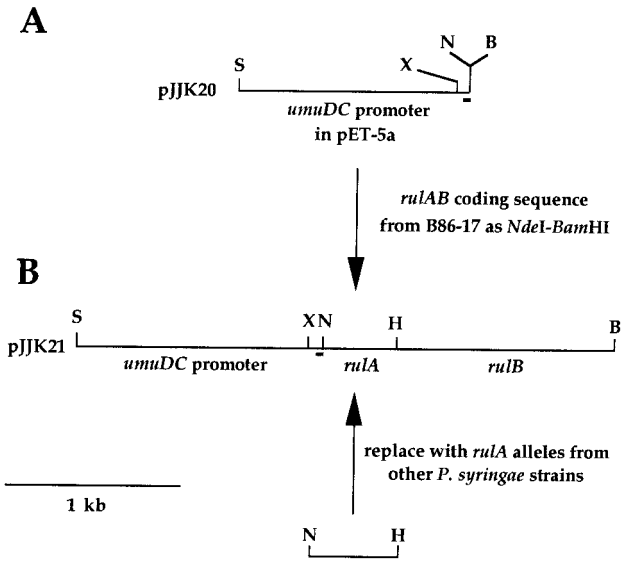


FIG. 1. Map and construction of chimeric *rulAB* expression clones. (A) Insertion of the *umuDC* promoter region from pRW154 as a *Sal*I-*Xba*I fragment into pET-5a creating pJJK20. (B) Insertion of the *rulAB* coding sequence from *P. syringae* pv. syringae B86-17 as an *Nde*I-*Bam*HI fragment, creating pJJK21, and replacement of the *rulA* allele with *rulA* alleles from other *P. syringae* strains inserted as *Nde*I-*Hin*dIII fragments. Restriction sites relevant for the construction are shown: B, *Bam*HI; H, *Hin*dIII; N, *Nde*I; S, *Sal*I; X, *Xba*I. The position of the Shine-Dalgarno sequence of pET-5a is indicated by the underscored bar.

UV lamp (Ultraviolet Products, Upland, Calif.) or to UV-C radiation (254 nm) from an XX-15S UV lamp. In either case, lamps were placed horizontally at a fixed height above the suspensions and turned on 15 min prior to use to allow for stabilization of the UV output. The output of the UV-B lamp was filtered through cellulose diacetate (Kodacel; Eastman Kodak, Rochester, N.Y.) to remove wavelengths below 290 nm. The energy output of the lamps was monitored with a UV-X radiometer fitted with the appropriate wavelength sensor (Ultraviolet Products) and determined to be 3.0 J $m^{-2}$ $s^{-1}$ for UV-B and 1.5 J $m^{-2}$ $s^{-1}$ for UV-C. Cells were continuously mixed during UVR exposure to eliminate survival due to shading. After irradiation, surviving cells were enumerated by dilution plating conducted under dark conditions to minimize photoreactivation.

**Genetic and molecular biology techniques.** Standard molecular biology techniques were utilized. Indigenous pPT23A-like plasmids were isolated from *P. syringae* strains as described previously (38). Nucleotide sequencing was done using the Big Dye kit (Applied Biosystems, Foster City, Calif.) following the instructions of the manufacturer; sequence reactions were run at the Genetic Technologies Center, Texas A&M University. Oligonucleotides were obtained from Life Technologies, Gaithersburg, Md. The *rulA* nucleotide and derived amino acid sequences were aligned using the program Clustal W (43). The derived amino acid sequences were analyzed phylogenetically using the Protpars program of PHYLIP (11) as previously described (23). The derived amino acid sequence of *rumA*, a closely related homolog of *rulA* from *Klebsiella pneumoniae* (25), was included as the outgroup.

**Genetic analysis of the *rulA* locus.** *rulA* was amplified from 14 *P. syringae* strains via PCR using plasmid preparations as the template and the primers *rul*2 (5′-CGTTAACTGTACGTCCATACAG-3′) and *rul*4 (5′-CGAATTGCAATCG ACCAG-3′). The *rul*2 primer encompassed the consensus LexA-binding site upstream of the *rulA* coding sequence, and the *rul*4 primer is the reverse complement of a sequence within *rulB* that is highly conserved among *rulB* homologs from *E. coli* (41). Standard PCR conditions were utilized (35), except the annealing temperature was lowered to 37°C to account for possible sequence divergence at the primer sites. The size of individual amplified fragments was checked on 1.2% agarose gels, and the fragments were ligated directly into the vector pCR2.1 (Invitrogen, Carlsbad, Calif.). The resulting clones were utilized as source material for nucleotide sequencing.

We made chimeric *rulAB* constructs utilizing pJJK21 (Fig. 1), a construct in the expression vector pET-5a (5) containing the *rulAB* coding sequence from *P. syringae* pv. syringae B86-17. *rulAB* was amplified via PCR from pB86-17A, the single indigenous plasmid harbored by strain B86-17, using the primers *rulAB Nde*I 5′ (5′-GGATTCCATATGAACGTCAAAATACTCGG-3′) and 3′ *rulB* TAA *Bam*HI (5′-GATCGGATCCTTACTTTACAACCCACAGCTG-3′), and ligated as an *Nde*I-*Bam*HI fragment into pJJK20. pJJK20 contains the SOS-inducible *umuDC* promoter from *E. coli*, which was amplified from pRW154, using the primers *umu* Pro 5′ *Sph*I (5′-GATCGCATGCGAGCAATTGCGTC GC-3′) and *umu* Pro 3′ (5′-GTACTCTAGACTGCCTGAAGTTATACTG-3′), and ligated as an *Sph*I-*Xba*I fragment into the expression vector pET-5a upstream of the *Nde*I site. The translational start site of the *rulA* coding sequence was the ATG sequence within the *Nde*I site; this site was located with optimal spacing from a Shine-Dalgarno sequence present on pET-5a.

We amplified *rulA* alleles from *P. syringae* pv. savastanoi 0886-21, *P. syringae* pv. syringae 5D425, 7B12, and BBS32-5, and *P. syringae* pv. tomato PT14 by PCR using the primers *rulA* 5′ *Nde*I (5′-GGATTCCATATGAACGTCAAAATACT CGG-3′) and *rul*4, digested them with *Nde*I-*Hin*dIII, and ligated them into pJJK21 without the *rulA* determinant originally present in this construct. We used primers 216 5′ *Nde*I (5′-GGAATTCCATATGAACGTAAAAATTCTCG GC-3′) and 216 *Hin*dIII 3′ *rulA* (5′-CCCAAGCTTGTTACGCCATGTCGCAC AACG-3′) to amplify the *rulA* locus from *P. syringae* pv. pisi 1086-2 because of extensive nucleotide sequence differences within *rulA*. This process altered one nucleotide in the *P. syringae* pv. pisi 1086-2 *rulA* sequence to generate the *Hin*dIII site used in the cloning. The presence of the correct *rulA* locus within each chimeric construct was confirmed by nucleotide sequencing. Each of the resulting cassettes containing the *umuDC* promoter, artificial Shine-Dalgarno sequence, and the chimeric *rulAB* locus was excised with *Sal*I and *Bam*HI, ligated into pJB321, and mobilized into *P. aeruginosa* PAO1 for analysis.

We determined the UV sensitivity of *P. aeruginosa* PAO1 containing each chimeric *rulAB* construct. The *E. coli umuDC* promoter is functional in PAO1 in a UV damage-inducible fashion (J. J. Kim and G. W. Sundin, unpublished data). UV sensitivity assays using UV-C radiation were done as described above, and each experiment was performed three times. The UV sensitivity data were evaluated using an analysis of variance based on UV-C dose, and differences among the survival values were assessed using the Student-Newman-Keuls test.

**Nucleotide sequence accession numbers.** The individual *rulA* nucleotide sequences generated in this study have been deposited in GenBank (accession numbers U43696 and AF251481 to AF251493).

TABLE 3. Relevant characteristics of *P. syringae* strains utilized in the present study relating to *rulAB* carriage and nucleotide sequence alterations of *rulA*

| Strain[a] | % survival following UV-B dose[b] | Nucleotide sequence differences with *rulA* from pPSR1 |
|---|---|---|
| Psl 1188-1 | 3 ± 1 | 50 |
| Psm 438 | 51 ± 12 | 37 |
| Psph 0886-19 | 62 ± 13 | 34 |
| Pspi 1086-2 | 2 ± 1 | 53 |
| Psv 0886-21 | 66 ± 13 | 31 |
| Pss A2 | 60 ± 17 | 0 |
| Pss BBS32-5 | 67 ± 17 | 23 |
| Pss B86-17 | 72 ± 19 | 23 |
| Pss 5D425 | 70 ± 18 | 26 |
| Pss 8B48 | 80 ± 15 | 28 |
| Pss HS191 | 71 ± 11 | 34 |
| Pss 4918 | 77 ± 12 | 30 |
| Pst OK-1 | 75 ± 23 | 34 |
| Pst PT14 | 60 ± 7 | 34 |

[a] Abbreviations: Psl, *P. syringae* pv. lachrymans; Psm, *P. syringae* pv. maculicola; Psph, *P. syringae* pv. phaseolicola; Pspi, *P. syringae* pv. pisi; Pss, *P. syringae* pv. syringae; Pst, *P. syringae* pv. tomato; Psv, *P. syringae* pv. savastanoi.

[b] The biologically effective (36) dose of UV-B radiation delivered (maximum output at 302 nm) was 590 J $m^{-2}$, delivered using an XX-15M lamp. Results are the means ± standard deviations of three experiments per strain.

## RESULTS

**Sequence diversity within *rulA* alleles.** We determined a UV tolerance phenotype for each strain except *P. syringae* pv. lachrymans 1188-1 and *P. syringae* pv. pisi 1086-2, which were both highly UV sensitive (Table 3).

At the nucleotide level, intrapathovar sequence divergence ranged from 5 to 8% and interpathovar divergence was as high as 13% when the *rulA* alleles were compared to *rulA* from *P. syringae* pv. syringae A2 (Table 3). Thirty-two of the 141 amino acids (23%) were polymorphic among the strains (data not shown). A twofold-larger proportion of polymorphic sites (39%) was observed within the first 25 amino acids of the *rulA* sequence than in the remaining 116 amino acids (19%). The

TABLE 4. Amino acid alterations between *rulA* alleles from 14 *P. syringae* strains

| Strain[b] | No. of amino acid alterations[a] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pss A2 | Pss BBS32-5 | Pss B86-17 | Pss 5D425 | Pss 8B48 | Pss 4918 | Pss HS191 | Psm 438 | Psph 0886-19 | Psv 0886-21 | Pst OK-1 | Pst PT14 | Psl 1188-1 | Pspi 1086-2 |
| Pss A2 | | 1+2 | 1+3 | 2+3 | 3+3 | 2+5 | 2+7 | 1+3 | 0+4 | 1+3 | 2+3 | 2+3 | 4+6 | 4+7 |
| Pss BBS32-5 | 1+2 | | 0+1 | 1+3 | 2+3 | 3+5 | 1+6 | 0+3 | 1+4 | 0+3 | 1+3 | 1+3 | 3+6 | 3+7 |
| Pss B86-17 | 1+3 | 0+1 | | 1+4 | 2+4 | 3+6 | 1+7 | 0+4 | 1+5 | 0+4 | 1+4 | 1+4 | 3+7 | 3+8 |
| Pss 5D425 | 2+3 | 1+3 | 1+4 | | 3+4 | 4+4 | 2+6 | 1+2 | 2+3 | 1+2 | 2+2 | 2+2 | 2+5 | 2+6 |
| Pss 8B48 | 3+3 | 2+3 | 2+4 | 3+4 | | 5+6 | 3+8 | 2+4 | 3+5 | 2+4 | 3+4 | 3+4 | 5+7 | 5+8 |
| Pss 4918 | 2+5 | 3+5 | 3+6 | 4+4 | 5+6 | | 4+8 | 3+4 | 2+3 | 3+4 | 4+4 | 4+4 | 6+7 | 6+8 |
| Pss HS191 | 2+7 | 1+6 | 1+7 | 2+6 | 3+8 | 4+8 | | 1+6 | 2+7 | 1+4 | 2+6 | 2+6 | 4+9 | 4+10 |
| Psm 438 | 1+3 | 0+3 | 0+4 | 1+2 | 2+4 | 3+4 | 1+6 | | 1+3 | 0+2 | 1+2 | 1+2 | 3+5 | 3+6 |
| Psph 0886-19 | 0+4 | 1+4 | 1+5 | 2+3 | 3+5 | 2+3 | 2+7 | 1+3 | | 1+3 | 2+3 | 2+3 | 4+6 | 4+7 |
| Psv 0886-21 | 1+3 | 0+3 | 0+4 | 1+2 | 2+4 | 3+4 | 1+4 | 0+2 | 1+3 | | 1+2 | 1+2 | 3+5 | 3+6 |
| Pst OK-1 | 2+3 | 1+3 | 1+4 | 2+2 | 3+4 | 4+4 | 2+6 | 1+2 | 2+3 | 1+2 | | 0+2 | 2+5 | 2+6 |
| Pst PT14 | 2+3 | 1+3 | 1+4 | 2+2 | 3+4 | 4+4 | 2+6 | 1+2 | 2+3 | 1+2 | 0+2 | | 2+5 | 2+4 |
| Psl 1188-1 | 4+6 | 3+6 | 3+7 | 2+5 | 5+7 | 6+7 | 4+9 | 3+5 | 4+6 | 3+5 | 2+5 | 2+5 | | 0+3 |
| Pspi 1086-2 | 4+7 | 3+7 | 3+8 | 2+6 | 5+8 | 6+8 | 4+10 | 3+6 | 4+7 | 3+6 | 2+6 | 2+4 | 0+3 | |

[a] The first number presented in each column entry is the number of sequence differences in the putative 25-amino-acid RulA leader region. The second number is the number of sequence differences in the remaining 116 amino acids of the protein. The sum of each column entry represents the total number of amino acid differences in the entire protein.

[b] Abbreviations: Psl, *P. syringae* pv. lachrymans; Psm, *P. syringae* pv. maculicola; Psph, *P. syringae* pv. phaseolicola; Pspi, *P. syringae* pv. pisi; Pss, *P. syringae* pv. syringae; Pst, *P. syringae* pv. tomato; Psv, *P. syringae* pv. Savastanoi.

amino acids $Ala_{25}$-$Gly_{26}$ define a putative cleavage site where homologs of RulA such as UmuD are truncated to an activated form (36), implying that alterations in the first 25 amino acids of RulA may be more tolerated. A matrix of amino acid substitutions among the strains showed that the minimum and maximum number of amino acid differences observed among single strain pairs was 1 (1%) and 14 (10%), respectively (Table 4). The average number of amino acid differences among the sequence collection was 9% within the putative 25-amino-acid leader region and 4% within the remainder of the sequence (Table 4). *rulA* alleles from some *P. syringae* pv. syringae strains were more similar to those from other pathovars than to other strains from within the pv. syringae (Table 4).

We generated a cladogram that differentiated the sequences into several subgroups (Fig. 2). Amino acid differences are shown as unique characters on the cladogram, and multiple changes may be associated with a single branching point (11). Three of the subgroups (*P. syringae* pv. tomato OK-1 and PT14, *P. syringae* pv. syringae A2 and 8B48, and *P. syringae* pv. syringae B86-17 and BBS32-5), contained strains that were isolated from the same plant host. In two other cases, however, subgroups (*P. syringae* pv. lachrymans 1188-1 and *P. syringae* pv. pisi 1086-2, *P. syringae* pv. phaseolicola 0886-19, and *P. syringae* pv. syringae 4918) contained strains from different pathovars and different hosts (Fig. 2).

**Functional analysis of individual *rulA* alleles.** We tested *rulA* alleles from strains representing six distinct branches of the *rulA* cladogram (Fig. 2). Since the constructs were identical except for the *rulA* allele, our assumption was that expression of *rulAB* from each construct was similar. Each chimeric *rulAB* construct had greater UV tolerance ($P = 0.05$) than that of PAO1 containing the vector pJB321 at each of five UV-C doses (Table 5). Significant differences in survival were observed among the alleles at four of the five UV-C doses employed (Table 5). The magnitude of survival differences increased with increasing UV-C dose, and the largest difference observed was a fourfold difference at 60 J $m^{-2}$ between the *rulA* alleles from *P. syringae* pv. syringae 5D425 and *P. syringae* pv. savastanoi 0886-21 (Table 5). The *rulA* allele from *P. syringae* pv. pisi 1086-2 was functional in these experiments, although its parental host strain is UV sensitive (Table 3).

## DISCUSSION

We compared *rulA* sequences from seven pathovars of *P. syringae*, as a prelude to larger-scale plasmid analyses, in an effort to define plasmid lineages within the *P. syringae* species. Considerable intra- and interpathovar sequence divergence was evident within the *rulA* gene of *P. syringae*, a gene which, together with *rulB*, encodes tolerance to UVR through a mu-

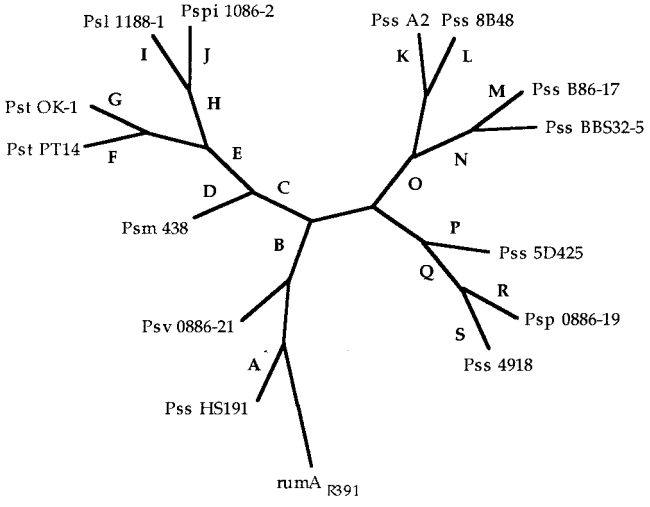


FIG. 2. Radial cladogram resulting from a Protpars program of PHYLIP (11) analysis based on amino acid dissimilarities of 14 sequences of *rulA* from *P. syringae* using the homolog *rumA* from plasmid R391 as an outgroup. Abbreviations Psl, *P. syringae* pv. lachrymans; Psm, *P. syringae* pv. maculicola; Psph, *P. syringae* pv. phaseolicola; Pspi, *P. syringae* pv. pisi; Pss, *P. syringae* pv. syringae; Pst, *P. syringae* pv. tomato; Psv, *P. syringae* pv. savastanoi. The letters (arbitrarily beginning with Pss HS191) indicate the amino acid change(s) from the A2 allele at the nearest branch point. Amino acid changes are noted in single letter code for the residue in the A2 *rulA* allele, followed by the residue in the alleles further away from the branching point: A, M1V, D58N, V74F, P95L, Q103H; B, L91V; C, V96L, S97C; D, R79A; E, L18F; F, F39S; G, D70G; H, C10S, T20S, K80T, P118A, E125D; I, V54A; J, F39S, H106D; K, S97 (unique amino acid to Pss A2, C in other alleles); L, N2K, R8W, Q37H, F139S; M, E140G; N, D59Y; O, V96 (unchanged from A2 allele); P, E126D; Q, I121V; R, A65G; S, P17S, Y19C, D42Y, H120Q.

TABLE 5. Comparison of UV sensitivities of *P. aeruginosa* PAO1 containing various chimeric *rulAB* constructs or the vector pJB321.

| Source of *rulA*[b] | Mean % survival following irradiation with 254 nm UV-C dose of [a]: | | | | |
|---|---|---|---|---|---|
| | 12 J $m^{-2}$ | 24 J $m^{-2}$ | 36 J $m^{-2}$ | 48 J $m^{-2}$ | 60 J $m^{-2}$ |
| Pathovar syringae 5D425 p205A-202B | 88 A | 67 AB | 49 A | 31 A | 14 A |
| Pathovar pisi 1086-2 p216A-202B | 86 A | 77 A | 47 A | 26 A | 9 B |
| Pathovar syringae A2 p241A-202B | 87 A | 77 A | 32 B | 21 B | 5 CD |
| Pathovar tomato PT14 p366A-202B | 85 A | 55 B | 29 B | 17 BC | 6 C |
| Pathovar savastanoi 0886-21 p244A-202B | 82 A | 50 B | 31 B | 17 BC | 4 D |
| Pathovar syringae B86-17 p202A-202B | 81 A | 50 B | 29 B | 13 C | 7 BC |
| pJB321 | 30 B | 4 C | 0.4 C | 0.1 D | 0.01 E |

[a] Within a column, means not followed by the same letter are significantly different at $P = 0.05$, following an analysis of variance and the Student-Newman-Keuls test. Calculated $R_2$ values ($\alpha = 0.05$) for each comparison (56 df) were as follows: for 12 J $m^{-2}$ 14.6; for 24 J $m^{-2}$, 15.4; for 36 J $m^{-2}$, 7.0; for 48 J $m^{-2}$, 5.4; for 60 J $m^{-2}$, 2.6.

[b] Six unique amino acid substitutions were noted among the *rulA* alleles examined (amino acid changes are noted in single-letter code for the original residue, followed by the residue in the unique allele): pathovar syringae 5D425, E125D; pathovar syringae A2, C97S; pathovar pisi 1086-2, K80T; pathovar savastanoi 0886-21, L91V; pathovar syringae B86-17, D58Y and E141G.

tagenic DNA repair system. The maximum nucleotide sequence differences observed were 12% within the pathovar syringae (8B48 and HS191) and 15% between two pathovars (*P. syringae* pv. syringae HS191 and *P. syringae* pv. pisi 1086-2). The minimum sequence difference observed was 1.2% between the two bean isolates of *P. syringae* pv. syringae, a value that is relatively high when other interstrain sequence comparisons of plasmid-borne *P. syringae* genes are considered (4, 44, 46). The percent divergence among *rulA* alleles observed in our study is similar to that observed for four chromosomal loci of *P. syringae* examined by Sawada et al. (33). In their study, comparisons of partial sequences of *gyrB, hrpL, hrpS*, and *rpoD* from 19 pathovars showed overall nucleotide differences ranging from approximately 4 to 15% (33).

Sequence comparisons of plasmid-borne *P. syringae* genes have been reported for the avirulence gene *avrD* (three pathovars), a 650-bp region internal to the coronafacate ligase (*cfl*) gene within the coronatine biosynthetic cluster (four pathovars), and the *efe* gene encoding the ethylene-forming enzyme (five pathovars) (4, 23, 44, 46). In contrast to results with *gyrB, hrpL, hrpS, rulA*, and *rpoD*, relatively few sequence differences were observed, even among pathovars. For example, the nucleotide sequence difference observed within the *cfl* gene ranged from <1 to 3%, and the *efe* alleles were virtually identical (< 1% sequence difference) with one exception (4, 44). Based on these comparisons, we think that the *avrD, cfl*, and *efe* genes have been disseminated more recently than *rulAB* among *P. syringae* pathovars. Alternatively, the *avrD, cfl*, and *efe* loci might be subject to strong selection pressure and unable to tolerate significant sequence alterations.

We utilized UV-B (290 to 320 nm) and UV-C (<290 nm) radiation interchangeably in our UV sensitivity analyses. The amount and spectral quality of UV-B (290 to 320 nm) radiation reaching the earth's surface is affected by geographic and physical factors and may range from 1 to >10 kJ $m^{-2}$ $day^{-1}$ (28). In contrast, higher-energy UV-C wavelengths are completely screened by the stratospheric ozone layer and do not reach the earth's surface; however, UV-C wavelengths more readily distinguish differences in the UV sensitivity of individual strains. In previous experiments, we have shown that *rulAB* confers a phenotype of UV tolerance to both UV-C and UV-B wavelengths (41, 42); such comparability is predicted because the biological effects of both UV-B and UV-C radiation are due mainly to direct DNA damage (13).

A UV-B dose of 590 J $m^{-2}$ differentially affected the survival of the *P. syringae* strain collection (Table 3). The *rulA* alleles from the UV-sensitive strains *P. syringae* pv. pisi 1086-2 and *P. syringae* pv. lachrymans 1188-1 differed by only three amino acids (2%), and possessed several unique subsititutions compared to the others (data not shown). The *rulA* from *P. syringae* pv. pisi 1086-2 could confer UV tolerance in conjunction with the *P. syringae* pv. syringae B86-17 *rulB* allele at levels similar to that of the other alleles in the experiment (Table 5). We concluded that the UV sensitivity of *P. syringae* pv. pisi 1086-2 was due to the presence of a nonfunctional *rulB* allele, analogous to the weakly functional *efe* allele borne on a plasmid in *P. syringae* pv. pisi (44). Alternatively, the UV sensitivity of strain 1086-2 could be due to the presence or absence of another genetic locus that increases the strain's UV sensitivity. Such a situation occurs on the IncJ plasmid R391, which bears the *rulAB* homolog $rumAB_{R391}$ (25) yet sensitizes its host bacterium to UV irradiation (31). Recently, a natural *rulB*-disruption mutant, containing a 4.5-kb insertion including the *avrPpiA1* gene, was discovered in a Race 2 strain of *P. syringae* pv. pisi (2). We are currently analyzing the *rulB* alleles from *P. syringae* pv. pisi 1086-2 and *P. syringae* pv. lachrymans 1188-1 to determine if similar insertions have occurred.

We found that the six *rulA* alleles conferred different levels of UV tolerance to the *P. aeruginosa* PAO1 host. The magnitude of differences in survival increased with increasing UV-C dose, suggesting that some chimeric *rulAB* alleles were functionally better equipped to handle an increased load of DNA damage. The significant differences in survival observed at higher UV-C doses among the *rulA* alleles from *P. syringae* pv. savastanoi 0886-21 and *P. syringae* pv. syringae 5D425, A2, and B86-17 could be due to unique amino acid alterations (e.g., L91V in 0886-21, E126D in 5D425, C97S in A2, and D58Y and E141G in B86-17) since these alleles were otherwise very similar. However, we did not include the *rulB* alleles from these strains; thus, it is possible that the differences in survival observed due to differences in *rulA* could be masked by sequence differences in the *rulB* alleles. We wish to determine if the differences in UV survival observed affect the area on a leaf surface that can be colonized. It is known that *rulAB* significantly affects strain survival on leaf surfaces in the presence of UV-B radiation (42). Our current focus is to determine if subtle differences in *rulAB* activity can significantly impact the phyllosphere fitness of competing $rulAB^+$ strains.

*rulAB* appears to be important to the general biology of a wide range of *P. syringae* strains, and *rulAB*-mediated UV tolerance enables *P. syringae* strains to maintain population size in the phyllosphere (42). The attainment of large population sizes

on individual leaves is an important prerequisite to disease incidence in some cases (19). Thus, from an ecological standpoint, it is likely that the ability of *P. syringae* to overcome UV stress is an important feature for its successful occupation of the phyllosphere habitat. That such a trait would be plasmid borne is consistent with Eberhard's hypothesis which states that plasmids tend to bear genes of importance in local adaptations (10). *rulAB*-mediated UV tolerance may be required only at certain times within the *P. syringae* life cycle, as strains are not constantly exposed to solar UVR.

Although extensive intrapathovar diversity occurs within *P. syringae*, and in particular within pathovar syringae (7, 9, 15, 26, 40, 45), some pathovars such as pathovars actinidiae and tomato are strikingly homogenous, with little observed genetic diversity (9, 34). Most *P. syringae* pathovars are distinct and readily distinguishable (27, 29). Indeed, comparison of sequence relatedness at the genome level has shown some pathovars (e.g., pathovars savastanoi and avellanae) are sufficiently different that reclassification into species separate from *P. syringae* has been proposed (14, 22). The *rulA* sequence variation is similar to that seen in previous studies utilizing chromosomal loci; i.e., there was considerable intrapathovar variation within *P. syringae* pv. syringae, and sequences from *P. syringae* pv. tomato were very closely related. Thus, we think that the *rulAB* locus has been evolving for a long period of time within *P. syringae*, probably mostly borne on plasmids of the pPT23A family. Further large-scale plasmid analyses are needed to unravel the evolutionary history of the pPT23A plasmid family, including identifying lineages and the pathovars that they occupy and examining the effect of residence within distinct pathovars and the effect of host plants on the ultimate composition of the plasmid genome.

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