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Differential Roles of the *Pseudomonas aeruginosa* PA14 *rpoN* Gene in Pathogenicity in Plants, Nematodes, Insects, and Mice

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We cloned the *rpoN (ntrA, glnF)* gene encoding the alternate sigma factor σ^{54} from the opportunistic multihost pathogen *Pseudomonas aeruginosa* strain PA14. A marker exchange protocol was used to construct the PA14 *rpoN* insertional mutation *rpoN*::Gen^r. PA14 *rpoN*::Gen^r synthesized reduced levels of pyocyanin and displayed a variety of phenotypes typical of *rpoN* mutants, including a lack of motility and the failure to grow on nitrate, glutamate, or histidine as the sole nitrogen source. Compared to wild-type PA14, *rpoN*::Gen^r was ca. 100-fold less virulent in a mouse thermal injury model and was significantly impaired in its ability to kill the nematode *Caenorhabditis elegans*. In an *Arabidopsis thaliana* leaf infectivity assay, although *rpoN*::Gen^r exhibited significantly reduced attachment to trichomes, stomata, and the epidermal cell surface, did not attach perpendicularly to or perforate mesophyll cell walls, and proliferated less rapidly in *Arabidopsis* leaves, it nevertheless elicited similar disease symptoms to wild-type *P. aeruginosa* PA14 at later stages of infection. *rpoN*::Gen^r was not impaired in virulence in a *Galleria mellonella* (greater wax moth) pathogenicity model. These data indicate that *rpoN* does not regulate the expression of any genes that encode virulence factors universally required for *P. aeruginosa* pathogenicity in diverse hosts.

In gram-negative bacteria, the alternate sigma factor σ^{54}, working in concert with a transcriptional activator that belongs to the NtrC superfamily, activates a variety of genes that are regulated in response to external stimuli (1). For example, in various bacteria, σ^{54} is required for expression of the enzymatic pathways responsible for nitrogen utilization, dicarboxylate transport, xylene degradation, and hydrogen utilization (6, 32, 39, 41, 61). σ^{54} is also involved in the regulation of virulence-related factors in both plant and animal pathogens, including pilin, flagellin, and alginate synthesis in *Pseudomonas aeruginosa* (19, 58, 60); capsular expression in *Klebsiella pneumoniae* (3); and regulation of *hrp* gene expression and coronatine biosynthesis in *Pseudomonas syringae* (23, 24).

Our laboratory has developed a bacterial pathogenicity model that utilizes a clinical isolate of *P. aeruginosa* (strain UCBPP-PA14 [referred to here as PA14]) that elicits severe soft-rot-like symptoms and proliferates when infiltrated into *Arabidopsis* leaves (52), kills the larvae of the wax moth caterpillar *Galleria mellonella* (30), causes lethal sepsis in a mouse full-skin-thickness burn model (52), and kills the nematode *Caenorhabditis elegans* (40, 56, 57). Interestingly, there is significant overlap among the PA14 virulence factors required for pathogenesis in plants, nematodes, insects, and mice. For example, among 21 genes identified as being involved in pathogenesis by screening transposon-induced PA14 mutants in plants and nematodes, 18, 17, 19, and 21 of these genes were required for pathogenicity in *Arabidopsis*, nematodes, wax moths, and mice, respectively (30, 40, 53, 57).

In other studies, we showed that *rpoN* is a key virulence factor for the plant pathogen *P. syringae* (23, 24). Specifically, molecular and genetic analysis showed that the *P. syringae rpoN* gene is required for expression of the *P. syringae hrp* gene cluster, a block of contiguous genes, some of which encode components of a type III secretory system (2, 18, 26, 45, 46, 59).

Given the facts that RpoN activates the expression of a wide variety of environmentally regulated genes and is required for virulence in a variety of pathogens, we hypothesized that RpoN would play a central role in the evolution of *P. aeruginosa*'s ability to be a pathogen of evolutionarily disparate hosts. In this study we describe the results of experiments that involved the construction of a *P. aeruginosa* PA14 *rpoN* mutant to study the role of σ^{54} in *P. aeruginosa* pathogenesis in a variety of plant and animal hosts. Surprisingly, we report that the *P. aeruginosa rpoN* gene is not a universal virulence factor required for multihost pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used and constructed in this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* strains were grown at 37°C in L broth, King's A (KA), King's B (KB) (33), or M9 minimal salts media. Nitrogen source utilization tests for PA14 *rpoN* mutants were performed in M9 salts minimal medium by replacing ammonium chloride with an alternative nitrogen source at 5 mM when required. Bacterial motility was tested on "swarm plates" (35). Pyocyanin assays (17) were carried out in KA broth containing 100 μM $FeCl_3$ (17, 33). Pyoverdin was assayed on KB plates as described previously (57). *C. elegans* killing assays were carried out on NG agar ("slow killing" [56]) or PGS agar ("fast killing" [56]) as described elsewhere. Antibiotic concentrations for *E. coli* strains were as follows: streptomycin, 150 μg/ml; kanamycin, 25 μg/ml; tetracycline, 12 μg/ml; gentamicin, 5 to 10 μg/ml; and spectinomycin, 20 μg/ml. Antibiotic concentrations for *P. aeruginosa* were as follows: streptomycin, 200 μg/ml; kanamycin, 200 μg/ml;

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype, phenotype, or role[a]	Source or reference
P. aeruginosa		
PAK N1	*rpoN*::Gen^r	S. Lory (28)
PA14	Wild type	M. Schroth (52)
PA14 *rpoN*::Gen^r	Contains Gen^r cassette inserted into *rpoN*	This study
PA14(pSMC21)	PA14 expressing GFP	56
PA14 *rpoN*::Gen^r(pSMC21)	*rpoN*::Gen^r expressing GFP	This study
E. coli		
DH5a	F$^-$ *lacZ*ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1* λ$^-$; host for cosmid library and other plasmids	Bethesda Research Laboratories (20)
MM294(pRK2013)	Donor of transfer functions for triparental crosses	14
Plasmids		
pJSR1	Ap^r, cosmid cloning vector	52
pBSK(+)	Ap^r, cloning vector	Stratagene, Inc.
pBR322	Ap^r Tet^r, cloning vector	New England Biolabs, Inc.
pKI11	Source of *P. aeruginosa* PAK *rpoN* gene	S. Lory (28)
pPAR4	4-kb fragment containing PA14 *rpoN* gene in pBSK(+)	This study
pPAR4SR	4-kb fragment containing PA14 *rpoN* gene in pJSR1	This study
pRPONgent	PA14 *rpoN*::Gen^r in pBR322	This study
pRPON10	PA14 *rpoN* gene in pJSR1	This study
pSMC21	Derivative of plasmid pSMC2; carries the *A. victoria* GFP	G. A. O'Toole (7)

[a] Ap^r, ampicillin resistance; Tet^r, tetracycline resistance.

tetracycline, 75 μg/ml; gentamicin, 30 μg/ml; nalidixic acid, 50 μg/ml; rifampin, 100 μg/ml, and carbenicillin, 300 μg/ml.

Bacterial genetics. pJSR1 derivatives were introduced into *Pseudomonas* strains via triparental matings with MM294/pRK2013 as the donor of transfer functions as described previously (14). Plasmid pSMC21 (7) containing the *Aequorea victoria* green fluorescent protein (GFP) was introduced into PA14 *rpoN*::Gen^r by electroporation.

Pyocyanin assays. Pyocyanin was measured at 520 nm in acidic solution by using a modified version of a previously described method (17). Cultures were grown from a 100-fold dilution of a log-phase culture in KA broth modified with 100 μM $FeCl_3$. After 20 h, 1 ml of the culture was extracted with 2 ml of chloroform and centrifuged for 5 min. The blue chloroform solution was transferred into a new tube containing 1 ml of 0.2 N HCl to extract pyocyanin into the acidic solution. The concentration was determined by measuring the optical density at 520 nm (OD_{520}).

Plant material and growth of plants. *Arabidopsis* ecotypes Llagostera (Ll-0) and Landsberg *erecta* (La-*er*) were obtained from the *Arabidopsis* Biological Resource Center, Columbus, Ohio. *Arabidopsis* plants were grown in Metro-Mix 2000 in either a climate-controlled greenhouse at 19°C under a 12-h light-dark cycle with supplemental fluorescent illumination or in a Percival AR-60L growth chamber at 20°C and 50% relative humidity.

***Arabidopsis* pathogenicity assays.** Six- to eight-week-old intact or detached *Arabidopsis* rosette leaves were used for pathogenicity assays. The pathogenicity of PA14 strains was tested by placing detached Ll-0 leaves on a 1.5% water agar surface with their petioles embedded into the agar and inoculating the leaves by growing lawns of PA14 or PA14 *rpoN*::Gen^r overnight on LB agar medium containing rifampin (PA14) and gentamicin (*rpoN*::Gen^r), cutting 3-mm-diameter agar cylinders from these plates, and placing the cylinders bacterial side down on one or both sides of the central vein in the top half of a leaf. The growth of PA14 or PA14 *rpoN*::Gen^r in *Arabidopsis* leaves was determined by infiltrating the leaves of intact La-*er* plants with 5×10^4 CFU/cm^2 of leaf area as described previously (52). The growth of PA14 or PA14 *rpoN*::Gen^r in detached *Arabidopsis* leaves was determined by infiltrating Ll-0 leaves with a bacterial suspension in 10 mM $MgSO_4$ at a density of 5×10^4 CFU/ml for 1 h under a slight vacuum at room temperature in the wells of a six-well microtiter plate. At days 0, 1, 2, 3, and 4, the titer of the bacteria in each of two punches in each of three leaves was determined as described previously (15).

Infection of *G. mellonella* larvae. The 50% lethal dose (LD_{50}) of PA14 strains in *G. mellonella* larvae was determined as described previously (30). In brief, overnight cultures grown in KB medium were diluted 1:100, allowed to grow until they reached an OD_{600} of 0.3 to 0.4, and resuspended in 10 mM $MgSO_4$. After dilution to an OD_{600} of 0.1 with 10 mM $MgSO_4$, serial 10-fold dilutions were made in 10 mM $MgSO_4$ containing 1 mg of rifampin/ml and 10 mg of carbenicillin/ml. A 10-μl Hamilton syringe was used to inject 5-μl aliquots into the hindmost left proleg of fifth-instar *G. mellonella* larvae purchased from Van der Horst Wholesale, St. Mary's, Ohio. Groups of 10 larvae infected with the same dose of bacteria were placed in petri dishes and incubated at 25°C for 60 h. Larvae were scored as dead when they no longer moved upon shaking of the petri dish or poking with a pipette tip.

Mouse full-skin-thickness burn model. Inbred AKR mice that had been subjected to a thermal burn injury were infected with PA14 strains as described previously (54). In brief, 6-week-old male mice were anesthetized by the injection of phenobarbital. The animals were then shaved, and a ventral skin fold was elevated. Two brass blocks preheated to 92 to 95°C were applied to the skin fold for 5 s to deliver a full-skin-thickness burn covering ca. 5% of the body surface area. The burn eschar was injected with 100 μl of bacterial suspension at a titer of 5×10^4 or 5×10^6 bacteria per ml. Bacteria for the inoculation were grown overnight in LB, diluted 1:100, allowed to grow until they reached an OD_{600} of 1.6 to 1.7, pelleted by centrifugation, and resuspended and diluted in 10 mM $MgSO_4$. The mouse protocol was reviewed and approved by the Animal Care Committee of the Massachusetts General Hospital.

***C. elegans* killing assay.** The killing kinetics of *C. elegans* strain Bristol N2 by PA14 strains were determined on PGS agar (fast-killing assay) or NG agar (slow-killing assay) as described previously (56).

Nucleic acid manipulations. Routine DNA manipulations such as DNA blots and plasmid DNA isolation were performed as described previously (4). Restriction enzymes, T4 DNA ligase, and calf intestine phosphatase were purchased from Boeringer Mannheim and New England BioLabs and used according to the manufacturers' specifications.

Cloning the *P. aeruginosa* PA14 *rpoN* gene and construction of a PA14 *rpoN* mutant. A cosmid clone, pRPON10, containing a presumptive *P. aeruginosa* PA14 *rpoN* gene was identified in a pJSR1 cosmid library (52) by colony hybridization with *P. aeruginosa* strain PAK *rpoN* gene on plasmid pKI11 as a hybridization probe. The presumptive PA14 *rpoN* gene was mapped to a 4.0-kb *Xho*I-*Eco*RI fragment and was subcloned into pBSK(+) to produce pPAR4. DNA sequence analysis showed 95% identity out of 141 nucleotides sequenced between the PA14 *rpoN* gene and the *P. aeruginosa* PAO1 *rpoN* gene (reference 31 and data not shown).

A mutant derivative of the PA14 *rpoN* gene was constructed by inserting a DNA cassette conferring gentamicin resistance (Gen^r) into the *Cla*I site of the PA14 *rpoN* gene in pPAR4 to create *rpoN*::Gen^r and then excising a 5.5-kb *Xho*I-*Spe*I fragment containing *rpoN*::Gen^r and ligating it into the *Eco*RI site of pBR322 to create pRPONgent. pRPONgent (containing *rpoN*::Gen^r) was conjugated into PA14 via a triparental mating by using pRK2013 (14), and

TABLE 2. Nitrogen utilization of *P. aeruginosa* strains

Nitrogen source	Nitrogen utilization of strain:				
	PA14			PAK	
	Wild type (plates[a])	*rpoN* mutant		Wild type (plates[a])	*rpoN* mutant (plates[a])
		Plates[a]	Liquid[b]		
Ammonia	2	4	+	2	4
Arginine	2	3	+	2	3
Glutamate	2	–	–	2	–
Glutamine	2	2	+	2	2
Histidine	2	–	–	2	–
Nitrate	2	–	–	2	–

[a] Measured as the number of days until the appearance of colonies. – indicates that only tiny colonies grew.
[b] Growth (+) or absence of growth (−) in liquid culture. Growth experiments were conducted twice with identical results

rpoN::Gen^r was marker exchanged into the PA14 genome by first selecting for gentamicin resistance and then screening for carbenicillin sensitivity.

Scanning electron and confocal microscopy. To monitor the attachment of PA14 GFP-labeled strains to the epidermal cell walls of Ll-0 leaves and to Ll-0 trichomes by confocal laser microscopy, 4-mm-diameter leaf disks were immersed in bacterial suspensions at a density of ca. 10^9 cells/ml under weak vacuum to remove possible air bubbles from the surface of the leaf disks and make the leaf surface more accessible to bacteria. Subsequently, leaf disks were incubated for 24 h in the bacterial suspensions with shaking under normal pressure and room temperature. The leaf disks were examined with a confocal laser spectrophotometer (Leica TCS NT) by excitation at 488 nm and by monitoring the emission intensity at 511 nm. To monitor the development of PA14 strains in Ll-0 parenchyma cells by scanning electron microscopy, 4-mm-diameter leaf disks were cut from the central portion of a leaf from either side of the central vein with a cork borer and then immersed in a PA14 or PA14 *rpoN*::Gen^r suspension at an OD_{600} of 0.02 in 10 mM $MgSO_4$ in the wells of a 24-well microtiter dish, followed by incubation at room temperature. Leaf disks were fixed at 1, 2, 3, and 4 days postinfection (dpi) in 4% paraformaldehyde, passed through an ethanol series (30, 50, 70, 96, and 100%), and freeze fractured in liquid nitrogen. The plant material was dried in a critical-point drying apparatus (Samdri-PVT-3B; Tousimis), mounted on stubs, coated with a 20- to 25-μm layer of gold-palladium in a Hummer II Sputter Coater (Technics), and studied by using an AMRAY 1000 scanning electron microscope.

RESULTS AND DISCUSSION

PA14 *rpoN*::Gen^r exhibits a variety of characteristic RpoN$^-$ phenotypes. As described in Materials and Methods, an interspecies hybridization method was used to identify and clone the *P. aeruginosa* PA14 *rpoN* gene. The PA14 *rpoN* gene was partially sequenced and over this region showed 95% identity to both *P. aeruginosa* PAO1 and PAK *rpoN* sequences (data not shown). Also, as described in Materials and Methods, a PA14 *rpoN* mutant (*rpoN*::Gen^r) was constructed by inserting a DNA cassette conferring gentamicin resistance into the *Cla*I site of the PA14 *rpoN* gene and transferring the disrupted gene into the PA14 genome by homologous recombination. The Gen^r cassette is inserted within the N-terminal 33% of *rpoN*, before the highly conserved carboxy-terminal region (31, 41).

Unlike wild-type PA14, *rpoN*::Gen^r exhibited a variety of characteristic RpoN$^-$ phenotypes. Similar to a previously described *P. aeruginosa* PAK *rpoN* mutant (strain PAK N1) (58), PA14 *rpoN*::Gen^r was nonmotile and did not grow well on glutamate, histidine, or nitrate as the sole nitrogen source (Table 2). Electron microscopic examination showed that PA14 *rpoN*::Gen^r cells were nonflagellated (data not shown). In contrast to a previous report that *P. aeruginosa* PAK *rpoN* (strain N1) is a glutamine auxotroph (58), we found that both PAK *rpoN* (strain N1) and PA14 *rpoN*::Gen^r grew slowly in the absence of glutamine. Restricted nitrogen utilization, rather than glutamine auxotrophy, is also observed with *rpoN* mutants of *P. syringae* and *P. putida* (36).

To confirm that the RpoN$^-$ phenotypes of PA14 *rpoN*::Gen^r were due to the *rpoN*::Gen^r insertion, a 4.0-kb DNA fragment containing a wild-type copy of PA14 *rpoN* on plasmid pPAR4SR was introduced into the PA14 *rpoN*::Gen^r strain. This plasmid restored motility and wild-type growth on all of the nitrogen sources listed in Table 2. Although this complementation experiment did not rule out the possibility that the *rpoN*::Gen^r insertion has a polar effect on downstream genes, it appears likely that the RpoN$^-$ phenotypes of PA14 *rpoN*::Gen^r are primarily due to the disruption of the *rpoN* gene. This conclusion is based on the conservation of the genomic region surrounding *rpoN* in *P. aeruginosa* strain PAO1 in comparison to the analogous genomic regions in several bacterial species (29, 31, 42, 44, 50, 55). Upstream of the PAO1 *rpoN* gene is an open reading frame (ORF) with homology to the ATP-binding component of an ABC transporter (21). Downstream of *rpoN* in PAO1 are four ORFs (55). Homologous downstream ORFs are found in several species (29, 31, 42, 44, 50, 55). The second ORF is homologous to the enzyme IIA domains of several proteins of the bacterial sugar phosphotransferase system (PTS) (31). The fourth ORF is homologous to the gene for the HPr protein, which is also a component of the PTS (50). ORF1 and -3 encode peptides that have no homology to proteins of known function. These downstream ORFs appear to play a role in modifying RpoN activity, although the effects are relatively minor. Disruption of the second downstream ORF in *P. aeruginosa* strain PAK reduced the ability of PAK to grow without glutamine, but overexpression of ORF1 or ORF2 by the *tac* promoter had no measurable effect (31). Mutation of either the first or second downstream ORFs in *K. pneumoniae* increased expression of a number of *rpoN*-regulated genes (42). In *Caulobacter crescentus* and *Rhizobium etli*, mutations of the downstream ORFs had only a minor effect on one *rpoN*-regulated gene, *fliK* (29), or had no measurable effect (44), respectively.

PA14 *rpoN*::Gen^r synthesizes reduced levels of pyocyanin. *P. aeruginosa* secretes a variety of compounds under nutrient-limiting conditions, including the yellow-green siderophore pyoverdin and the blue microbial toxin pyocyanin (10, 43). Pyoverdin may be a virulence factor in burn wound infections (5, 43), and pyocyanin has been implicated in pulmonary artery injury (9, 22). KA Fe^{3+} medium or swarm plates turned noticeably blue when PA14 was grown to stationary phase,

TABLE 3. Lethality of PA14 in a mouse burn model

P. aeruginosa strain	Mortality ratio (no. of animals that died/total no. of animals [%]) at 10 days post burn	
	Low dose[a]	High dose[b]
PA14	7/7 (100)	8/8 (100)
PA14 *rpoN*::Gen^r	1/7 (14)	5/8 (62)

[a] Six-week-old male AKR/J mice (Charles River Laboratories) were injected with 5×10^3 cells as described previously (54).
[b] Injected with 5×10^5 cells.

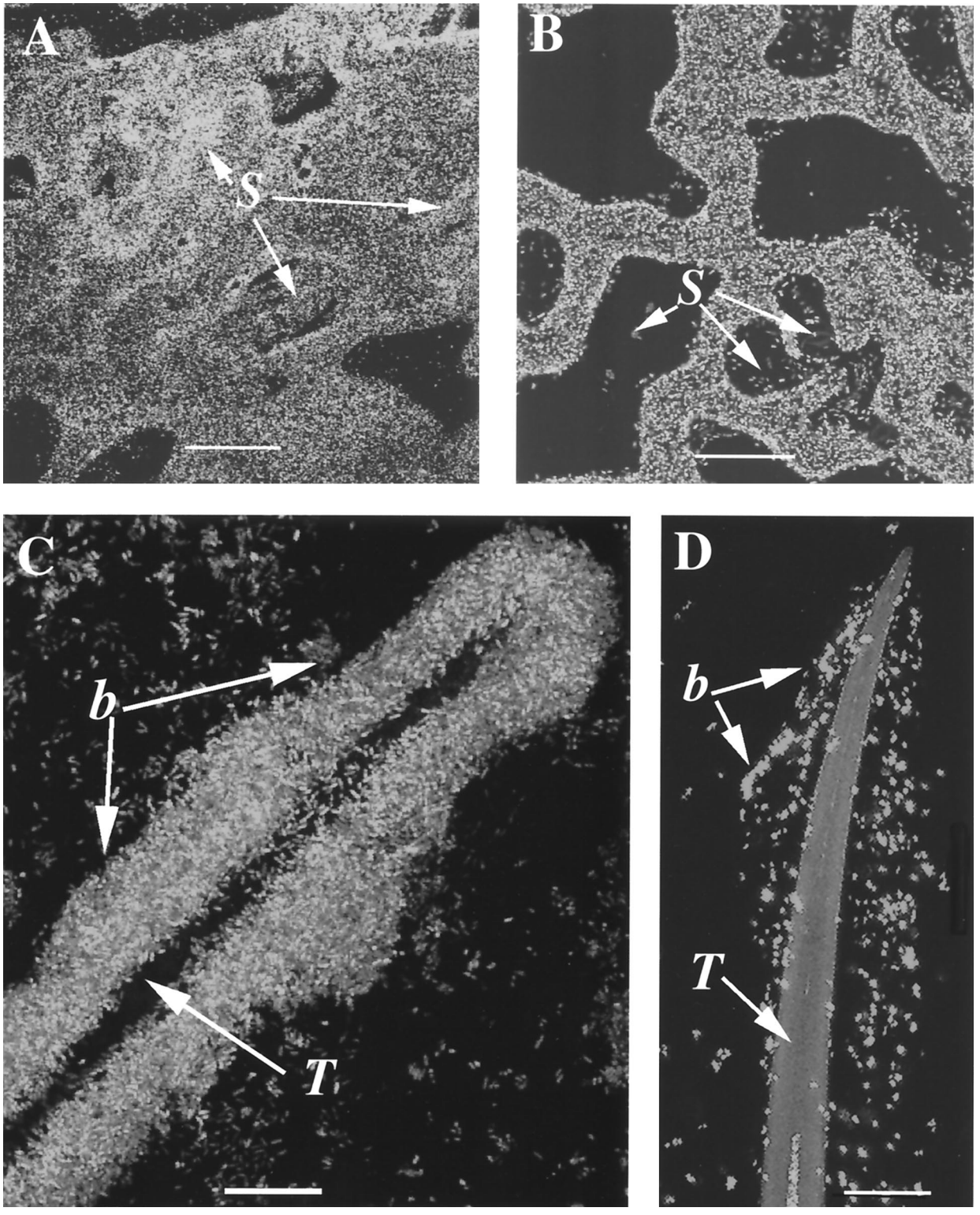

FIG. 1. *P. aeruginosa* adheres to leaf surfaces. Confocal scanning microscopy of PA14 or *rpoN*::Gen^r expressing GFP in association with *Arabidopsis* ecotype Ll-O. (A) PA14(pSCM21) adheres to the entire surface of the leaf, including the stomatal (*S*) openings. Bar, 20 μm. (B) PA14 *rpoN*::Gen^r(pSCM21) cells primarily attached to the grooves formed at cell junctions; very few of the mutant bacteria were located above the stomata (*S*). Bar, 20 μm. (C) PA14(pSCM21) (*b*) formed large multilayered clusters on trichomes (*T*). Bar, 10 μm. (D) PA14 *rpoN-GFP*::Gen^r(pSCM21) did not attach to the trichome (T) surface and concentrated loosely around them. Bar, 10 μm. Leaf disks were incubated with PA14/pSMC21 (A and C) or PA14 *rpoN*::Gen^r/pSMC21(B and D) in bacterial suspensions at a density of ~10^9 CFU/ml under slight vacuum for 1 h, followed by incubation for 24 h in an orbital shaker at room temperature, and then examined by confocal laser microscopy as described in Materials and Methods.

whereas no blue color was observed after the growth of PA14 *rpoN*::Gen^r. Quantitative measurement showed that PA14 *rpoN*::Gen^r produced 56% ± 13% less pyocyanin than did the wild type and that pyocyanin production was restored to wild-type levels in PA14 *rpoN*::Gen^r(pPAR4SR). Pyoverdin production appeared to be unaffected in PA14 *rpoN*::Gen^r. PA14 colonies grown on KB plates express pyoverdin, which can be detected by fluorescence when the colonies are exposed to long-wavelength UV light. Visual inspection revealed no noticeable difference between wild-type and PA14 *rpoN*::Gen^r colonies.

PA14 *rpoN*::Gen^r exhibits reduced pathogenicity in mice. As shown in Table 3, PA14 *rpoN*::Gen^r was significantly less pathogenic than wild-type PA14 in a mouse burn model. A high percentage of lethality was observed with PA14 at both a relatively high dose of 5×10^5 cells (100% lethality) and a relatively low dose of 5×10^3 cells (~80% lethality). In contrast, only five of eight and one of seven animals died when inoculated with 5×10^5 and 5×10^3 PA14 *rpoN*::Gen^r, respectively. The use of two different doses allows a better estimation of the mutation's effects on virulence. In the case of *rpoN*, even the high dose was not as lethal as wild type at a 100-fold-lower dose, demonstrating a significant reduction in virulence. These burn model data are consistent with previous reports demonstrating that *P. aeruginosa rpoN* mutants or *P. aeruginosa* mutants which contain lesions in RpoN-regulated genes exhibit reduced colonization and virulence in a number of other model systems. *rpoN* mutants showed a reduced ability to colonize in a chronic murine intestinal mucosal model (48) or in human respiratory epithelial xenografts (11) and exhibited reduced virulence in a murine corneal scratch model (51) or in a murine model of acute pneumonia (12). One limitation of these studies, as well as of our own, however, is the fact that *rpoN* mutants are nonmotile, and it is not known to what extent the nonmotile phenotype of the *rpoN* mutants contributes to the loss of virulence. Nonmotile mutants of *P. aeruginosa* strains M2, PA01, and MT1200 exhibited significant reduction in virulence in a mouse burn model (16). The reduction was at the same level or greater than that seen with the PA14 *rpoN* mutant. Thus, the entire effect on virulence of the *rpoN* mutant in *P. aeruginosa* PA14 in the mouse burn model could be due to the loss of motility. RpoN is also an important virulence factor for virulence of *Vibrio anguillarum* in fish (47) and *Vibrio cholerae* in an infant mouse colonization model (13, 34). In the case of *V. cholerae*, it appears that unknown *rpoN*-regulated genes, in addition to the flagellum genes, are required for colonization.

PA14 *rpoN*::Gen^r exhibits reduced pathogenicity in *Arabidopsis*. We have developed several assays to assess the pathogenicity of *P. aeruginosa* in *Arabidopsis* leaves, comparing attachment to plant leaf surfaces and monitoring growth rate and symptom development. We previously showed (49) that when *Arabidopsis* ecotype Ll-0 leaf disks are incubated in a dense bacterial suspension (i.e., ~10^9 cells/ml) for 24 h, wild-type PA14 cells attached to the entire leaf epidermal cell surface, congregated above most of the stomata, and formed large, multilayered clusters on the trichomes (Fig. 1A and C). In contrast, as shown in Fig. 1B and D, PA14 *rpoN*::Gen^r cells primarily attached to the grooves formed at cell junctions, and very few of the mutant bacteria were located above the stomata or associated with trichomes.

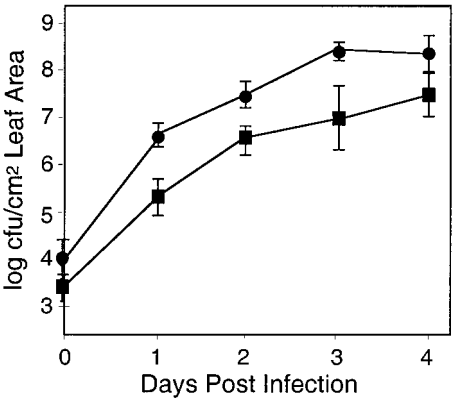

FIG. 2. Growth of PA14 and PA14 *rpoN*::Gen^r in *Arabidopsis* leaves. Detached Ll-0 leaves were vacuum infiltrated with bacterial suspensions as described in Materials and Methods. The initial titers were ca. 5×10^4 CFU/cm^2 leaf area. Bacterial titers in the infiltrated leaves were measured immediately after infiltration and at 1, 2, 3, and 4 days after infiltration as described in Materials and Methods. Each value represents the average of three replicates. Similar results were obtained when the leaves of intact 6-week-old La-*er* plants were infiltrated at a titer of 5×10^4 CFU/cm^2 of leaf area and incubated as described in Materials and Methods (not shown). Symbols: ●, PA14; ■, PA14 *rpoN*::Gen^r.

As shown in Fig. 2, PA14 *rpoN*::Gen^r showed an ~10-fold decrease in its ability to proliferate in detached *Arabidopsis* Ll-0 leaves compared to PA14 after gentle vacuum infiltration of a relatively low inoculum of bacteria. Similar results were obtained when intact La-*er Arabidopsis* plants were hand infiltrated with a 1-ml syringe without a needle (data not shown). With either mode of infection, PA14 *rpoN*::Gen^r ultimately elicited severe soft-rot symptoms.

We also tested the ability of PA14 *rpoN*::Gen^r to infect Ll-0 leaves and elicit disease symptoms without being forced into the leaf by a syringe or vacuum infiltration. Lawns of PA14 or PA14 *rpoN*::Gen^r were grown on LB-rifampin agar. Agar cylinders 3 mm in diameter were cut and placed bacterial side down on detached *Arabidopsis* Ll-0 leaves as described in Materials and Methods. Both PA14 and PA14 *rpoN*::Gen^r formed lesions under these assay conditions, but at relatively early stages of infection (4 days postinfection [dpi]) the wild-type strain produced significantly larger lesions than PA14 *rpoN*::Gen^r. However, by 7 dpi, we did not find any significant differences in the symptoms produced by PA14 and PA14 *rpoN*::Gen^r. At 4 dpi, the PA14 lesions were, on average, 6.3 ± 1.45 mm in diameter, whereas the diameter of the PA14 *rpoN*::Gen^r-elicited lesions were 4.4 ± 1 mm. At 7 dpi, the mean diameters of the PA14 and PA14 *rpoN*::Gen^r lesions were 7.3 ± 0.85 and 6.91 ± 1.14 mm, respectively. Interestingly, chlorotic rings around the lesions formed by the less-virulent PA14 *rpoN*::Gen^r mutant were significantly larger than around PA14 lesions (data not shown).

Finally, we showed in a recent publication that one of the hallmarks of PA14 pathogenesis of *Arabidopsis* is perpendicular attachment of PA14 cells to the leaf epidermis and to mesophyll cell walls (49). Moreover, as shown in Fig. 3B and C,

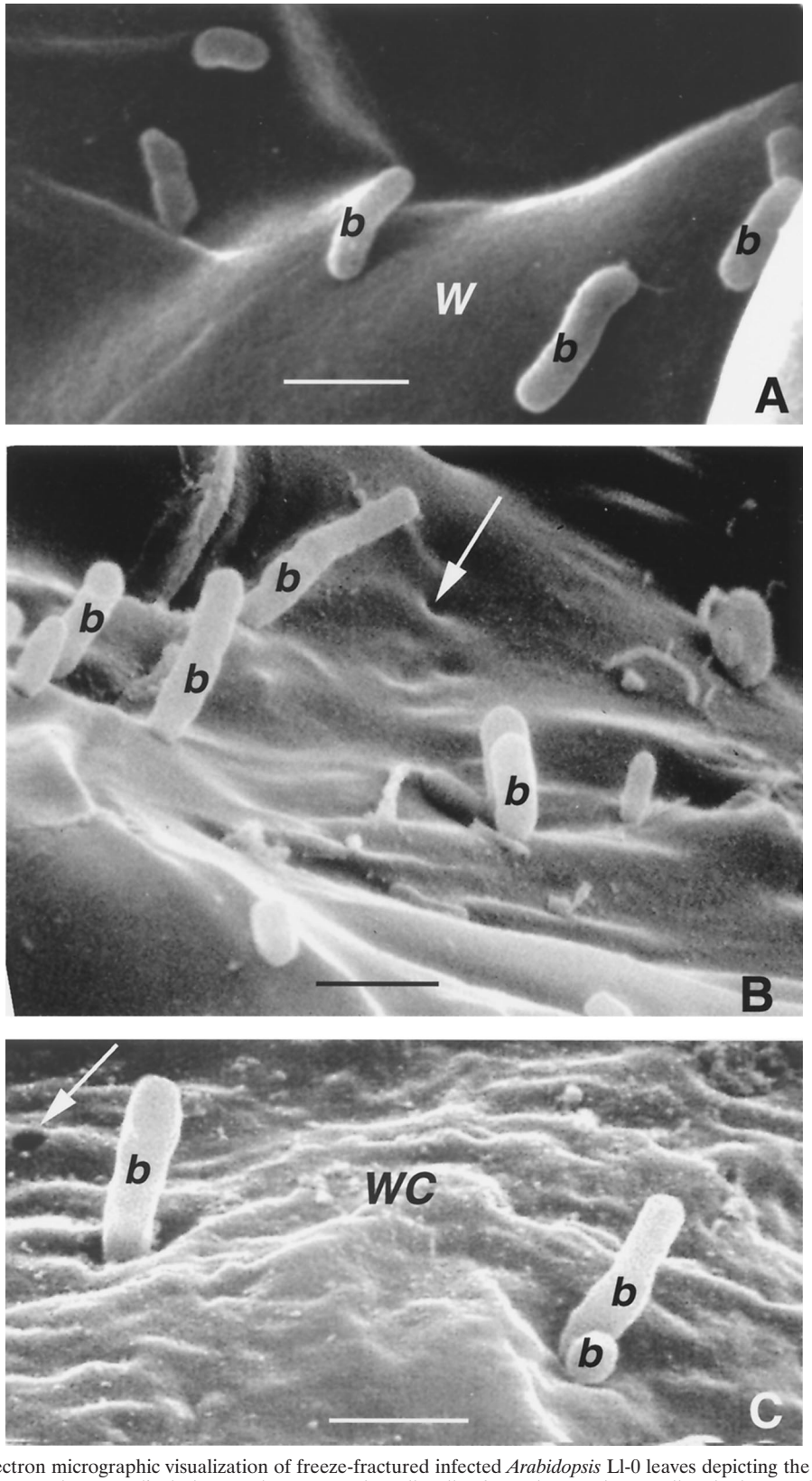

FIG. 3. Scanning electron micrographic visualization of freeze-fractured infected *Arabidopsis* Ll-0 leaves depicting the failure of *P. aeruginosa* PA14 *rpoN*::Gen^r cells to attach perpendicularly to and penetrate the cell walls of vessel parenchyma cells. The leaves of intact Ll-0 plants were infiltrated under vacuum, the infected plants were incubated for 72 h, and then examined by scanning electron microscopy as described in Materials and Methods. *b,* bacterium, *W*, plant wall; *WC*, wall convolution. (A) PA14 *rpoN*::Gen^r cells parallel to the surface of a smooth cell wall of a parenchyma vessel cell. Bar, 1 μm. (B and C) PA14 cells attached perpendicularly to the surface of a highly convoluted cell wall of a parenchyma vessel cell. Holes (arrows) in the plant cell walls with diameters similar to that of the bacteria are readily visible. Bars, 1 μm.

PA14 causes the convolution of and the formation of small holes in mesophyll cell walls. The diameter of these holes is approximately the same as the diameter of the bacteria. In contrast to the wild type, PA14 *rpoN*::Gen^r cells did not attach perpendicularly under the conditions examined to the leaf epidermal surface 24 h postinfection (data not shown) and, as shown in Fig. 3A, *rpoN*::Gen^r cells oriented themselves parallel to mesophyll cell walls and did not cause the formation of the small holes. Thus, although we found that PA14 *rpoN*::Gen^r has diminished ability to attach to plant cell surfaces, it still has the ability to cause soft-rot symptoms. In contrast, we have shown that the *P. syringae rpoN* gene is absolutely required for full pathogenicity in *Arabidopsis*, most likely because it is required for *hrp* gene expression (23, 24). The *hrp* genes encode the components of a type III secretory system related to the *Yersinia* species Yop secretion system (37). *P. aeruginosa* has recently been shown to contain a type III secretion system as well, which is required for the export of exotoxin S (62). Our data indicate that the *P. aeruginosa* type III system is either *rpoN* independent or that a type III secretion system is not absolutely required for *P. aeruginosa* pathogenicity in plants.

PA14 *rpoN*::Gen^r exhibits reduced *C. elegans* killing. When the nematode *C. elegans* is fed a lawn of PA14 grown on solid medium, the nematodes die in two characteristic ways depending on the medium on which PA14 is grown (56). On a high-osmolarity but low-phosphate medium (e.g., PGS), the nematodes die rapidly (fast killing) over the course of 24 h. In contrast, nematodes that are fed PA14 grown on a low-osmolarity but high-phosphate medium (NGM) are killed at a slower rate (slow killing), dying after 2 to 3 days. Previous studies have shown that the killing of *C. elegans* by PA14 under these two different conditions is mediated by distinct molecular mechanisms (40, 56, 57). As shown in Fig. 4, there was significantly less killing of *C. elegans* by PA14 *rpoN*::Gen^r compared to killing by wild-type PA14 under both the fast- and slow-killing conditions. The killing of PA14 *rpoN*::Gen^r in the fast-killing assay was restored to wild-type levels by pPAR4SR, which carries wild-type *rpoN*, indicating that the loss of pathogenicity phenotype of PA14 *rpoN*::Gen^r was due to the disruption of the *rpoN* gene (data not shown). The decrease in fast killing by PA14 *rpoN*::Gen^r is consistent with the results described above showing that PA14 *rpoN*::Gen^r synthesizes reduced levels of pyocyanin. Previous results from our laboratory showed that pyocyanin is an important toxin mediating the fast-killing process (40).

PA14 *rpoN*::Gen^r does not exhibit reduced pathogenicity in *G. mellonella*. A single PA14 cell is sufficient to kill a greater wax moth caterpillar when PA14 is injected into the hemolymph (30). We found that PA14 and PA14 *rpoN*::Gen^r were indistinguishable in their ability to kill fifth-instar wax moth larvae (data not shown). The LD_{50} in both cases was approximately one bacterial cell. This is interesting in light of the decreased virulence of PA14 *rpoN*::Gen^r in the mouse burn model. Microbial defense in insects shows interesting parallels to innate immunity in mammals and plants (8, 25, 27). Members of the cecropin class of antibacterial peptides have been found in both mammals and insects, and cecropin A in *Drosophila melanogaster* is regulated by a cascade similar to the one involving NF-κB activation in the mammalian inflammatory response (38). The reduction in virulence of PA14 *rpoN*::Gen^r in mice but not in wax moths suggests that *rpoN* does not play a significant role in the regulation of virulence factors that affect components of the host innate immune system conserved between insects and mammals.

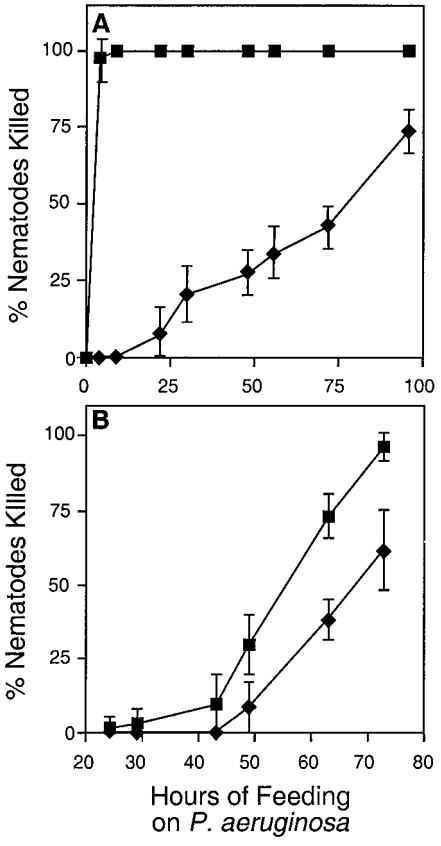

FIG. 4. PA14 *rpoN*::Gen^r killing of *C. elegans* under fast- and slow-killing conditions. (A) Fast killing. L4 stage worms were seeded onto bacterial lawns on PGS medium, and mortality was recorded as described earlier (40). Each value is the mean for three replicates. Symbols: ■, PA14; ◆, PA14 *rpoN*::Gen^r. (B) Slow killing. L4 stage worms were seeded onto bacterial lawns on NGM media, and mortality was recorded as described previously (56). Each value is the mean for six replicates. Symbols: ●, PA14; ◆, *rpoN*::Gen^r.

Conclusions. Previous work with PA14 in our laboratory has shown that PA14 utilizes a common set of virulence factors in evolutionarily disparate hosts (30, 40, 53, 57). Given this and the highly pleiotropic nature of *rpoN* mutants, we expected that disruption of *rpoN* would result in a significant impairment in plant, nematode, insect, and mouse pathogenicity. Surprisingly, a major reduction in pathogenicity of the *P. aeruginosa* PA14 *rpoN* mutant was observed only in *C. elegans* killing and in the elicitation of sepsis in the mouse burn model. Although decreased lesion size and a 10-fold reduction in in planta growth was observed in *Arabidopsis* at early stages of an infection (3 to 4 dpi), at later stages (7 dpi) the *P. aeruginosa rpoN* mutant elicited disease symptoms that were indistinguishable from those caused by the wild type. This contrasts with the absolute requirement for RpoN function in *P. syringae* for pathogenicity (23, 24). No effect of the *rpoN* mutation was observed in *G. mellonella* killing. Thus, although it has been previously reported that *P. aeruginosa rpoN* mutants exhibit

impaired virulence in mice, the results reported here provide a more complete perspective concerning the role of RpoN in the diverse pathogenic interactions that *P. aeruginosa* has with several evolutionarily disparate hosts. The major conclusion is that, in contrast to our expectations, *rpoN* does not appear to regulate any genes that are likely to encode virulence factors universally required for pathogenicity irrespective of the host.

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