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Virulence of the Phytopathogen *Pseudomonas syringae* pv. Maculicola Is *rpoN* Dependent

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We cloned the *rpoN* (*ntrA* and *glnF*) gene encoding σ^{54} from the phytopathogen *Pseudomonas syringae* pv. maculicola strain ES4326. The *P. syringae* ES4326 *rpoN* gene complemented *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella aerogenes rpoN* mutants for a variety of *rpoN* mutant phenotypes, including the inability to utilize nitrate as sole nitrogen source. DNA sequence analysis of the *P. syringae* ES4326 *rpoN* gene revealed that the deduced amino acid sequence was most similar (86% identity; 95% similarity) to the σ^{54} protein encoded by the *Pseudomonas putida rpoN* gene. A marker exchange protocol was used to construct an ES4326 *rpoN* insertional mutation, *rpoN*::Km^r. In contrast to wild-type ES4326, ES4326 *rpoN*::Km^r was nonmotile and could not utilize nitrate, urea, C_4-dicarboxylic acids, several amino acids, or concentrations of ammonia below 2 mM as nitrogen sources. *rpoN* was essential for production of the phytotoxin coronatine and for expression of the structural genes encoding coronamic acid. In addition, ES4326 *rpoN*::Km^r did not multiply or elicit disease symptoms when infiltrated into *Arabidopsis thaliana* leaves, did not elicit the accumulation of several *Arabidopsis* defense-related mRNAs, and did not elicit a hypersensitive response (HR) when infiltrated into tobacco (*Nicotiana tabacum*) leaves. Furthermore, whereas *P. syringae* ES4326 carrying the avirulence gene *avrRpt2* elicited an HR when infiltrated into *Arabidopsis* ecotype Columbia leaves, ES4326 *rpoN*::Km^r carrying *avrRpt2* elicited no response. Constitutive expression of ES4326 *hrpL* in ES4326 *rpoN*::Km^r partially restored defense-related mRNA accumulation, showing a direct role for the *hrp* cluster in host defense gene induction in a compatible host-pathogen interaction. However, constitutive expression of *hrpL* in ES4326 *rpoN*::Km^r did not restore coronatine production, showing that coronatine biosynthesis requires factors other than *hrpL*.

The *rpoN* gene encodes the alternate sigma factor σ^{54}, which works in conjunction with the NtrC class of transcriptional activators to control the expression of many genes in response to nutritional and environmental conditions (2, 54). For example, genes involved in nitrogen, hydrogen, and catabolite utilization are frequently regulated by σ^{54} (6, 35, 48, 95). In the case of pathogenic bacteria, *rpoN* mediates expression of virulence-related factors such as pilin in *Pseudomonas aeruginosa* and flagellin in *Vibrio anguillarum* (24, 61, 86).

For some phytopathogenic bacteria, *rpoN* has been implicated indirectly as a regulator of pathogenicity-related genes known as the *hrp* gene cluster (17, 18). *Pseudomonas syringae* pv. syringae strain 61, for example, contains a 25-kb *hrp* cluster consisting of several complementation groups comprising at least 27 genes (8, 26). Several *hrp* genes encode proteins that have a high degree of homology to components of the type III secretory pathway of *Yersinia* species which are responsible for translocating *Yersinia* outer membrane proteins into mammalian cells (13, 15, 55, 83). By analogy, it is proposed that *hrp*-encoded proteins in phytopathogenic bacteria are involved in the transport of pathogenicity-related factors into plant cells.

The acronym *hrp* stands for hypersensitive response and pathogenicity. *hrp* genes are required not only for pathogenicity of a virulent pathogen but also for the elicitation of the hypersensitive response (HR) which occurs on some hosts (44, 45). The HR involves rapid, but localized, programmed plant cell death and is believed to restrict pathogen spread (1, 37). There is mounting evidence that the elicitation of an HR is mediated by the specific interaction between the products of a plant resistance gene (*R* gene) and a pathogen avirulence (*avr*) gene (43, 80, 85). It appears likely that at least some *avr* genes encode pathogenicity-related factors (34, 47, 71, 84) that are transported into plant cells via the *hrp*-encoded transport machinery (58, 59, 90). In the absence of a corresponding *R* gene product, the *avr* product enhances virulence; however, in hosts which have the corresponding *R* gene, recognition of the *avr* gene product enhances host resistance. Interestingly, most *avr* genes are also coordinately regulated with genes in the *hrp* cluster (27, 29, 46, 67, 71, 77, 97).

The HR is accompanied by the induction of defense-related genes (7, 91) that are differentially expressed depending on the particular pair of *avr* and *R* gene products eliciting the HR (70, 72). Defense gene induction also occurs in the absence of the HR during compatible pathogen-host interactions, although usually later and at lower levels than those occurring during an HR (11, 32, 66). Furthermore, *hrp* mutations that presumably block the export of *avr* gene products have been found to reduce, but not eliminate, defense gene induction (60). Collectively, these results suggest that there are a variety of signaling pathways that activate host responses.

In *P. syringae* the circuitry of *hrp* regulation appears to involve a transcriptional activation cascade. At the top of the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
E. coli		
DH5α	F$^-$ *lacZ*ΔM15 *endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1* λ$^-$	Bethesda Research Laboratories (20)
MM294		G. Walker (4)
Th1 λgln101	*lac*Δ *rpoN*Δ *glnA-lacZ*	D. Ow (62)
K. aerogenes HG63 *rpoN*	*rpoN*	D. Ow (62)
P. aeruginosa PAK N1	Gm^r cassette in *rpoN*	S. Lory (31)
P. syringae pv. maculicola		
ES4326	Wild-type	K. Davis (9)
ES4326 *rpoN*::Km^r	Km^r cassette in *rpoN*	This study
P. syringae pv. tomato		
DC3000	Wild type	D. Cuppels (57)
DC3661	COR-defective mutant	D. Cuppels (57)
P. syringae pv. glycinea		
PG4180.N9	COR-producing strain	C. Bender (88)
PG4180.P2	Gm^r cassette in *corR*	C. Bender (65)
Plasmids		
pRK2013	Km^r Tra$^+$, mating helper	G. Ditta (10)
pPH1	Gm^r Sp^r, chasing plasmid	
pLAFR3	Tc^r cosmid cloning vector	B. Staskawicz (81)
pBluescript SK(+)	Ap^r, cloning vector	Stratagene, Inc., La Jolla, Calif.
pGEM-7Zf(+)	Ap^r, cloning vector	Promega Inc., Madison, Wis.
pUC4K	Source of Km^r cassette from Tn*903*	Pharmacia, Inc., Bridgewater, N.J.
pJSR1	Ap^r, cosmid cloning vector	J. Shao (68)
pKI11	Source of *P. aeruginosa rpoN* gene	S. Lory (31)
pLH12	1.4-kb *Sal*I fragment containing *avrRpt2* in pLAFR3	R. Innes (93)
pPG101	17.2-kb insert of ES4326 DNA carrying *rpoN* in pLAFR3	This study
pPG102	4.5-kb *Bam*HI-*Eco*RI fragment containing ES4326 *rpoN* in pLAFR3	This study
pLAFR-RK	pLAFR3 containing *rpoN*::Km^r	This study
pRN5; pNR9	pBluescript SK(+) carrying 4.5-kb *Hin*dIII fragment containing ES4326 *rpoN* gene in opposite orientations	This study
pRG960sd	Sm^r Sp^r, contains promoterless *uidA* with start codon and Shine-Dalgarno sequence	C. Bender (89)
pRGMU7	Sm^r Sp^r, contains 1.5-kb *Pst*I-*Aat*I fragment containing *cmaABT* promoter inserted into pRG960sd	C. Bender (87)
pHRPLC	*lacZ-hrpL* transcriptional fusion in pLAFR3	E. Hendrickson (23)

cascade are two regulatory genes, *hrpR* and *hrpS*, which are required for expression of the remaining *hrp* genes in the cluster (12, 18). Both *hrpR* and *hrpS* encode proteins consist almost exclusively of the domain conserved among transcriptional activators such as NtrC, DctD, and NifA that work in concert with σ^{54} (21, 22; reviewed in references 2 and 54). The *hrpR* product activates *hrpS* expression, and the *hrpS* product activates *hrpL* transcription (17, 18). HrpL is an alternate sigma factor homologous to AlgU of *P. aeruginosa* and is thought to activate transcription of the remaining genes in the *hrp* cluster (96, 97). The factor(s) involved in the regulation of *hrpRS* remains obscure. Nevertheless, the central role of *hrpS* in this cascade and the HrpS-NtrC homology predicts that *rpoN* would be required for activation of the *hrp* gene cluster in *P. syringae*.

Despite the highly conserved and clustered nature of *hrp* genes among phytopathogenic bacteria, transcriptional regulation of the *hrp* genes is achieved by different mechanisms in different species. In *Ralstonia solanacearum*, HrpB, an AraC-like transcriptional activator, controls *hrp* gene expression (14). Similarly, in *Xanthomonas campestris* pv. vesicatoria, *hrp* gene expression is regulated by an OmpR homolog, HrpG, which in turn activates HrpX, another AraC-like activator that activates the remaining *hrp* genes (79, 92). This latter regulatory cascade is consistent with the fact that *rpoN* is not required for *hrp* expression or pathogenicity in *X. campestris* pv. vesicatoria (25).

The experiments described here utilize a pathogenicity system that involves the infection of *Arabidopsis thaliana* with *P. syringae* pv. maculicola strain ES4326. Strain ES4326 belongs to the leaf spotting class of phytopathogenic pseudomonads (78), proliferates extensively in *Arabidopsis* ecotype Columbia leaves, and causes the development of water-soaked disease lesions (9, 11). In contrast, ES4326 carrying the avirulence gene *avrRpt2* elicits a visible HR about 16 h after infiltration and proliferates 50- to 100-fold less than the wild-type strain ES4326 (11). Using this system, we describe experiments that examine the role of σ^{54} in the pathogenicity of *P. syringae* ES4326. Our results indicate that σ^{54} is an important virulence factor for *P. syringae* and is required for the elicitation of an HR by *P. syringae* in both host and nonhost plants.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this work are listed in Table 1. *P. syringae* pv. maculicola strain ES4326 and its derivatives were grown at 28°C in L broth (50), minimal M9 salts media (50), or King's B medium (36). *Escherichia coli*, *Klebsiella aerogenes*, and *P. aeruginosa* strains were grown at 37°C in L broth or M9 minimal salts medium. For clarity, ES4326 carrying plasmid pLH12 (which carries the avirulence gene *avrRpt2*) is referred to as ES4326 (*avrRpt2*). Carbon and nitrogen source utilization tests for ES4326 *rpoN* mutants were performed in M9 salts minimal medium by providing a

carbon source at 10 mM and by replacing ammonium chloride with an alternative nitrogen source at 5 mM when required. Bacterial motility was tested on "swarm plates" consisting of 0.3% agar, 0.5% NaCl and 0.5% tryptone (38). Antibiotic concentrations for *E. coli* and *P. syringae* strains were as follows: streptomycin, 150 μg/ml; kanamycin, 25 μg/ml; tetracycline, 12 μg/ml; gentamicin, 20 μg/ml; and spectinomycin, 20 μg/ml. Interspecies complementation tests of the *E. coli rpoN* mutant by ES4326 *rpoN* were carried out on M9 minimal salts agar supplemented with 0.2% glutamine and 20 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml (Sigma).

Bacterial genetics. pLAFR3 derivatives were introduced into *Pseudomonas* strains via triparental matings with MM294(pRK2013) (10). Aspartate-utilizing pseudorevertants of strain ES4326 *rpoN*::Km^r were obtained by plating approximately 10^9 CFU on M9 agar plates containing succinate and aspartate as carbon sources at 10 mM and the appropriate antibiotics (streptomycin and kanamycin).

Plant pathogenicity assays. *Arabidopsis* ecotype Columbia was germinated, grown, and inoculated with ES4326 strains. Bacterial strains were grown overnight in King's B, subcultured and grown to mid-log phase, resuspended in 10 mM $MgSO_4$, and inoculated into the underside of the leaf at a titer of 10^4 CFU/cm^2 using a disposable syringe. Growth of *P. syringae* strains in leaves was measured by individually grinding four to six 0.2-cm^2 leaf punches (excised with a no. 2 cork borer) in 10 mM $MgSO_4$, plating appropriate dilutions on King's B medium containing the appropriate antibiotics, and counting the CFU. For RNA blot analysis, entire *Arabidopsis* leaves infiltrated with ES4326 bacterial suspensions were harvested, frozen in liquid nitrogen at the indicated times, and stored at −80°C until needed (see below). *Nicotiana tabacum* (tobacco) cultivar Xanthi was grown under greenhouse conditions and inoculated with ES4326 strains and assayed for the HR as previously described (82).

Cosmid library constructions. Total bacterial genomic DNA was prepared from strain ES4326 as described previously (3), partially digested with *Sau*3A, and size fractionated on a 14-ml sucrose gradient (50). DNA fragments of approximately 20 kb were purified and ligated with linearized pLAFR3 that had been prepared to promote the formation of concatemers (50). Packaging, infection, and plating of the cosmid clones were performed using the Giga Gold packaging kit according to the manufacturer's specifications (Stratagene, La Jolla, Calif.).

Nucleic acid manipulations. Routine manipulations such as DNA blots and plasmid DNA isolation were performed as described earlier (3). Restriction enzymes, T4 DNA ligase, and calf intestine phosphatase were purchased from Boehringer Mannheim and New England BioLabs and used according to the manufacturer's specifications. Deletions in plasmids were created using the Erase-a-Base kit (Promega, Madison, Wis.). Isolation of *Arabidopsis* mRNA and RNA blot analysis was carried out as described previously (11). Hybridizations were performed at stringent conditions (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 65°C) as described earlier (50). ^{32}P-labeled DNA probes for use in hybridizations were prepared as described previously for the *Pal1*, *PR1*, *BGL2* (*PR2*), *PR5*, and *GST1* pathogen-induced genes (16, 73).

Cloning and sequencing the ES4326 *rpoN* gene. DNA blot analysis data indicated that ES4326 contained a single 4.5-kb *Hin*dIII fragment that hybridized to a 4.2-kb *Xho*I fragment in plasmid pKI11 containing the *P. aeruginosa* strain PAK *rpoN* gene (31). Approximately 1,400 clones from a cosmid library of strain ES4326 DNA were screened by colony hybridization using the *rpoN* probe derived from *P. aeruginosa*. A hybridizing clone, pPG101, carrying a 17.2-kb insert was identified and shown to contain the 4.5-kb *Hin*dIII fragment previously detected by Southern blot analysis (data not shown).

E. coli strain TH1 λgln101, which contains deletions in *rpoN* and *lacZ* and a *glnA-lacZ* reporter construct, was used as an assay for functional ES4326 *rpoN* clones by plating subclones of pPG101 onto M9 medium containing 0.2% glutamine and 20 μg of X-Gal per ml. The 4.5-kb *Hin*dIII fragment from pPG101 contained a functional *rpoN* gene that complemented the *E. coli rpoN* mutation in TH1 λgln101. The *rpoN* gene in this construct presumably contained its own promoter since this fragment activated the *glnA-lacZ* fusion in TH1 λgln101 when cloned in the *Hin*dIII site of pBluescript SK(+) in either orientation (plasmids pRN5 and pRN9). For subsequent use in *Pseudomonas* spp., the 4.5-kb fragment containing ES4326 *rpoN* was subcloned into cosmid pLAFR3. A 4.5-kb *Hin*dIII fragment from pRN5 was subcloned into pGem7Zf. Using the pGem7Zf polylinker sites, the *rpoN* gene was recloned as a 4.5-kb *Eco*RI-*Bam*HI fragment in pLAFR3 to produce pPG102. Plasmids pRN5 and pRN9 were used to derive a series of nested deletions starting from either end of the 4.5 *Hin*dIII fragment. This analysis showed that the ES4326 *rpoN* gene was located near the left end of the 4.5-kb fragment (data not shown).

DNA sequence analysis was initiated at the middle *Xho*I site in Fig. 1 and continued in both directions using synthetic oligonucleotides for a total of approximately 1,900 bp. A single large open reading frame that encodes a protein that is highly homologous to σ^{54} in other bacterial species was identified in the 1,900-bp region.

Insertional mutagenesis of the ES4326 *rpoN* gene. pGem7Zf containing the 4.5-kb *rpoN* fragment was digested with *Pst*I, and a 1.24-kb fragment encoding the aminoglycoside 3′-phosphotransferase activity of Tn*903* (Pharmacia) from pUC4K was ligated to the *Pst*I-digested ends. A 5.8-kb *Eco*RI-*Bam*HI fragment from this plasmid was then subcloned into pLAFR3. The resulting plasmid (pLAFR-RK) was used to recombine the mutated *rpoN* gene (referred to as *rpoN*::Km^r) into strain ES4326 by first mobilizing pLAFR-RK into ES4326 and then introducing plasmid pPH1, which confers gentamicin (Gm) resistance. Cultures were grown under selection for Km^r and Gm^r, and individual colonies were screened for tetracycline sensitivity (76). Southern blot analysis of chromosomal DNA prepared from a putative *rpoN* mutant confirmed the insertion of Km^r into the *rpoN* gene (data not shown).

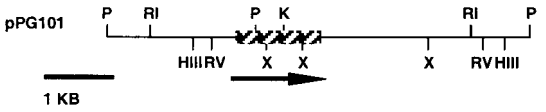

FIG. 1. Restriction map of the ES4326 *rpoN* region. The stippled box indicates the region sequenced. The horizontal arrow indicates the direction of transcription. Restriction enzyme sites: HIII, *Hin*dIII; RI, *Eco*RI; RV, *Eco*RV; P, *Pst*I; K, *Kpn*I; X, *Xho*I.

COR preparation and assay. Coronatine (COR) synthesis by strain ES4326 was assayed using two approaches. In the first procedure (74), a 5-ml culture of ES4326 grown overnight in King's B medium was used to inoculate 50 ml of Woolley's liquid medium (94), where potassium nitrate was replaced with 5 mM arginine to facilitate rapid growth of the ES4326 *rpoN*-Km^r mutant. Cultures (50 ml) were shaken at 20°C for 6 days, at which point the optical density at 600 nm (OD_{600}) was measured, and the cells were pelleted and weighed. The supernatants were acidified to pH 2.0 with HCl and extracted with 50 ml of ethyl acetate. The organic phase was lyophilized to dryness, and the residue was resuspended in 2.0 ml of H_2O/g of wet bacterial pellet. Then, 10-μl droplets containing dilutions of either purified COR or the COR preparation described above were inoculated into *Arabidopsis* and tomato leaves. Elicitation of red anthocyanin pigments on *Arabidopsis* leaves and chlorosis on tomato leaves was assayed 3 to 7 days later.

In the second method, *P. syringae* strains were grown at 18°C in Hoitink-Sinden medium optimized for COR production (HSC) (63), and the supernatants were analyzed for COR production by high-pressure liquid chromatography (HPLC) 5 days after inoculation (63). Each strain was inoculated into three replicate aliquots (10 ml) of HSC medium for evaluation of COR production, and each experiment was repeated.

Nucleotide sequence accession number. The *rpoN* sequence from *P. syringae* pv. maculicola has been assigned accession number AF199600 in the GenBank database.

RESULTS

Cloning the strain ES4326 *rpoN* gene. An interspecies hybridization strategy was used to isolate pPG101, a cosmid clone that carried a presumptive *P. syringae* ES4326 *rpoN* gene. Plasmid pPG101 complemented the inability of *E. coli*, *K. aerogenes*, and *P. aeruginosa rpoN* mutants (strains TH1 λgln101, HG63 *rpoN*, and PAKN1, respectively) to utilize 10 mM nitrate and 10 mM ammonia as sole nitrogen sources; the inability of the *E. coli rpoN* mutant to utilize histidine, arginine, or proline as sole nitrogen sources; and the lack of motility of the *P. aeruginosa rpoN* mutant. As described in Materials and Methods and as illustrated in Fig. 1, the ES4326 *rpoN* gene on pPG101 was mapped to a 4.5-kb-*Hin*dIII fragment, and a 1,900-bp region containing the presumptive *rpoN* gene was sequenced. DNA sequence analysis (Fig. 2) revealed that the presumptive ES4326 *rpoN* gene encodes a protein with 86% identity and 95% similarity to the *rpoN* gene of *P. putida*.

Construction and metabolic phenotypes of a strain ES4326 *rpoN* insertional mutant. An ES4326 *rpoN* mutant was constructed by subcloning *rpoN* from pPG101, inserting a DNA cassette conferring kanamycin resistance into the *Pst*I site (located at codon 162) of *rpoN*, transferring the mutated *rpoN* gene to pLAFR3, and marker exchanging the mutant gene into the ES4326 genome (see Materials and Methods). ES4326 *rpoN*::Km^r exhibited an array of phenotypes typical of *rpoN* mutants, including the inability to grow on nitrate and urea as sole nitrogen sources, lack of motility, and inability to grow on a variety of C_4-dicarboxylic acids as sole carbon sources, including aspartate, succinate, and fumarate, as well as the tricarboxylic acid intermediate α-ketoglutarate (data not shown). Unlike *rpoN* mutants of enteric bacteria (49, 86), however,

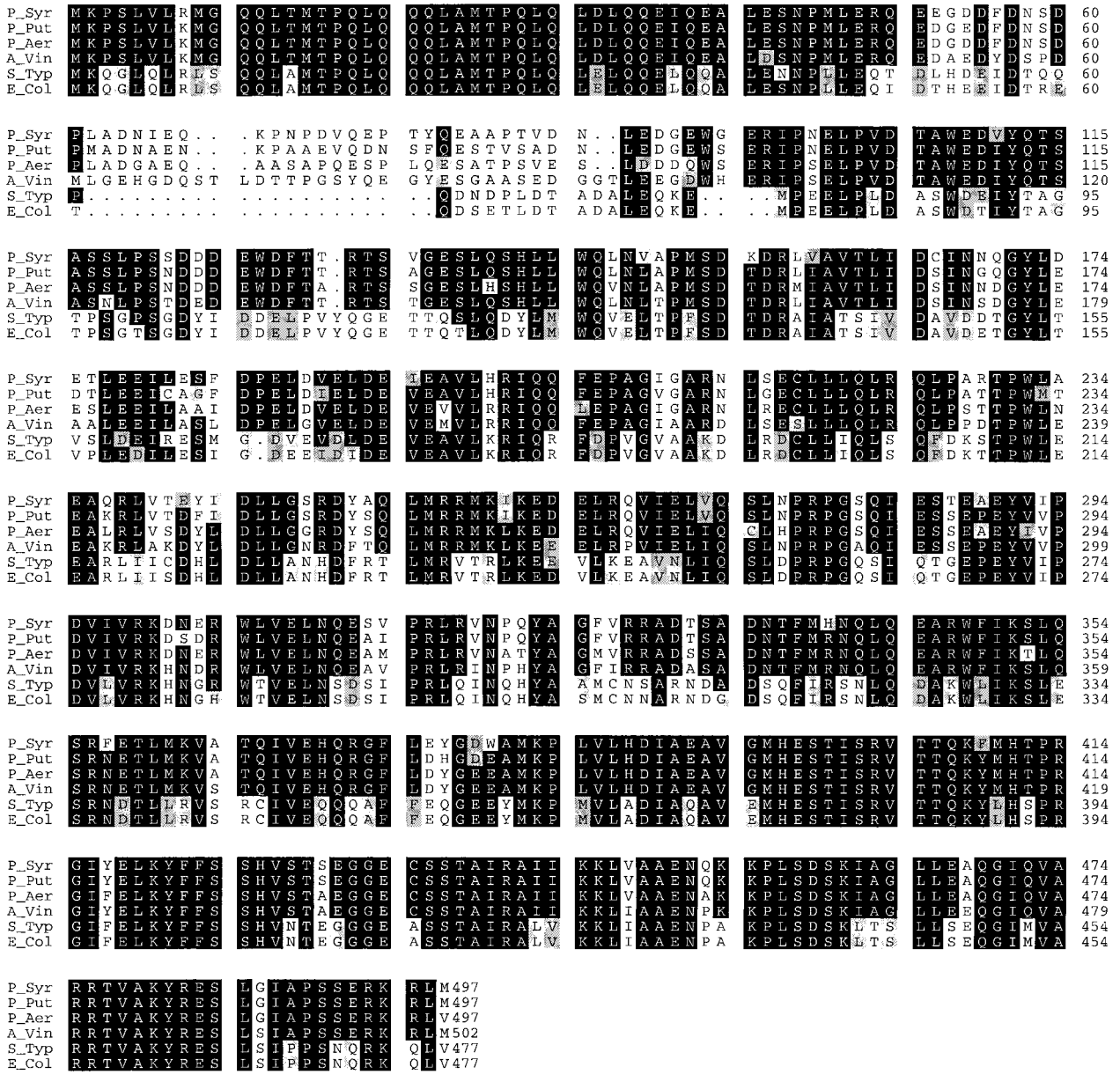

FIG. 2. Amino acid alignment of the ES4326 protein with σ^{54} from diverse bacterial species. The sequences are listed in decreasing order of conservation with σ^{54} from *P. syringae* pv. maculicola. (P_Syr). Abbreviations and references: P_Put, *Pseudomonas putida* (30); P_Aer, *Pseudomonas aeruginosa* (33); A_Vin, *Azotobacter vinelandii* (53); S_Typ, *Salmonella enterica* serovar Typhimurium (66); E_Col, *Escherichia coli* (28). Shading: black, conserved amino acids; gray, conservative substitutions.

ES4326 *rpoN*::Km^r was able to grow on glucose and ammonia as the sole carbon and nitrogen sources if ammonia was present at concentrations higher than 2 mM.

The ability of ES4326 *rpoN*::Km^r to utilize a variety of amino acids as nitrogen sources was tested on minimal M9 solid medium. The *rpoN* mutant failed to utilize aspartate, proline, histidine, and methionine as nitrogen sources, which supported growth of large colonies of wild-type ES4326. Both wild-type and the *rpoN* mutant formed large colonies on arginine, lysine, asparagine, glutamine, and glutamate, although the *rpoN* mutant grew somewhat slower than the wild type (5 days to form a large colony compared to 3 days for the wild type). The wild-type ES4326 formed small colonies, and the *rpoN* mutant formed even smaller colonies on serine, leucine, threonine, isoleucine, or alanine. Phenylalanine and cysteine were not utilized by the wild type or the *rpoN* mutant. As in the case of *P. putida rpoN* mutants, all amino acids that served as a sole nitrogen source for the ES4326 *rpoN*::Km^r mutant also served as sole carbon source with the exception of lysine, which served only as a nitrogen source. The best growth rate for ES4326 *rpoN*::Km^r was observed when the medium was supplemented with glutamate, where the growth was equivalent to the wild-type strain.

Strain ES4326 *rpoN* is nonpathogenic on *Arabidopsis* and cannot elicit an HR. As described previously (11), infiltration of *Arabidopsis* leaves with ES4326 at a titer of 10^3 to 10^4

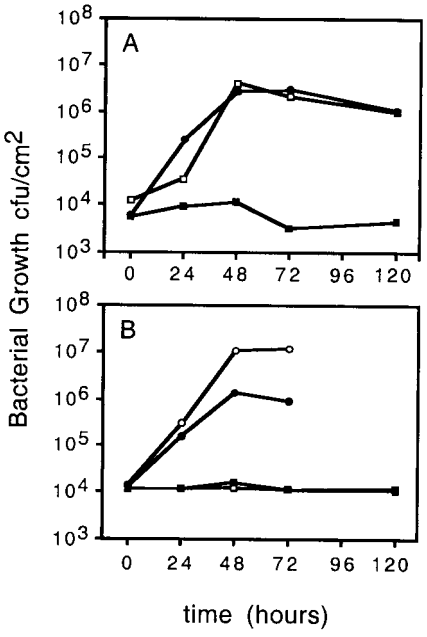

FIG. 3. Growth of ES4326 *rpoN*::Km^r in *Arabidopsis* leaves. Six-week-old *Arabidopsis* (Columbia) seedlings were infiltrated with bacterial suspensions at a titer of 10^4 CFU/cm^2 of leaf area, and bacterial populations were determined as described in Materials and Methods. Each value represents the average of at least four leaf discs. (A) Symbols: □, ES4326(pLAFR3); ■, ES4326 *rpoN*::Km^r (pLAFR3); ●, ES4326 *rpoN*::Km^r (pPG102) (*rpoN*$^+$). The experiment was repeated with similar results. (B) Symbols: ○, ES4326(pLAFR3); ●, ES4326 (*avrRpt2*); ■, ES4326 *rpoN*::Km^r (pLAFR3); □, ES4326 *rpoN*::Km^r (*avrRpt2*). The experiment was repeated with similar results.

CFU/cm^2 of leaf area resulted in the development of characteristic disease symptoms, including spreading chlorosis, water-soaked lesions, and growth of the infiltrated bacteria to a titer of approximately 10^7 CFU/cm^2 (Fig. 3A). In contrast, *Arabidopsis* leaves infiltrated with ES4326 *rpoN*::Km^r exhibited no symptoms, even when inoculated with 10^8 CFU/cm^2. Furthermore, the titer of ES4326 *rpoN*::Km^r in *Arabidopsis* leaves remained consistently low for the duration of the experiment (Fig. 3A). ES4326 *rpoN*::Km^r carrying pPG102, which carries wild-type *rpoN*, exhibited the same pathogenic phenotype as ES4326, and its growth in *Arabidopsis* leaves was indistinguishable from that of the wild type (Fig. 3A). This result indicated that the nonpathogenic phenotype of ES4326 *rpoN*::Km^r was due to the disruption of the *rpoN* gene. However, because the complementing plasmid contains about 2,000 bp downstream of *rpoN*, these data do not rule out the possibility that the insertion in *rpoN* exerts polarity on a downstream gene and that this downstream gene is required for pathogenicity.

ES4326 carrying *avrRpt2* on plasmid pLH12 [ES4326 (*avrRpt2*)] elicits an HR on *Arabidopsis* ecotype Columbia instead of disease symptoms, and the HR is accompanied by a 10- to 100-fold reduction in bacterial growth compared to ES4326 in infiltrated leaves (Fig. 3B). In contrast, ES4326 *rpoN*::Km^r (*avrRpt2*) failed to elicit an HR or any other visible symptom, even when the inoculum was 20 times higher than ES4326 (*avrRpt2*) (data not shown). As shown in Fig. 3B, ES4326 *rpoN*::Km^r (*avrRpt2*), like ES4326 (*avrRpt2*), failed to multiply in *Arabidopsis* leaves. Infiltration of ES4326 into tobacco leaves also results in the elicitation of an HR (not shown). In confirmation of the results obtained in *Arabidopsis*, infiltration of tobacco leaves with ES4326 *rpoN*::Km^r did not result in the appearance of any visible symptoms (not shown).

TABLE 2. COR production and *cor* gene transcriptional activity in selected strains of *P. syringae*[a]

Strain	COR production (mg/g of protein)	Transcriptional activity (U of GUS/mg of protein)[b]
P. syringae pv. maculicola		
ES4326	51.0	347.0
ES4326 *rpoN*	0.3	8.5
ES4326 *rpoN* + pHRPLC	0.0	ND
P. syringae pv. *glycinea*		
PG4180.N9	43.4	390.0
PG4180.P2	0.3	12.6

[a] Values for COR and glucuronidase (GUS) activity represent the average of two experiments with three replicates each. Means within each column were analyzed using Duncan's multiple-range test. The protein content in the cell lysates was determined with the Bio-Rad protein assay kit as recommended by the manufacturer.

[b] Transcriptional activity in the COR biosynthetic gene cluster was evaluated by introducing pRGMU7 into each strain and measuring the glucuronidase activity in the resulting transconjugants. ND, not determined.

The pathogenicity defect of ES4326 *rpoN*::Km^r is not solely due to the inability to assimilate aspartate. Because genes encoding dicarboxylic acid permease are regulated by DctD, an NtrC homolog, *rpoN* mutants in a variety of species cannot utilize dicarboxylic acids as carbon or nitrogen sources. *R. meliloti* mutants defective in aspartate aminotransferase cannot utilize aspartate and are defective in symbiosis, suggesting that aspartate may be a major carbon source for symbiotic bacterial cells (69). Therefore, we tested whether ES4326 *rpoN*::Km^r is nonpathogenic solely because it cannot assimilate aspartate. We selected a ES4326 *rpoN*::Km^r pseudorevertant, as described in Materials and Methods, that was able to utilize aspartate as a carbon source. This revertant still had an RpoN$^-$ phenotype with respect to its inability to use nitrate and succinate and its lack of motility. The Asp$^+$ revertant failed to grow in *A. thaliana* or to elicit an HR in tobacco or *Arabidopsis* (data not shown).

Strain ES4326 *rpoN*::Km^r fails to synthesize the phytotoxin coronatine. One possible explanation for the reduced virulence of ES4326 *rpoN*::Km^r is the inability to produce virulence factors such as toxins. Many *P. syringae* pathovars, including ES4326, produce a chlorosis-inducing phytotoxin, coronatine, which is composed of an ethyl cyclopropyl amino acid linked to a polyketide moiety (19). COR production is regulated by a modified two-component regulatory system that controls the expression of essential COR biosynthetic genes. The regulators CorR and CorP are related to response regulators of the ROIII group, while CorS is similar to the corresponding histidine protein kinase sensors. To determine whether COR biosynthesis requires *rpoN*, crude COR was extracted from strains ES4326, ES4326 *rpoN*::Km^r, the COR-producing strain *P. syringae* pv. tomato DC3000, and DC3661, a COR$^-$ mutant of DC3000. The COR extracted from DC3000 and ES4326 elicited typical COR-induced symptoms, chlorosis and anthocyanin accumulation, respectively, on tomato and *Arabidopsis* leaves. These symptoms were not detected with organic acids extracted from ES4326 *rpoN*::Km^r or DC3661. Further characterization was carried out by quantitatively analyzing COR production using HPLC. As shown in Table 2, ES4326 produced 51 mg of COR/g of protein, a level comparable to that produced by *P. syringae* pv. glycinea strain PG4180.N9, a high-

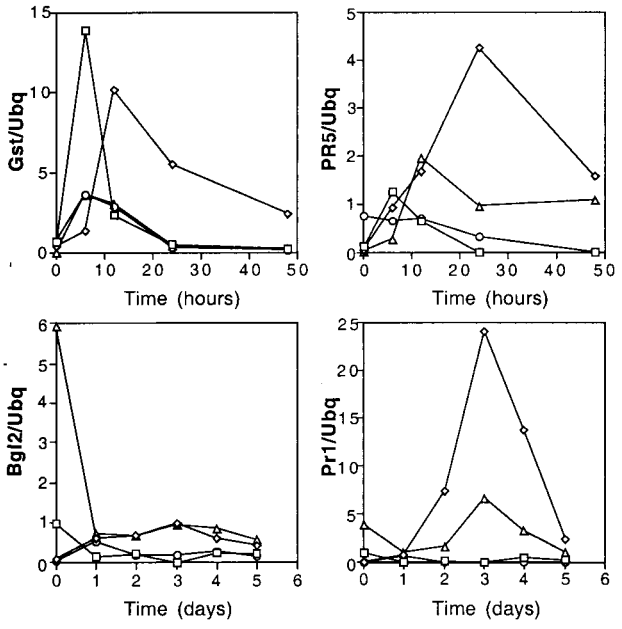

FIG. 4. Induction of *Arabidopsis* defense-related mRNAs following infiltration of ES4326 *rpoN*::Km^r. Leaves were infiltrated with bacterial suspensions normalized to 0.1 OD_{600} as described in Materials and Methods. Filters containing 5 μg of total RNA per lane were probed with radiolabeled DNA fragments encoding the *Arabidopsis GST1*, *PR5*, *BGL2*, and *PR1* genes. The filters were also probed with a radiolabeled probe corresponding to the *Arabidopsis* ubiquitin gene *UBQ*. Phosphorimager data are expressed as a ratio of defense gene to *UBQ* gene induction. Symbols: □, 10 mM $MgSO_4$; ◇, ES4326; ○, ES4326 *rpoN*::Km^r; △, ES4326 *rpoN*::Km^r (pHRPLC).

yielding COR producer which has been used in many genetic investigations (65, 88). However, ES4326 *rpoN*::Km^r produced only 0.3 mg of COR, a level comparable to PG4180.P2, a *corR* mutant of PG4180.N9 which is considered completely defective in COR production (65).

The structural genes encoding COR production belong primarily to two distinct transcriptional units: the *cmaABT* transcript which is essential for production of coronamic acid and the *cfl-CFA* transcript which encodes coronafacic acid (5). Coronamic acid and coronafacic acid function as the two key intermediates in the biosynthetic pathway to coronatine (5). The effect of the *rpoN* mutation on transcriptional activity in the COR biosynthetic gene cluster was investigated by measuring β-glucuronidase activity from pRGMU7, a construct containing the *cmaABT* promoter fused to *uidA* (87). As shown in Table 2, when pRGMU7 was introduced into the two COR-producing strains, ES4326 and PG4180.N9, transcriptional activity from the *cmaABT* promoter was comparable (347 and 390 U GUS, respectively). However, β-glucuronidase activity in the *rpoN* mutant containing pRGMU7 was extremely low and was comparable to the low level of expression in PG4180.P2(pRGMU7). It is important to note that PG4180.P2 is defective in *corR*, a gene which encodes a positive transcriptional activator of the *cmaABT* transcript (64). The present data suggest that a functional *rpoN* is required for expression of the *cmaABT* transcript in *P. syringae* pv. maculicola.

In the accompanying study (23), we demonstrate that *rpoN* in ES4326 is required for the expression of *hrpL*, which encodes an alternative sigma factor and is required for expression of the ES4326 *hrp* genes and *avrRpt2* (12, 17, 29, 97). We also show that constitutive expression of *hrpL* on plasmid pHRPLC restores the ability of ES4326 *rpoN*::Km^r to elicit disease symptoms in *A. thaliana* and an HR in tobacco. However, pHRPLC did not restore COR production to ES4326 *rpoN*::Km^r (Table 2), indicating that COR biosynthesis is not dependent on *hrpL* in ES4326 but on a separate regulatory pathway that also requires *rpoN*.

Strain ES4326 *rpoN*::Km^r fails to activate high-level expression of *Arabidopsis* defense-related genes. The infiltration of *Arabidopsis* leaves with ES4326 normally leads to the accumulation of mRNAs corresponding to a variety of *Arabidopsis* defense-related genes, including *PR2* (*BGL2*), *GST1*, *PR5* and *PR1*, which encode β-1,3-glucanase, glutathione *S*-transferase, a thaumatin-like protein, and a protein with unknown activity, respectively (42, 51, 52, 91). Each of these genes shows a different induction pattern. In general, *GST1* and *PR5* are induced within several hours after infection, whereas *PR1* and *BGL2* are induced later than 24 h postinfection. In contrast to ES4326, very little accumulation of the *BGL2*, *GST1*, *PR1*, and *PR5* transcripts was seen following infiltration with ES4326 *rpoN*::Km^r (Fig. 4), which gave results similar to those for the $MgSO_4$ control. This demonstrated that a factor under *rpoN* control is necessary for defense gene induction.

Even more rapid induction of defense-related genes is seen accompanying the HR in incompatible interactions (11, 32, 66). Two such genes, *Pal1* and *Gst1*, which encode phenylalanine ammonia lysase and glutathione *S*-transferase, respectively, show early, high-level accumulation of mRNAs in response to the avirulence gene *avrRpt2* (11, 16). ES4326 *rpoN*::Km^r carrying the *avrRpt2* gene induces little accumulation of *Pal1* or *Gst1* mRNA compared to ES4326 (*avrRpt2*) (Fig. 5). Again, the *rpoN* mutant gave results similar to those for the $MgSO_4$ control.

In the accompanying study we report that constitutive expression of the sigma factor HrpL restores disease and HR phenotypes to ES4326 *rpoN*::Km^r (23). Curiously, however, constitutive expression of *hrpL* did not restore in planta growth to ES4326 *rpoN*::Km^r. As seen in Fig. 4, constitutive expression of *hrpL* in ES4326 *rpoN*::Km^r restored partial activation of the

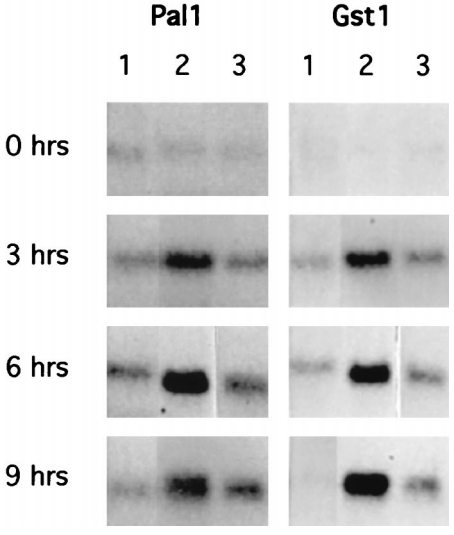

FIG. 5. Induction of *Arabidopsis* defense-related mRNAs following infiltration of ES4326 *rpoN*::Km^r carrying the avirulence gene *avrRpt2*. Leaves were infiltrated with bacterial suspensions normalized to 0.1 OD_{600} as described in Materials and Methods. Filters containing 5 μg of total RNA per lane were probed with radiolabeled DNA fragments encoding the *Arabidopsis Pal1* gene or the *Arabidopsis GST1* gene. Lanes: 1, 10 mM $MgSO_4$; 2, ES4326 (*avrRpt2*); 3, ES4326 *rpoN*::Km^r (*avrRpt2*).

PR1, *PR2* (*BGL2*), and *PR5* genes in infiltrated plants, suggesting that defense gene activation and the elicitation of disease symptoms are a consequence of HrpL-dependent bacterial factors rather than growth of the pathogen per se. One difficulty encountered in this experiment was the instability of pHRPLC in wild-type ES4326 in planta (23). Thus, it was not possible to examine *PR* gene expression in response to ES4326 (pHRPLC).

DISCUSSION

ES4326 *rpoN* gene. The amino acid sequence of the ES4326 σ^{54} protein is most closely related to the *P. putida* σ^{54} protein, and the phenotype of the ES4326 *rpoN* mutant resembles the phenotype of *P. putida rpoN* mutants (38). Both *P. syringae* and *P. putida rpoN* mutants grow more slowly than their wild-type counterparts in all media tested and were unable to utilize several uncharged amino acids as substrates. Neither is a glutamine auxotroph, and both can utilize NH_4 as a sole nitrogen source. They can also utilize lysine as a nitrogen source though not as a carbon source. In *P. putida*, the inability of the *rpoN* mutant to grow on lysine is probably due to the fact that lysine decarboxylase is under σ^{54} control (38). The major phenotypic difference that we observed between the *P. putida* and the *P. syringae rpoN* mutants was that the *P. syringae rpoN* mutant could not utilize proline and histidine as nitrogen sources, whereas the *P. putida rpoN* mutant could.

Lack of growth of strain ES4326 *rpoN* on low concentrations of ammonia and other nitrogen sources. The inability of ES4326 *rpoN*::Km^r to grow on low concentrations of ammonia (<1 mM) and various amino acids is presumably caused by the lack of *glnA* expression (which encodes glutamine synthetase), which is under σ^{54} control in several other bacterial species (54, 86). On the other hand, the ES4326 *rpoN* mutant is not a glutamine auxotroph, since it grew well when high concentrations of ammonia (>20 mM) or several amino acids other than glutamine were supplied as the sole nitrogen source. One explanation for these results is that *P. syringae*, like *Rhizobium* and *Bradyrhizobium* species, has more than one gene encoding glutamine synthetase, one that is expressed at high levels under σ^{54} control and a second copy that is expressed at low levels and is σ^{54} independent (41). Alternatively, *P. syringae* could have a single gene encoding glutamine synthetase, which requires σ^{54} for the high-level expression needed for ammonia assimilation but which has sufficient basal expression to prevent auxotrophy. *rpoN* mutants of most soil bacteria, including *Azotobacter vinelandii*, *P. putida*, and *Agrobacterium tumefaciens*, are not glutamine auxotrophs (39, 40, 53, 74, 75).

Growth rate of strain ES4326 *rpoN*. The ES4326 *rpoN* mutant displayed slower growth rates than the wild type in each medium examined, including M9 supplemented with 0.2 mM glutamine. Thus, it appears unlikely that the slower growth of the *rpoN* mutant can be explained solely on the basis of decreased levels of *glnA* expression. While it is possible that the growth deficit is due to a secondary mutation, plasmid pPG102, which carries the wild-type *rpoN* gene, fully complemented every *rpoN*-related phenotype tested, including growth and pathogenesis in *Arabidopsis* leaves.

Nonpathogenic phenotype of strain ES4326 *rpoN*. Given the pleiotropic phenotype of *rpoN* mutants, it is not possible to state precisely why the ES4326 *rpoN* mutant failed to elicit disease symptoms and to grow in *Arabidopsis* leaves or to elicit an HR. In the accompanying study we show that the absence of a functional σ^{54} in ES4326 blocks the transcription of *hrp* genes downstream of *hrpRS* (23), which would account for the nonpathogenic and HR-deficient phenotypes. However, we also report that the constitutive expression of *hrpL* in ES4326 *rpoN*::Km^r restored the elicitation of disease symptoms but failed to restore growth of ES4326 *rpoN*::Km^r in planta, implying that the absence of *hrp* functions is not the sole reason for the nonpathogenic phenotype of ES4326 *rpoN*::Km^r.

Our experiments using a pseudorevertant of the ES4326 *rpoN* mutant that was able to utilize aspartate eliminated the possibility that the *rpoN* mutant is nonpathogenic solely due to its inability to utilize this amino acid. However, it is possible that ES4326 *rpoN*::Km^r has another metabolic defect that contributes to the nonpathogenic phenotype. The *rpoN* mutant is also unable to utilize proline, histidine, and methionine, and if ammonia serves as the main nitrogen source during infection then leaf concentrations of less than 1 to 2 mM would likely stop the growth of ES4326 *rpoN*::Km^r. Finally, as discussed in the next section, *rpoN* is involved in the production of at least one known toxin, COR, which could help explain the reduced virulence.

***rpoN*-mediated regulation of COR synthesis.** The data in Table 2 demonstrate that ES4326 *rpoN*::Km^r does not produce COR, which contributes to lesion expansion, chlorosis, and bacterial multiplication in *Arabidopsis* (56). Although a COR$^-$ mutant is not available for *P. syringae* pv. maculicola, pHRPLC, which expresses *hrpL* constitutively, restored some disease symptoms but not COR production to ES4326 *rpoN*::Km^r. However, because pHRPLC failed to restore in planta growth to the mutant (23), it remains possible that some of the growth defect in ES4326 *rpoN*::Km^r could be caused by loss of COR production.

The data in Table 2 show that *rpoN* is also required for the expression of the *cmaABT* transcript, which encodes proteins that produce coronamic acid, an intermediate in the COR pathway (5). This was surprising since a conserved −24(GG)/−12(GC) motif is lacking upstream of the *cmaA* transcriptional start site (87). Thus, σ^{54} control of *cmaABT* expression is probably mediated indirectly through another regulatory gene whose expression is directly controlled by σ^{54}. Possible candidates for σ^{54} control inside the COR gene cluster include *corP* and *corR*, which encode response regulators with uncharacterized upstream sequences. Alternatively, σ^{54} might control the expression of regulatory genes unlinked to the COR biosynthetic gene cluster.

Reduced induction of the host defense response by ES4326 *rpoN*. ES4326 *rpoN*::Km^r failed to induce defense gene induction in *Arabidopsis* during both compatible and incompatible interactions. These results contrast with those reported previously for *hrp* mutants. A nonpathogenic *hrp* deletion mutant of a compatible *X. campestris* pv. campestris strain elicited the expression of a variety of defense genes in the turnip to approximately 50% of their normal expression levels (60). Similarly, an incompatible *X. campestris* pv. armoraciae strain with an *hrp* deletion did not induce an HR in the turnip but still induced defense gene expression (60). In the *P. syringae* pv. phaseolicola-bean interaction, approximately the same level of defense gene induction occurred with incompatible wild-type and *hrp* deletion strains (32). One way to explain the discrepancy observed in defense gene induction by *rpoN* and *hrp* mutants is that important factors for defense gene induction may lie outside of the *hrp* pathway but under *rpoN* control. While there are reasons to believe that phytotoxins and *avr* genes may contribute to defense gene induction, they seem unlikely explanations for this phenomenon (5, 34, 47, 71). Although production of the phytotoxin COR is *rpoN* dependent, in ES4326, COR$^-$ mutants of DC3000 elicited more defense gene induction than the wild-type strain (56). Similarly, *avr* gene products are thought to require a functioning *hrp* cluster for activity and are therefore unlikely candidates for

hrp-independent defense induction factors (34, 47, 71, 84). Finally, the fact that *X. campestris* pv. vesicatoria *rpoN* mutants are fully pathogenic (25) indicates that, at least in the case of this species, *rpoN* does not regulate any essential pathogenicity factors.

Our experiments also indicate an important role for *hrp*-dependent factors in defense gene induction. When *hrpL* was constitutively expressed in ES4326 *rpoN*::Km^r, both *hrp* gene expression (23) and defense gene induction (Fig. 4) were restored. This result indicates that at least some inducing factors require genes downstream of *hrpL* for expression, function, or both. As mentioned above, *avr* genes are a likely source of *hrp*-dependent defense-inducing factors. We also report that the partial restoration of defense gene induction by *hrpL* is accompanied by restoration of disease symptoms and host cell death (23). This restored host cell death may also play a role in the activation of host defense responses. *P. syringae* products secreted by the Hrp system could result in necrotic or programmed cell death which in turn activates defense gene induction in neighboring cells.

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