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Suppression of a Sensor Kinase-Dependent Phenotype in *Pseudomonas syringae* by Ribosomal Proteins L35 and L20

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The *lemA* gene of *Pseudomonas syringae* pv. syringae encodes the sensor kinase of a bacterial two-component signal transduction system. Phenotypes that are *lemA* dependent in *P. syringae* include lesion formation on bean and production of extracellular protease and the antibiotic syringomycin. Recently, the *gacA* gene has been identified as encoding the response regulator of the *lemA* regulon. To identify additional components that interact with LemA, suppressors of a *lemA* mutation were sought. A locus was identified that, when present in multiple copies, restores extracellular protease production to a *lemA* insertion mutant of *P. syringae* pv. syringae. This locus was found to encode the *P. syringae* homologs of translation initiation factor IF3 and ribosomal proteins L20 and L35 of *Escherichia coli* and other bacteria. Deletion analysis and data from Western immunoblots with anti-IF3 antiserum suggest that protease restoration does not require IF3. Deletion of both the L35 and L20 genes resulted in loss of protease restoration, whereas disruption of either gene alone increased protease restoration. Our results suggest that overexpression of either L20 or L35 is sufficient for protease restoration. It is unclear how alteration of ribosomal protein expression compensates in this instance for loss of a transcriptional activator, but a regulatory role for L20 and L35 apart from their function in the ribosome may be indicated.

Strain B728a of the bacterium *Pseudomonas syringae* pv. syringae causes brown spot disease of bean (*Phaseolus vulgaris* L.). Mutagenesis of B728a with Tn*5* led to the identification of the *lemA* gene as a positive regulator of lesion formation in *P. syringae* pv. syringae (35). DNA sequence analysis of *lemA* suggests that it encodes a sensor kinase with homology to members of bacterial two-component regulatory systems (15). Sensor proteins such as LemA are typically transmembrane proteins that respond to environmental stimuli by autophosphorylation, followed by transfer of the phosphate to a cognate response regulator component. The response regulator may then activate or repress transcription of genes directly by binding to adjacent DNA sequences or indirectly by regulating the expression or activity of other transcriptional regulators (25). The *gacA* gene has been identified as encoding the cognate response regulator in the *lemA* system (28).

Homologs of the *lemA* and *gacA* genes have been identified in a number of pseudomonads, in which they control a variety of phenotypes. Among pathogens, the role of *lemA* is varied. In strain B728a, production of protease and the antibiotic syringomycin, while not strictly required for lesion formation, is positively regulated by *lemA* (13, 14). In three other pseudomonad plant pathogens, *lemA* homologs were necessary for disease formation and production of toxin and protease (11); toxin production but not lesion formation (3); or neither toxin production nor lesion formation (27). The *lemA*/*gacA* system is also required by *Pseudomonas fluorescens* for biocontrol of fungal diseases of various plants and for the production of numerous antibiotics (7, 10b).

From the above, it is difficult to assign a general role for the *lemA*/*gacA* regulatory system. Although the regulation of secondary metabolism appears to be a frequent theme of the *lemA*/*gacA* regulon, there is wide variation in the particular metabolites affected. The suggestion that *gacA* is a general stationary-phase regulator in *P. fluorescens* (30) is not universally true, since the *lemA*-dependent production of tabtoxin and transcription of a gene required for tabtoxin production (*tblA*) in *P. syringae* BR2 occur constitutively throughout the growth cycle (2). Many "two-component" systems in other bacteria that have been carefully examined contain components in addition to the sensor kinase and response regulator. To identify additional components of the *lemA*/*gacA* system which could contribute to the observed species specificity, suppressors of a *lemA* mutation within *P. syringae* pv. syringae B728a were isolated. This work describes the analysis of a suppressor to the protease-deficient phenotype of a *lemA* mutant.

MATERIALS AND METHODS

Bacterial plasmids, strains, and growth. Bacterial plasmids, with the exception of those depicted in Fig. 5, are listed in Table 1. The *P. syringae* pv. syringae strains used in this study are also listed in Table 1. Growth in liquid culture was done at ambient temperature in King's medium B (KB) (17) or NYG medium (8) containing 1% skim milk. Plate cultures were maintained at 28°C on KB agar or NYG agar containing 10% skim milk. The antibiotic concentrations used were 100 μg/ml for rifampicin, 25 μg/ml for ampicillin, 10 μg/ml for tetracycline, and 10 to 15 μg/ml for kanamycin. *Escherichia coli* DH5α (Bethesda Research Laboratories) and derivatives were grown on Luria broth and agar (31); the antibiotic concentrations were 200 μg/ml for ampicillin, 10 μg/ml for tetracycline, and 50 μg/ml for kanamycin. Mutagenesis with ethylmethane sulfonate (EMS; Sigma) was done as described by Miller (23) except that cells were grown at 28°C. Triparental matings with pRK2013 as a helper plasmid have been described before (10).

General DNA manipulations and analysis. The preparation of genomic DNA from *P. syringae* and of cosmid libraries in pRK7813 has been described previously (18). Restriction endonucleases, T4 DNA ligase, and the Klenow fragment

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
P. syringae pv. syringae		
B728a	Rif^r, causal agent of brown spot of bean	S. Hirano, University of Wisconsin-Madison
NPS3136	Rif^r Kan^r *lemA1*::Tn*5*, derived from B728a	35
BUVS1	Rif^r Spc^r Δ(*recA*)*51*::Ω, derived from B728a	15
NUVS1	Rif^r Kan^r Spc^r Δ(*recA*)*51*::Ω *lemA1*::Tn*5*, derived from NPS3136	15
SupP27	Rif^r Kan^r EMS-generated mutant of NPS3136, restored for protease and syringomycin production and lesion formation on bean	This study
NPS3136::pSPH5	NPS3136 derivative containing pSPH5 integrated into the chromosome	This study
NPS3136::pSPH6	NPS3136 derivative containing pSPH6 integrated into the chromosome	This study
Plasmids		
pBluescriptKS$^+$	Amp^r	Stratagene
pRK2013	Kan^r, mobilization plasmid	10
pRK7813	Tet^r, cosmid vector	15a
pLVC18	Tet^r Amp^r, pBR322 derivative containing pRSF1010 *mob*	G. J. Warren
pTOK2	Tet^r, integration vector derived from pLVC18	This study
p27-8	Tet^r, cosmid constructed from SupP27 genomic DNA in pRK7813	This study
pSPH1	Tet^r, *Hin*dIII subclone of p27-8 in pRK7813	This study
pSPH5, pSPH6	Tet^r, *Hin*dIII subclones of p27-8 in both orientations in pTOK2	This study
pSPV1, pSPV2	Tet^r, 2-kb *Eco*RV fragment of pSPH1 in both orientations in pRK7813	This study
pWPV1	Tet^r, identical to pSPV1 except derived from B728a rather than SupP27 genomic DNA	This study
pKSPV1	Amp^r, 2-kb insert of pSPV1 in pBluescriptKS$^+$	This study

of *E. coli* DNA polymerase were purchased from New England Biolabs or Promega and used according to the manufacturer's specifications. Mutagenesis with Tn*lacZ* was done by the method of Manoil (21). DNA sequence was determined directly from cosmids or from subclones in the vector pBluescriptII KS$^+$ with Sequenase version 2.0 and 7-deaza-GTP (United States Biochemical). Protein sequence alignments were determined with the Genetics Computer Group program GAP (9). Codon usage profiles were determined with Codon Use software (freeware by Conrad Halling).

Construction of pSPV1 plasmid derivatives. The subclones pSPVE1 and pSPVE2 were created by digesting pSPV1 with *Eco*RI, dilution, and ligation. Other subclones containing simple deletions were constructed by ligating pSPV1 restriction fragments, made blunt if necessary by end filling with the Klenow fragment of DNA polymerase (United States Biochemical) in the presence of all four deoxynucleoside triphosphates (dNTPs), to *Bam*HI-digested, end-filled pRK7813. To create an in-frame deletion in *infC*, pSPV1 was digested with *Pml*I and *Hin*dIII. The resultant 1.5- and 13-kb fragments were electroeluted and collected separately. The 13-kb fragment was dephosphorylated with bacterial alkaline phosphatase (United States Biochemical). The 1.5-kb fragment was digested with *Bst*EII and *Sst*II. These fragments were ligated to the 13-kb fragment and to two complementary oligonucleotides containing an introduced *Bam*HI site and ends compatible with *Bst*EII and *Sst*II (5′-GGATCCGAAG-3′ and 5′-GTCACCTTCGGATCCGC-3′) to create pSPVΔ1. For the *rpmI* deletion in plasmid pSPVΔ2, a 5-kb *Mlu*I fragment, a 9-kb *Mlu*I-*Sal*I fragment, and a 135-bp *Sph*I-*Mlu*I fragment were collected from separate digestions of pSPV1 by electroelution. After dephosphorylation of the 5-kb fragment, the fragments were ligated to two complementary oligonucleotides containing a *Bam*HI site and ends compatible with *Sal*I and *Sph*I (5′-TCGAGGATCCACCGCATG-3′ and 5′-CGGTGGATCC-3′). Both constructs were checked by restriction analysis and DNA sequence analysis. A 4-bp insertion in *rplT* was created by first digesting pKSPV1 with *Mlu*I, end filling, and ligating. A *Bam*HI-*Hin*dIII fragment containing the insertion was then ligated to pRK7813 previously digested with *Bam*HI and *Hin*dIII to create pSPV::M.

Generation of an integrating plasmid. Plasmid pLVC18, a pBR322 derivative containing the *mob* region from pRSF1010, was generously provided by G. J. Warren. To eliminate *Hin*dIII and *Bam*HI sites within the tetracycline resistance (*tet*) region, pLVC18 was first digested with *Bam*HI and treated with S1 nuclease (Promega) and then with the Klenow fragment of DNA polymerase to produce blunt ends. This DNA was further digested with *Cla*I, and the 5.5-kb fragment containing pLVC18 without the first ~375 bp of the *tet* region was collected by electroelution. PCR was used to amplify the deleted region of pLVC18 with pLVC18 as a template and the primers 5′-GCTTATCATCGATATGCTTT-3′ and 5′-AATCCACAGGACGGGTG-3′, which introduce single base substitutions within the *Bam*HI and *Hin*dIII restriction sites. The PCR product was digested with *Cla*I, and the ~375-bp fragment was electroeluted and ligated to the 5.5-kb fragment of pLVC18. The resultant plasmid, designated pTOK1, was digested with *Eco*RI and *Dra*I followed by S1 nuclease. The ~4.8-kb fragment containing pTOK1 without the *bla* gene was electroeluted and ligated to a ~645-bp *Hae*II fragment (made blunt by S1 nuclease digestion) containing the multiple cloning site and *lacZ*α gene from pBluescript II KS$^+$ (Stratagene) to produce pTOK2.

Protein analysis. Cell lysates were measured for protein content by the bicinchoninic acid method (Pierce) with bovine serum albumin as a standard. Seventy-five micrograms of protein was loaded into each lane. Electrophoresis and analysis by Western immunoblot were done as described previously (28). Polyclonal rabbit antiserum raised against *E. coli* IF3 was kindly provided by J. W. B. Hershey (University of California, Davis). Molecular weight determination was done by comparison with prestained, broad-range molecular weight markers (New England Biolabs).

Quantification of protease production. NUVS1 transconjugants were transferred to replicate NYG plates containing 10% skim milk and tetracycline (10 μg/ml). Each plate contained one replica of each transconjugant. After incubation for 1 day at 28°C, the plates were photographed with a DCS420 digital camera (Kodak). The density slicing function of NIH Image software (by Wayne Rasband at the U.S. National Institutes of Health; available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, part number PB93-504868) was used to select and measure the area of proteolytic clearing around colonies in the digitized gray scale images as well as the area occupied by each colony. Protease production was calculated as the area of clearing surrounding each colony divided by the area occupied by the colony. Individual measurements were combined, and the means were divided by the mean value obtained for NUVS1(pSPV1). Propagation of error was determined as described before (33).

Plant assays. Pod and foliar pathogenicity assays on bean cultivar "Bush Blue Lake 274" (Roger Bros. Seeds) were performed as previously described (35).

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence of the *P. syringae* pv. syringae *infC-rpmI-rplT* region is U44118.

RESULTS

Identification of a locus that restores protease activity to a *lemA* mutant. To isolate extragenic suppressors of a *lemA* mutation, the *lemA1*::Tn*5* mutant NPS3136 (deficient in protease and syringomycin production and lesion formation) (35) was mutagenized with EMS and screened for restoration of extracellular protease production. Of 43,000 colonies examined, 53 were restored for protease production, as evidenced by an increased zone of clearing on skim milk plates. A single strain, designated SupP27, was restored for the additional *lemA*-dependent phenotypes of syringomycin production and lesion formation on bean (unpublished data).

To identify the genes responsible for restoration of the *lemA*-dependent phenotypes in SupP27, a cosmid library was constructed from SupP27 genomic DNA and the cosmid vector

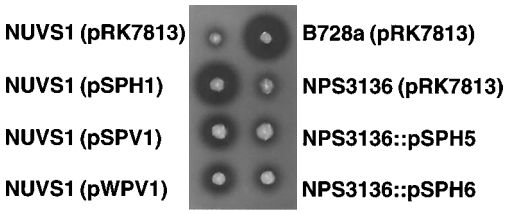

FIG. 1. Proteolysis by *P. syringae* strains on skim milk plates. The strains indicated were stabbed into NYG–10% skim milk plates with a wooden stick, incubated for 1 day at 28°C, and then photographed against a dark background. Dark halos around colonies indicate proteolytic clearing.

pRK7813 and mobilized into the *recA* derivative of NPS3136, NUVS1 (35). Twenty-two distinct cosmids that restored protease activity to NUVS1 in *trans* were isolated. By restriction digestion and Southern blot analysis, the cosmids were placed into four families, with members of a given family containing overlapping inserts. Transconjugants bearing cosmids from each family were tested for syringomycin production and lesion formation. None of the transconjugants were restored for either phenotype (data not shown). We chose for further examination a cosmid, designated p27-8, from the family of cosmids that provided the greatest degree of protease restoration. A 13-kb subclone of p27-8 in the vector pRK7813, designated pSPH1, retained the protease-restoring activity (Fig. 1).

To further define the region responsible for restoration, pSPH1 was mutagenized with the transposon Tn*lacZ* (21). Figure 2 depicts p27-8, pSPH1, and the locations of Tn*lacZ* insertions in pSPH1. Two adjacent insertions, numbers 11 and 21, eliminated protease restoration by pSPH1, whereas all others had no effect (Fig. 2). Both insertions were contained within a 2-kb *Eco*RV fragment, which was again subcloned into pRK7813. The subclone designated pSPV1 (Fig. 2) restored protease activity to the *lemA* mutant NUVS1, although the halo of clearing was slightly smaller than that produced by pSPH1 in NUVS1 (Fig. 1). A plasmid containing the same insert as pSPV1 in the other orientation (pSPV2) failed to restore activity (data not shown).

The 2-kb *Eco*RV fragment restores protease production by copy number effects. To determine whether pSPV1 contains an EMS-generated mutation necessary for protease restoration, we sought to clone the homologous *Eco*RV fragment from the wild-type parent of SupP27 and NPS3136, B728a. The pSPH1 insert was used to probe a cosmid library of B728a DNA. A hybridizing 2-kb *Eco*RV fragment was identified in one of the cosmids and subcloned into pRK7813 to produce pWPV1. Figure 1 shows that pWPV1 is comparable to pSPV1 in restoration of proteolysis in NUVS1, suggesting that chemical mutagenesis of the locus from SupP27 was not necessary to produce the protease-restoring phenotype. To determine the effect of locus copy number on restoration, the 13-kb *Hin*dIII fragment from pSPH1 was cloned in both orientations into pTOK2 (a ColE1 vector that does not replicate in *P. syringae*) and mobilized into the *recA*$^+$ parent of NUVS1, NPS3136, to create NPS3136::pSPH5 and NPS3136::pSPH6. Transconjugants were verified to contain a single copy of either integrated plasmid by Southern blot (data not shown) and were indistinguishable from NUVS1 and NPS3136 in protease activity (Fig. 1). This result suggests that restoration by pSPV1 is mediated solely by copy number effects.

Identification of the *infC*, *rplT*, and *rpmI* genes in the protease activity-restoring locus. The DNA sequence of the pSPV1 insert was determined and found to contain four open reading frames in one orientation that were consistent with *P. syringae* codon usage (Fig. 3). Comparison with nonredundant nucleotide and protein databases with the BLAST protocol (1) revealed that these open reading frames resemble in sequence and organization the locus containing *thrS*, *infC*, *rpmI*, and *rplT* in *E. coli* and other bacteria, encoding threonyl-tRNA synthetase, translation initiation factor 3 (IF3), and ribosomal proteins L35 and L20, respectively. In *E. coli*, the AUU start codon for *infC* (29) is separated by 3 bp from the UAA stop codon of *thrS*. Three consecutive AUU codons (indicated in boldface in Fig. 3) are located at the beginning of the *P. syringae infC* open reading frame, with the first overlapping the UAA stop codon of *thrS*. The *infC*, *rpmI*, and *rplT* genes of *E. coli* are separated by 94 and 52 bp, respectively, whereas 60 and 29 bp separate the homologous genes in *P. syringae*. Figure 4 shows alignments of the predicted translational products of the *P. syringae infC*, *rpmI*, and *rplT* genes (IF3, L35, and L20, respectively) with their *E. coli* counterparts. The amino acid sequences show 65% identity for IF3, 52% for L35, and 82% for L20. Figures 3 and 5 indicate the organization of the genes within pSPV1. The *thrS* gene is incomplete, lacking the first 1,242 bp of the corresponding *E. coli* gene (which is 1,926 bp in length [22]), whereas the *infC*, *rpmI*, and *rplT* genes are present in their entirety. In *E. coli*, the latter three genes are transcribed as an operon, with most transcription originating from a promoter identified as pO′ (4) or as P_{I2} (34) 188 bp upstream from the *infC* start codon (within *thrS*). This arrangement is paralleled in *P. syringae*, which contains −35 and −10 elements ~185 bp upstream from the putative *infC* start codon that are identical in sequence and spacing to the *E. coli* elements (Fig. 3 and 5). DNA sequence analysis of Tn*lacZ* insertions falling within the 2-kb *Eco*RV fragment indicates that insertions 5 and 17, which do not interfere with protease restoration, lie within the *thrS* gene, with insertion 17 creating a translational fusion with β-galactosidase. The insertions that prevent restoration, 11 and 21, are within the genes encoding IF3 (*infC*) and L35 (*rpmI*), respectively (Fig. 3 and 5).

To better define the gene(s) responsible for restoration, deletions were made from both ends of the cloned DNA extending inward. The resulting plasmids were introduced into NUVS1, and transconjugants were evaluated for protease production on skim milk plates. Figure 5 indicates the extent of the deletions and a qualitative assessment of the protease activity by transconjugants bearing the plasmids shown. As indicated, deletion of the *thrS* gene up to the first *Eco*RI site did not interfere with restoration by pSPVE2. Further deletion to the *Dra*I site (pSPVD1 [Fig. 3 and 5]) removes the −35 element and 1 bp of the −10 element of the pO′-like promoter (4) and eliminates restoration. Changing the orientation of this

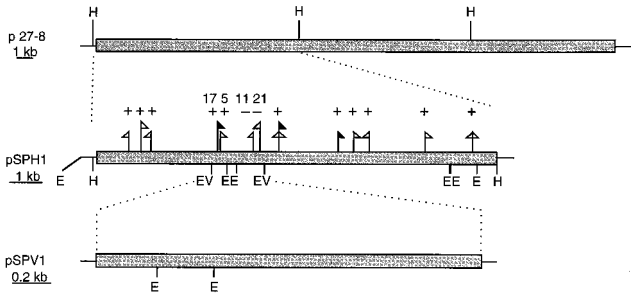

FIG. 2. Restriction and transposon insertion maps of protease-restoring clones. Restriction sites are H, *Hin*dIII; E, *Eco*RI; and EV, *Eco*RV. Tn*lacZ* insertions in pSPH1 are marked with flags. Solid flags indicate insertions that produce translational fusions with β-galactosidase. −, loss of protease-restoring activity from the insertion, +, no loss of restoration.

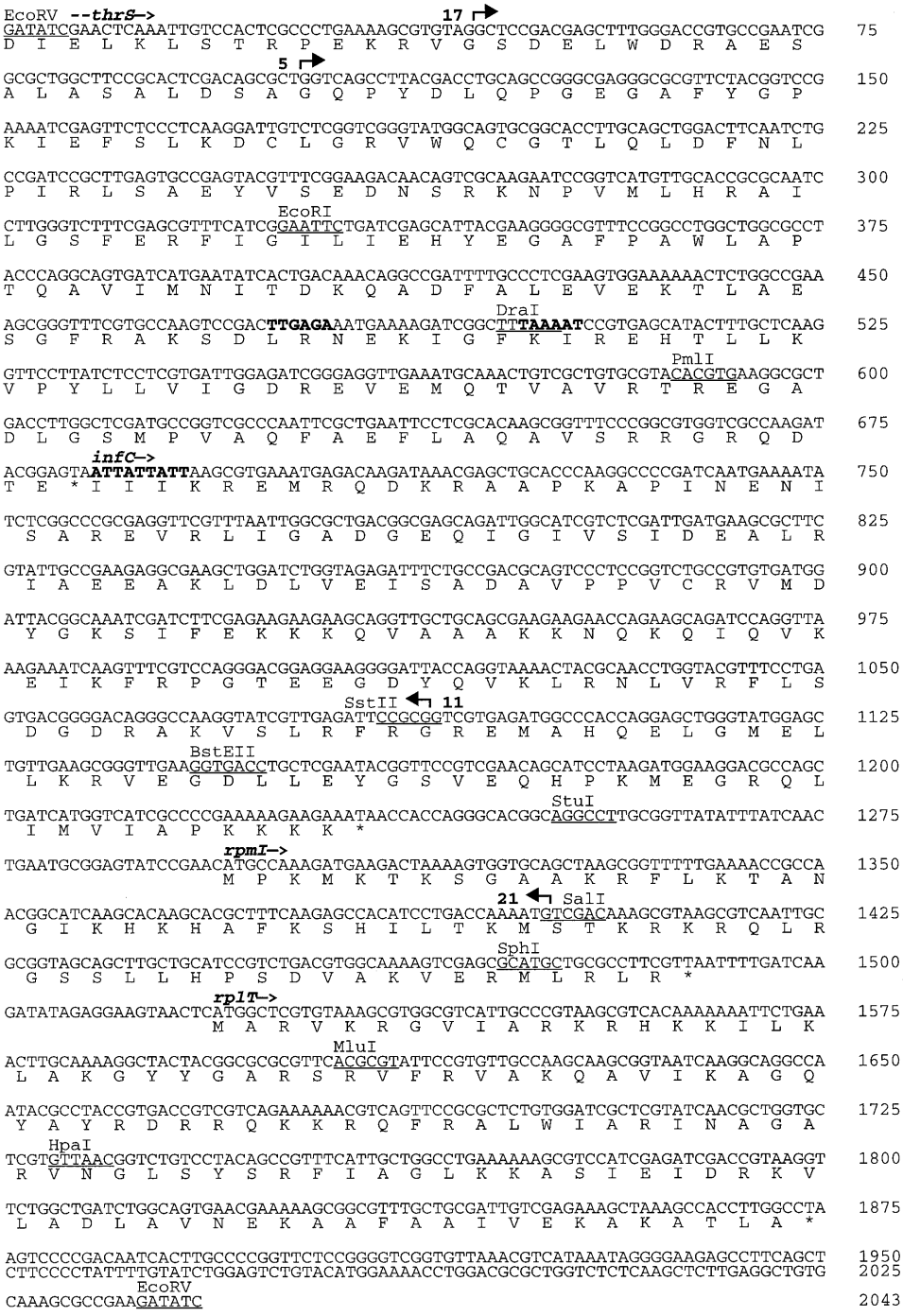

FIG. 3. DNA sequence of 2-kb *Eco*RV fragment and predicted amino acid sequences of the *thrS*, *infC*, *rpmI*, and *rplT* open reading frames. Restriction sites are underlined. Numbered arrows indicate the sites and orientations of Tn*lacZ* insertions. The −35 and −10 elements of the putative *infC* promoter as well as potential start codons for IF3 are indicated in boldface.

insert relative to the vector had no effect on protease restoration (pSPVD2; not shown). From the other end of the insert, deletion to the *Hpa*I site in *rplT* does not eliminate protease restoration by pSPVH1, although it does cause transconjugants to form smaller colonies on skim milk plates (data not shown). Further deletion prevents protease restoration (Fig. 5).

The deletion and transposon data suggested that both *infC* and *rpmI* might be necessary for restoration of protease production, whereas *rplT* was dispensable. To test this hypothesis, in-frame deletions were created in the *infC* and *rpmI* genes, removing 16 and 22 codons, respectively, from their open reading frames (Fig. 4). Also, because the large deletion in pSPVH1 (removing part of *rplT*) appeared to impair the growth of transconjugants, a second mutagenesis of *rplT* was performed by the creation of a frameshift. A 4-bp insertion at the *Mlu*I site of *rplT* resulted in pSPV::M, which encodes a

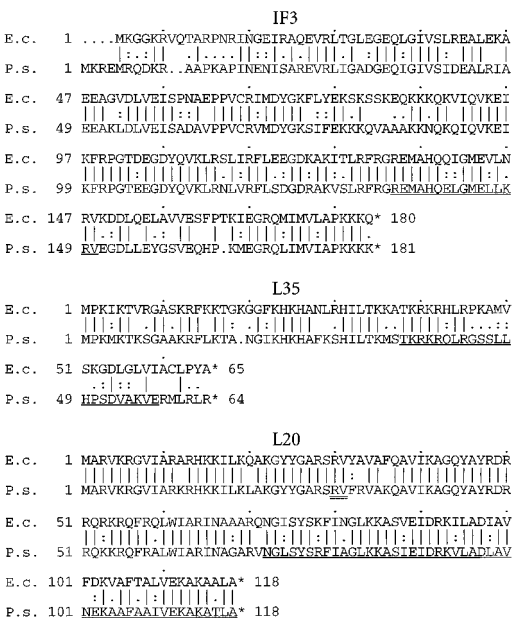

FIG. 4. Comparison of the predicted amino acid sequences of *E. coli* (E.c.) and *P. syringae* (P.s.) IF3, L35, and L20. The predicted *P. syringae* IF3 sequence assumes the use of the third consecutive AUU codon for the start codon (see Fig. 3). Bars indicate identity, while double and single dots indicate similarity according to the GAP program default settings (9). Single underlines indicate amino acids deleted in pSPVΔ1 (IF3), pSPVΔ2 (L35), and pSPVH1 (L20). The double underline indicates the position of the 4-bp insertion in pSPV::M (see Fig. 5).

protein containing the first 30 of L20's 118 amino acids fused to 19 amino acids encoded by a different reading frame (Fig. 4 and 5). The *infC* deletion (pSPVΔ1 [Fig. 5]) had little effect on protease production, while the *rpmI* deletion in pSPVΔ2 and the frameshift in *rplT* seemed to increase proteolysis. To quantify protease production, cell-free supernatants from liquid cultures were assayed for relative azocasein digestion, with proteinase K as a standard. The plasmid bearing the *rpmI* deletion (pSPVΔ2) significantly increased proteolysis by both BUVS1 (*lemA*$^+$) and NUVS1 (*lemA*) cultures; however, all other plasmids had no significant effect on proteolysis. Because the liquid assay could not be used to distinguish among the effects of most of the plasmids tested, an effort was made to more accurately determine relative protease production by colonies of transconjugants growing on plates containing skim milk. The area of proteolytic clearing surrounding each colony was measured. To account for differences in cell number, each value obtained was divided by the value of the area occupied by the corresponding colony. The results obtained from replicate measurements are presented in the last two columns of Fig. 5. The deletion in *rpmI* (pSPVΔ2) and the frameshift in *rplT* (pSPV::M) result in increased proteolysis compared with pSPV1, while the *infC* deletion (pSPVΔ1) does not produce a significant effect. With the exception of pSPVE2, the remaining plasmids were much less effective than pSPV1 in restoring proteolysis to NUVS1. These results suggest that either *rpmI* or *rplT* is necessary for protease restoration, but not both genes.

The role of IF3 in protease restoration is not definitive from these data, since it could be argued that the in-frame deletion in pSPVΔ1 did not eliminate IF3 activity. To address this, BUVS1 and NUVS1 transconjugants were examined by Western blot with anti-*E. coli* IF3 antiserum. Figure 6 indicates that this antiserum reacts chiefly with a single protein of 20.7 kDa, which compares favorably with a predicted molecular mass of 20.6 kDa for *P. syringae* IF3 (assuming use of the third AUU codon as a start codon, as in Fig. 4). The only exception is found in cells containing the in-frame *infC* deletion in pSPVΔ1. In this case, the 20.7-kDa protein (presumably the product of the chromosomal *infC* gene) is joined by a protein of 18.1 kDa (IF3Δ in Fig. 6), approximately the expected size of the deleted IF3, 18.7 kDa. Although exact quantification is not possible in this experiment, it is apparent that IF3 levels are similar in transconjugants containing protease-restoring plasmids pSPV1 and pSPVΔ2 and the nonrestoring plasmid pSPVS1. This suggests that alteration of IF3 abundance is not responsible for protease restoration.

DISCUSSION

We have identified the *P. syringae infC-rpmI-rplT* locus as a partial, multicopy suppressor of a mutation in the *lemA* gene. The regulation of this locus has been studied thoroughly in *E. coli*, in which the gene organization is identical to that in *P. syringae*. In *E. coli*, transcription of all three genes originates mostly from a promoter within the *thrS* gene (Fig. 3), although a second promoter upstream of *thrS* also contributes significantly (20, 34). A terminator downstream from *infC* reduces transcription of *rpmI* and *rplT* by 50 to 70% compared with *infC* (34). IF3, the product of the *infC* gene, represses its own translation by a mechanism that depends upon its unusual start codon, AUU (4, 5). Ribosomal protein L20 represses expression of L35, probably by binding to a site within the *infC* transcript (20). By a process that appears to involve the formation of a secondary structure encompassing the L20 ribosome-binding site, this binding also results in autoregulation of L20 expression (19). If this region is regulated similarly in *P. syringae*, we can predict the effects of several of our manipulations. The Tn*lacZ* insertions 11 and 21 (Fig. 3 and 5) would be expected to be polar, eliminating expression of downstream genes as well as inactivating the genes in which they are inserted. Inactivation of L20 by deletion or frameshift should relieve repression of L35. The deletion within *rpmI* (pSPVΔ2 [Fig. 5]) would be expected to alleviate L20 autoregulation by preventing secondary-structure formation (19). Thus, the construct pSPVΔ2 would be expected to overexpress L20, whereas pSPV::M would overexpress L35. Translational reporters within the genes encoding L20 and L35 or antibodies to the gene products would be necessary to test these predictions.

Although IF3 has been identified as a suppressor of a *recJ* mutation in *E. coli* (12), overproduction of *P. syringae* IF3 is apparently neither necessary nor sufficient for protease restoration. Figures 5 and 6 together show that IF3 levels are not correlated with levels of protease production. Furthermore, pSPVΔ1 (containing an in-frame deletion in IF3 [Fig. 5]) is not markedly different from pSPV1 in its ability to restore protease activity to NUVS1. Our results suggest, however, that the pO′-like promoter upstream of the IF3 gene may be necessary for the restoration phenotype. The construct that lacks this promoter, pSPVD1, does not restore proteolysis. Surprisingly, placing the vector *lacZ* promoter in front of the operon in addition to (pSPV2) or instead of (pSPVD2) the pO′-like promoter results in no restoration of protease production. This may indicate that the start site of the transcript is critical for protease restoration.

Another explanation of the results presented in Fig. 5 is that suppressing plasmids exert their function through the production of antisense RNAs, since the *infC* operon carried on pSPV1 and the other protease-restoring plasmids is oriented

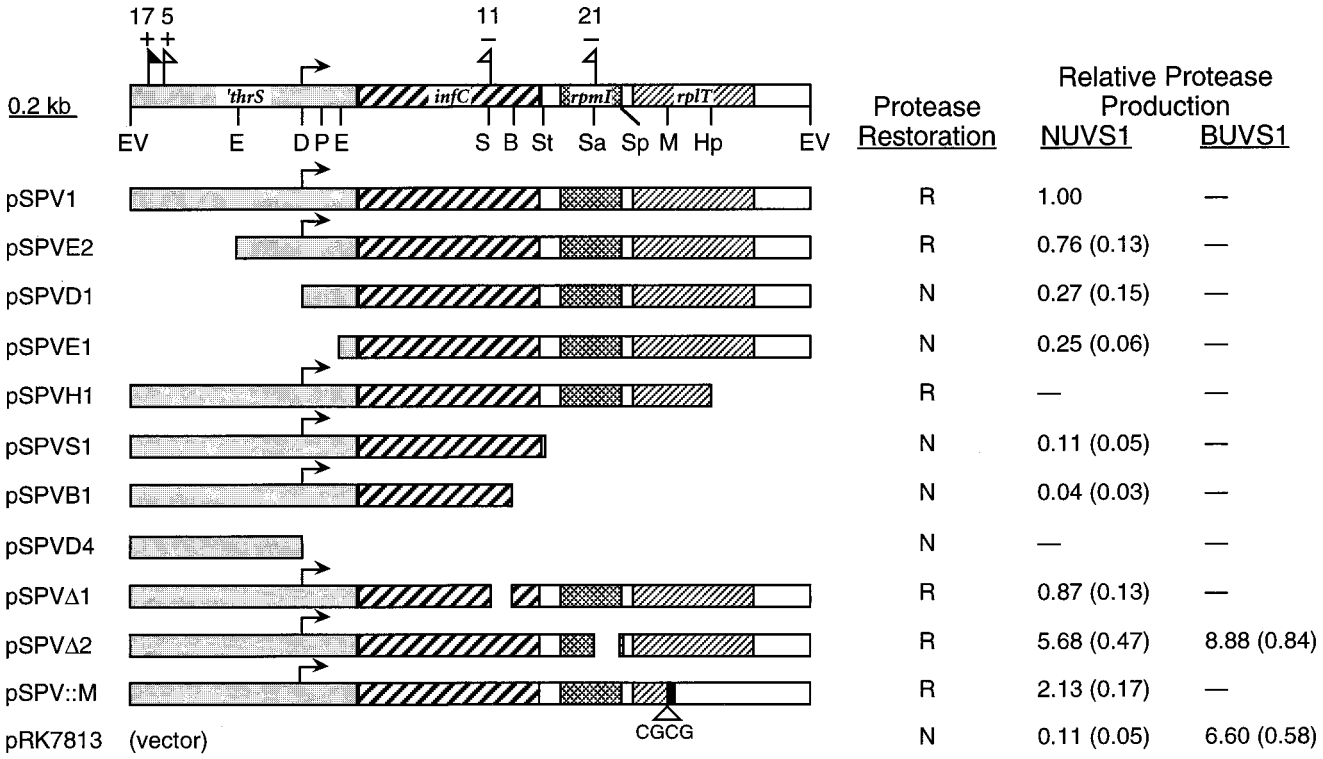

FIG. 5. Structure and protease-restoring ability of pSPV1 and derivatives. Numbered flags indicate the positions and orientations of Tn*lacZ* insertions mapped to this region of pSPH1 (see Fig. 2). The arrow indicates the putative *infC* promoter. Restriction sites shown are EV, *Eco*RV; E, *Eco*RI; D, *Dra*I; P, *Pml*I; S, *Sst*II; B, *Bst*EII; St, *Stu*I; Sa, *Sal*I; Sp, *Sph*I; M, *Mlu*I; and Hp, *Hpa*I. Protease production by NUVS1 containing the plasmids shown to the left is indicated qualitatively as R (restoration) or N (no restoration). Relative protease production on skim milk plates by NUVS1 and BUVS1 containing the plasmids shown is indicated in the last two columns, with the amount produced by NUVS1(pSPV1) set equal to 1. Numbers in parentheses indicate the standard error of the mean. —, not done.

opposite the direction of transcription of the vector *lacZ* promoter. Were this true, the deletion data would dictate that the antisense RNA produced from restoring plasmids extend at least from *rpmI* through the pO′-like promoter 5′ to *infC*, with the expected result that IF3 levels would drop. It is clear from Fig. 6 that protease-restoring plasmids increase rather than decrease the cellular level of IF3 compared with the level in BUVS1 and NUVS1 containing only the vector.

The combined results of these studies provide indirect evidence that protease restoration is mediated by increased production of ribosomal protein L20 or L35. If IF3 levels can be used as a measure of expression of the entire *infC* operon, we can reject the most direct explanation for suppression—that increased L20 and L35 production compensates for decreased production of these proteins in *lemA* mutants. Figure 6 indicates that IF3 levels are comparable in BUVS1(pRK7813) and the *lemA* mutant NUVS1(pRK7813).

We are not aware of previous reports of ribosomal protein genes acting as suppressors of mutations in transcriptional regulators. One explanation to account for this finding is that suppression involves known functions of ribosomal proteins. Perhaps alteration of the balance of ribosomal proteins causes more efficient translation of a protease message that is present in reduced amounts in a *lemA* mutant. In this model, suppression could result from the overexpression of other large-subunit ribosomal proteins rather than L20 and L35. This hypothesis is consistent with our findings that the *lemA* mutant NPS3136 produces a small but detectable level of protease (13) (Fig. 1) and that pSPVΔ2 increases protease production by both *lemA*$^+$ and *lemA* cells (Fig. 5), as well as cells containing mutations in the *lemA* response regulator *gacA* (data not shown). Furthermore, at least three other uncharacterized loci were found to restore protease production to a *lemA* mutant when present in multiple copies. These loci could contain other ribosomal protein genes. We are not aware, however, of any previous reports of ribosomal protein overexpression causing increased translational efficiency. Suppression may occur, instead, at the level of protease secretion. A mutant ribosomal protein gene has been identified previously as a suppressor of a secretion mutation (32). Assays of protease activity present in

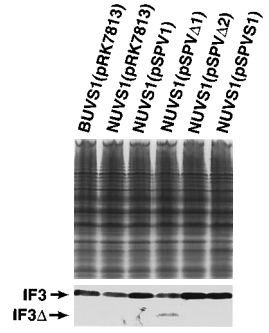

FIG. 6. Polyacrylamide gel electrophoresis of *P. syringae* lysates followed by staining with Coomassie brilliant blue (upper panel) or by Western blot analysis with antiserum to *E. coli* IF3 (lower panel). The position of intact IF3 in the Western blot is indicated, as is the position of the internally deleted protein (IF3Δ) encoded by pSPVΔ1.

cell lysates, however, suggest that NPS3136 suffers from a defect in protease expression rather than export (13).

Alternatively, L35 and L20 may serve regulatory roles apart from their function in the ribosome. Support for the latter hypothesis may come from a study which addresses another intriguing aspect of this work—that the L20 and L35 genes are interchangeable for protease restoration. The only commonality that we can find between L20 and L35, other than their being components of the same (large) ribosomal subunit and being encoded by genes in the same operon, is the report that both *E. coli* proteins are enzyme inhibitors in vitro (16). In this report (in which L35 was first identified and is referred to as X1 protein), free L20 and L35 were found to be more potent inhibitors of ornithine decarboxylase activity in vitro than all other ribosomal proteins. Although an in vivo role for L20 and L35 in regulating ornithine decarboxylase is doubtful (16, 24), whatever common structural determinant is responsible for enzyme inhibition in vitro may allow binding of L20 and L35 to some other protein in vivo. Binding to a transcription factor, repressor, or RNA polymerase could suppress the effects of loss of LemA or GacA as well as increase transcription of a protease gene in their presence. Another study, performed before L35 was identified, determined that L20 was one of two ribosomal proteins that disappear entirely from ribosomes but persist undiminished elsewhere in the cell during postexponential growth of *E. coli* in L-broth (26). This suggests that L20 may be available for interaction with other proteins during the time at which protease is expressed in *P. syringae*, during stationary phase (13). The introduction of other ribosomal protein genes in multiple copies in *P. syringae* would be necessary to determine whether L20 and L35 play a specific role in regulating protease production.

The restoration of one of three known *lemA*-dependent phenotypes by the *infC* operon in multiple copies is reminiscent of the report by Carbonetti et al. (6), who found that overexpression of the α subunit of RNA polymerase reduced transcription of two genes under the control of the *bvgA*/*bvgS* two-component system while leaving other *bvg*-controlled genes unaffected. In both cases, overexpression of proteins involved in general cellular processes had specific effects on gene expression. Although L20 and L35 may exert a chance effect on a *lemA*-controlled gene, either within the ribosome or separately, it is also possible that components of the translational machinery are intimately linked to the *lemA* system. This possibility is supported by the finding that a Tn*5* insertion within a region of the *P. syringae* B728a chromosome containing genes for initiation factor 2 (*infB*) and two tRNAs (*leuU* and *metY*) results in loss of lesion formation and syringomycin production, two *lemA*-dependent phenotypes (36).

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