Involvement of the *lemA* Gene in Production of Syringomycin and Protease by *Pseudomonas syringae* pv. *syringae*

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The *lemA* gene of *Pseudomonas syringae* pv. *syringae* has been proposed to encode a transmembrane protein related to a large family of bacterial regulatory proteins. A *lemA* mutation in *P. s.* pv. *syringae* isolate B728a eliminates lesion development in brown spot disease of bean. In this study, we report that the *lemA* gene is also required for *P. s.* pv. *syringae* to produce normal levels of extracellular protease and of the toxin, syringomycin, but not for motility or the production of pyoverdin or bacteriocin. The role of protease and syringomycin in lesion development was investigated after isolation of mutants affected in production of either activity. One class of syringomycin mutants was identified that produced no syringomycin as measured by *in vitro* bioassay, but retained wild-type levels of pathogenicity on bean. In addition, protease mutants were isolated that had lost most of their proteolytic activity and remained pathogenic on bean. These results suggest that protease or syringomycin production by *P. s.* pv. *syringae* does not play a major role in the process of lesion formation on bean.

Additional keywords: metalloprotease, pathogenicity, *Phaseolus vulgaris*, regulation, toxin.

Extracellular products such as toxins, enzymes, extracellular polysaccharides, and hormones are synthesized by many bacterial plant pathogens. Some of these compounds have either been correlated with or proven to be involved in disease development by different pathogens on their host plants (for review, see Daniels *et al.* 1988).

Syringomycin is a nonspecific toxin produced by many isolates of *Pseudomonas syringae* pv. *syringae*. The toxin is a lipodepsinona peptide consisting of a macrocyclic ring of nine amino acids with a fatty acid side chain. Three of the amino acids are uncommon: 4-chlorothreonine, 3-hydroxyaspartic acid, and 2,3-dehydrothreonine (Fukuchi *et al.* 1990; Mitchell 1991; Segre *et al.* 1989). The biosynthetic pathway for syringomycin is unknown but is expected to be complex due to the requirements for the unusual amino acids and for a ring structure. By analogy with other structurally related antibiotics, syringomycin is probably synthesized nonribosomally (Kleinkauf and von Dohren 1990). The mode of action of syringomycin is thought to involve increased plant cell uptake of calcium (Takemoto *et al.* 1991), a second messenger in signal transduction pathways in eukaryotes.

Syringomycin has been implicated as a contributing factor in the virulence of strains of *P. s.* pv. *syringae* that cause holcus spot of maize (Gross and DeVay 1977) and bacterial canker of peach and other stone fruits (DeVay *et al.* 1968; Sinden *et al.* 1971; Xu and Gross 1988a). However, this correlation is not always absolute and some isolates that produce no detectable syringomycin by *in vitro* bioassay are still fully pathogenic (Backman and DeVay 1971). Efforts to clarify the role of syringomycin in disease development have been complicated by difficulties in the detection of syringomycin *in planta* (Gross and DeVay 1977) and it remains uncertain whether some pathogenic strains that do not produce syringomycin *in vitro* might be induced to produce the toxin in the plant environment.

The role of proteases in plant disease development has not been well studied. Although protease genes have been cloned from *Erwinia* spp. and *Xanthomonas campestris* pv. *campestris* (Allen *et al.* 1986; Barras *et al.* 1986; Tang *et al.* 1987; Wandersman *et al.* 1987), the role of protease in disease development has been extensively investigated only with the latter pathogen. A *X. c.* pv. *campestris* mutant deficient in the production of the two major extracellular proteases was found to be delayed in disease development on turnip (Dow *et al.* 1990).

In a previous study, we reported that the *lemA* gene is required by *P. s.* pv. *syringae* for lesion formation on bean (*Phaseolus vulgaris* L.) (Willis *et al.* 1990). Recently, we have shown that the predicted protein product of the *lemA* gene is similar to members of a family of prokaryotic two-component regulatory proteins (Hrabak and Willis 1992). Mutations in regulatory loci often result in a pleiotropic phenotype since a regulatory gene usually controls transcription of several other genes. To determine if other *P. s.* pv. *syringae* phenotypes were affected by a *lemA* mutation, a wild-type and a *lemA* mutant strain were compared for production of a number of extracellular products. In this report, we present evidence that a *lemA* mutation affects production of both syringomycin and protease. In

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addition, we present results on the isolation and partial characterization of new mutants of *P. s.* pv. *syringae* strain B728a deficient in either syringomycin or protease production and determine their effect on pathogenicity. Preliminary accounts of some of these results have been published previously (Hrabak *et al.* 1989; Hrabak and Willis 1990).

RESULTS

The *lemA* gene is required for production of wild-type levels of syringomycin and protease.

Based on its sequence, it is probable that the *lemA* gene product is involved in the regulation of genes required for disease development (Hrabak and Willis 1992). In an attempt to identify products from such genes, we investigated whether the wild-type *P. s.* pv. *syringae* isolate B728a and a *lemA* mutant such as NPS3136 differed in motility or in their ability to produce any of a variety of extracellular compounds. Both B728a and NPS3136 were motile as determined by light microscopy and spreading ability in motility agar media. Comparison of B728a and NPS3136 showed that the *lemA* mutation did not affect the production of bacteriocin or of the fluorescent siderophore, pyoverdin (data not shown).

Preliminary findings indicated that a functional *lemA* gene was required for the production of extracellular protease and the toxin syringomycin by *P. s.* pv. *syringae* (Hrabak *et al.* 1989; Hrabak and Willis 1990). The *lemA* mutant strain NPS3136 produces neither protease or syringomycin. However, this strain is restored to protease and syringomycin production by the introduction of the plasmid pEMH97 carrying the wild-type *lemA* gene. In this paper, we confirm these findings using *recA* mutant derivatives of B728a and NPS3136 (designated as BUVS1 and NUVS1, respectively). Figure 1 shows the production of protease and syringomycin by BUVS1. The introduction of the *lemA1*::Tn*5* mutation into this genetic background (strain NUVS1) resulted in the loss of observable extracellular production of syringomycin and protease on SRM and NYGM media, respectively (Fig. 1). Introduction of pEMH97 into NUVS1 restored the ability of this *lemA* mutant to produce both syringomycin and protease (Fig. 1). The presence of the *recA* mutation in NUVS1 ensured that this complementation was not due to homologous recombination between the plasmid and the chromosomal *lemA* gene. Taken as a whole, these results demonstrated that, under our assay conditions, the *lemA* gene was required for production of syringomycin and protease by *P. s.* pv. *syringae* isolate B728a, but not for motility or for the production of pyoverdin or bacteriocin.

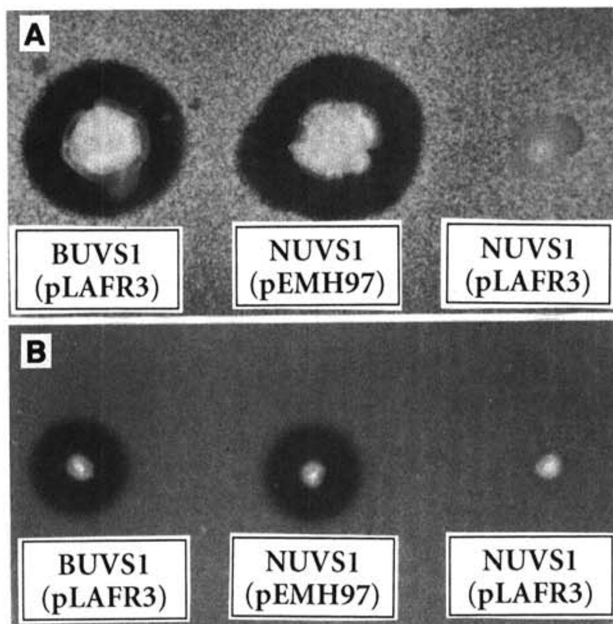

Fig. 1. Complementation of the *lemA* mutation in *P. s.* pv. *syringae* by a *lemA* clone. BUVS1 and NUVS1 are *recA* deletion strains derived from wild-type B728a and the *lemA* mutant NPS3136, respectively. pEMH97 contains the wild-type *lemA* gene cloned in pLAFR3 (Staskawicz *et al.* 1987). The cloning vector pLAFR3 was introduced into NUVS1 as a control. **A,** Syringomycin assay on SRM medium containing 10 mg/ml Tet to increase plasmid stability during the assay. **B,** Protease assay on NYGM medium.

Assay conditions for syringomycin and protease.

Several parameters were tested to determine optimal assay conditions for syringomycin and protease. Syringomycin production was assayed at 16, 20, 24, 28, 32, and 36° C on both SRM and PDA media. Optimal syringomycin production by the wild-type B728a, as determined by size of the zone of growth inhibition of the indicator strain, occurred between 24 and 32° C. Syringomycin production declined as temperature decreased below or increased above the optimal range. No syringomycin production by the *lemA* mutant NPS3136 was detected at any temperature or on any medium tested. Based on these results, SRM medium and an incubation temperature of 28° C were used in all syringomycin assays unless otherwise noted.

The wild-type *P. s.* pv. *syringae* isolate B728a and the *lemA* mutant NPS3136 were grown in six different broth media, as described in the Materials and Methods, and filter-sterilized broth supernatants were assayed for protease activity on NYGM plates. Only supernatants from cultures of *P. s.* pv. *syringae* isolate B728a grown in media that included skim milk were found to contain proteolytic activity (data not shown). The presence of skim milk did not result in the appearance of proteolytic activity in cultures of the *lemA* mutant. To investigate qualitatively whether protease induction was affected by medium composition, bacteria were inoculated into plates of the standard protease detection medium NYGM or into plates of KB supplemented with 10% skim milk and incubated overnight. Protease production by B728a could be induced on both media, but the size of the proteolysis zone was much smaller on KB + milk than on NYGM (data not shown). NPS3136 did not produce protease on either medium. Protease was produced by *P. s.* pv. *syringae* isolate B728a on NYGM plates at 16, 20, 24, 28 and 32° C. The largest zone of proteolysis occurred at 28° C, followed by 24° C and 20 and 32° C. No proteolysis was apparent when plates were incubated at 36° C, perhaps because *P. s.* pv. *syringae* grows poorly at this temperature. The *lemA* mutant NPS3136 did not produce protease on

NYGM agar medium at any temperature tested. Based on these results, NYGM medium and incubation overnight at 28° C was used for all protease assays.

Characterization of mutants affected in syringomycin production.

Prototrophic Tn*5* mutants of *P. s.* pv. *syringae* isolate B728a were screened for their ability to produce the toxin syringomycin by an *in vitro* bioassay on SRM medium (Fig. 2). All of the mutants that were reduced or deficient in syringomycin production retained proteolytic activity on NYGM agar and incited a hypersensitive reaction (HR) when inoculated into a nonhost plant such as tobacco (data not shown). Some of the mutants that did not produce syringomycin on SRM did produce small zones of inhibition when assayed on PDA (Table 1), while two syringomycin mutants (KW311 and KW327) produced small amounts of syringomycin when assayed on SRM but not on PDA.

Chromosomal DNA isolated from each syringomycin mutant was used in Southern blot hybridization experiments with three different DNA probes. First, when the internal *Hin*dIII fragment of Tn*5* was used as a probe, all syringomycin mutants were found to contain only a single copy of the transposon (data not shown). Second, to confirm that none of the transposon insertions in the syringomycin mutants had occurred in or near the *lemA* gene, DNA from all of the syringomycin mutants was probed with pKW351 or pEMH97, cosmid clones containing the *lemA* gene and some flanking DNA. Hybridization signals similar in size to those of the wild-type strain were detected in DNA samples from all of the syringomycin mutants, indicating that none of the syringomycin mutations had occurred in the *lemA* gene or the DNA immediately adjacent to it (data not shown). Third, DNA from each syringomycin mutant was probed with pYM101, containing the *syrB, syrC,* and *syrD* genes which are required for syringomycin production (Xu and Gross 1988b; N. Quigley and D. Gross, personal communication). Three different patterns of hybridization with pYM101 were observed. In the first type, the hybridization pattern with pYM101 was not different from the wild-type hybridization pattern. This was the case for the majority of the syringomycin mutants. Two syringomycin mutants (KW327 and KW329) were of the second type and had transposon insertions in one of the DNA fragments that hybridized to pYM101 (data not shown). Finally, three of the syringomycin mutants (KW319, KW321, and KW332) either partially or totally lacked hybridization to pYM101 (Table 1). Nine other mutants of this type have been identified independently (J. J. Rich and D. K. Willis, unpublished data).

The mutants that were affected in syringomycin production could also be subdivided on the basis of their ability to cause a typical pathogenic, water-soaked response after injection into bean pods (Table 1). The wild-type isolate B728a caused characteristic sunken, water-soaked lesions when suspensions containing from 10^5 to 10^8 cells per milliliter were injected. A nonpathogenic mutant, NPS-3136, produced a faint pod reaction only at 10^7 to 10^8 cells per milliliter injected. The faint browning and water-soaking were never accompanied by sunken lesions and were quite distinct from the wild-type reaction. Syringomycin mutants produced pod reactions that were grouped into three classes: 1) nonpathogenic (i.e., equivalent to the nonpathogenic mutant NPS3136); 2) fully pathogenic (i.e., not distinguishable from the wild-type response); and 3) intermediate pathogenicity (a response between the nonpathogenic and fully pathogenic). Mutants having intermediate pathogenicity were defined as those that caused no visible reaction at 10^5 bacteria per milliliter and no reaction or only a faint reaction at 10^6 bacteria per milliliter injected. Mutants with intermediate pathogenicity still caused sunken, water-soaked lesions at high inoculum levels. Pathogenicity assays on each mutant were repeated at least twice.

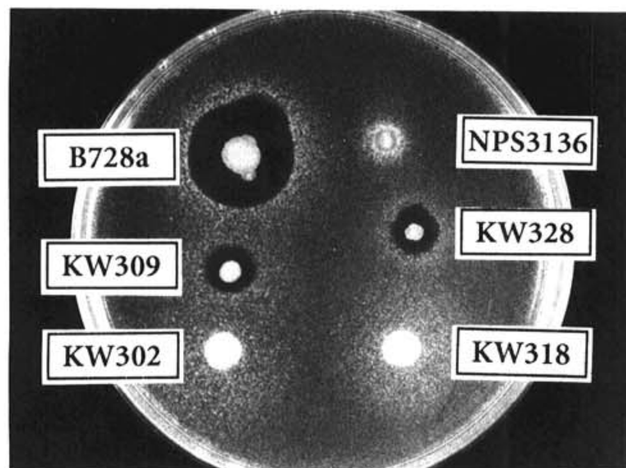

Fig. 2. Syringomycin assay of the wild-type isolate B728a, the *lemA* mutant NPS3136 and six representative syringomycin mutants on SRM medium. Clear zones around some colonies are due to growth inhibition of the toxin-sensitive indicator *Rhodotorula pilimanae.*

Table 1. Phenotypic classes of syringomycin mutants assayed on SRM medium

Syringomycin phenotype	Pathogenicity on bean pods	Mutant
R[a]	–	KW21
R	I[b]	KW327[c]
R	+	KW309, KW311, KW314, KW325, KW328
–	–	NPS3136[d], KW301[e]
–	I	KW302, KW319[c], KW321[c], KW331
–	+	KW304, KW306, KW318, KW320, KW322[e], KW323, KW324[e], KW326[e], KW329[c], KW330, KW332[c]

[a] R indicates a reduced zone of inhibition in the syringomycin bioassay compared to the wild-type isolate B728a.
[b] Indicates an intermediate reaction on bean pods, as defined in the text.
[c] Strains are deleted for, or have transposon insertions in, DNA contained in pYM101.
[d] NPS3136 is a *lemA* mutant and is also protease-negative. All other syringomycin mutants are protease-positive.
[e] Mutants produce some syringomycin on PDA plates.

The nonpathogenic phenotype of two of the syringomycin mutants (KW21 and KW301) was confirmed with a leaf lesion formation assay. Wild-type isolate B728a produced typical brown spot lesions in 5–7 days (Fig. 3). As expected, similar lesions were observed after inoculation with two of the pathogenic syringomycin mutants (KW304 and KW309) as controls. Although fewer lesions were produced by mutant KW304 in the experiment shown in Figure 3, this result represents normal variation in this type of qualitative assay. Nonpathogenic mutants KW21 (data not shown), NPS3136, and KW301 (Fig. 3) did not form brown spot lesions under the assay conditions. KW21 and KW301 were also found to be affected in motility in agar media, although it is not known if this reduced motility is the cause of the nonpathogenic phenotype.

Characterization of mutants affected in protease production.

Prototrophic Tn*5* mutants of *P. s.* pv. *syringae* isolate B728a were screened for attenuation of their ability to produce extracellular protease detectable by the skim milk plate assay. Five mutants were identified that were reduced or deficient in the size of the clearing zones produced on NYGM plates (Fig. 4). All of these protease mutants produced wild-type levels of syringomycin by bioassay and a HR when inoculated into a nonhost plant such as tobacco (data not shown). Southern blot analysis indicated that each protease mutant contained a single copy of the transposon Tn*5* and that this insertion had not occurred in the *lemA* region. Each protease mutant was assayed for lesion formation on bean pods and all of the protease mutants were fully pathogenic in the bean pod assay (data not shown). The pathogenic phenotype of two of the protease mutants, KW336 and KW338, was confirmed with the leaf lesion assay (data not shown). Growth of B728a, the *lemA* mutant NPS3136, and the protease mutants was monitored in NYGM broth. With one exception, all strains grew at comparable rates. KW339 divided very slowly in NYGM broth and after 2 days of growth had not yet reached an optical density or population density similar to that of wild-type strain B728a. In addition, in some protease assays, KW339 produced a clearing zone almost as large as the wild-type strain B728a (Fig. 4). Because of its pleiotropic phenotype, mutant KW339 was not examined further.

Proteolytic activity contained in concentrated extracellular culture supernatants was determined for the wild-type isolate B728a, the *lemA* mutant NPS3136, and the four remaining protease mutants. Initially, samples were tested by the skim milk plate assay (Table 2). Zones of clearing on NYGM medium produced by the concentrated supernatants were relatively proportional to the size of clear zones produced by the colonies on NYGM plates (Fig. 4). In an effort to quantitate protease activity, Azocoll colorimetric assays were performed on the samples. The Azocoll assay was also used to confirm that the proteolytic

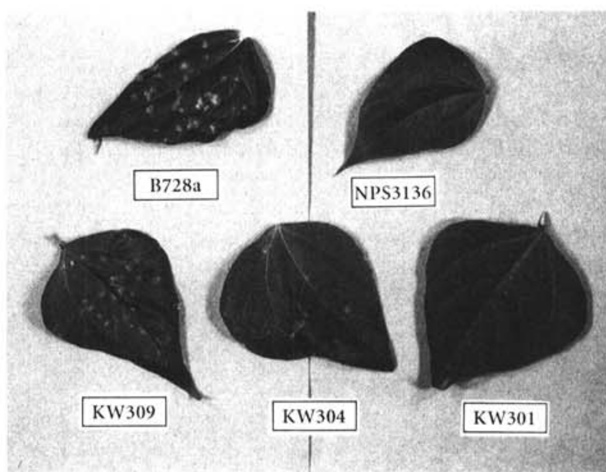

Fig. 3. Brown spot disease lesion formation on bean leaves. Bean plants were sprayed with bacterial suspensions and incubated under humid conditions. Typical necrotic lesions with chlorotic halos are visible on leaves from plants inoculated with the wild-type B728a and syringomycin mutants KW304 and KW309. The *lemA* mutant NPS3136 and the syringomycin mutant KW301 did not form lesions on bean leaves under these conditions.

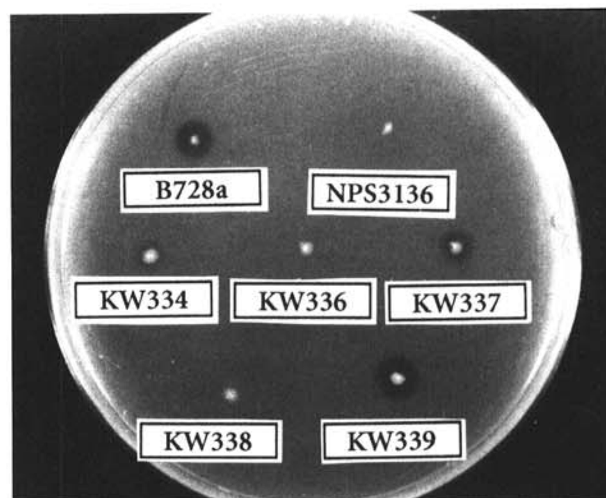

Fig. 4. Protease assay on NYGM medium. Cells of the wild-type isolate B728a, the *lemA* mutant NPS3136, and the five protease mutants were inoculated into NYGM plates and incubated overnight. Zones of clearing around colonies indicate proteolysis of milk proteins.

Table 2. Results of skim milk agar (NYGM) and Azocoll assays of protease mutants and other strains

Strain or mutant	Protease activity determined by NYGM agar[a] (mm)	Protease activity determined by Azocoll assay[b] (units)
B728a	5.8 (0.4)[c]	173.5 (76.4)
NPS3136[d]	0	0.7 (0.2)
KW334	1.9 (0.1)	2.6 (0.6)
KW336	1.5 (0.2)	2.9 (0.5)
KW337	3.7 (0.2)	20.0 (3.4)
KW338	0	1.5 (0.2)

[a] Radius of clear zone around well in NYGM plate containing 25 ml of protease sample. Plates were incubated overnight at 28° C. This reaction is not linear.

[b] One unit of protease activity is defined as: mg proteinase K equivalents/ml times a correction factor to standardize samples to similar cell numbers.

[c] Values are the mean followed by the standard error in parentheses.

[d] NPS3136 is a *lemA* mutant and is also syringomycin-negative. All other protease mutants are syringomycin-positive.

activity detected on NYGM plates was not an artifact due to increased solubility of proteins by lipids in the samples (Keen *et al.* 1969) or to some other factor. Results from Azocoll assays generally confirmed the results from skim milk plates except that samples from the *lemA* mutant NPS3136 and the protease mutant KW338, which did not produce any detectable clearing after overnight incubation on skim milk plates, contained a small amount of proteolytic activity detectable with the Azocoll assay (Table 2).

To further characterize the proteolytic activity, 50 mM $CaCl_2$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), or 10 mM EDTA were added to enzyme samples from wild-type cells and preincubated for 15 min before assaying for protease by the Azocoll assay. The results (data not shown) indicated that calcium either stabilized or stimulated enzyme activity, while EDTA almost completely inhibited activity. Slight inhibition was observed with DTT or PMSF. These results indicated that the majority of the collagen-degrading proteolytic activity excreted by wild-type cells is probably a metalloprotease (Barrett 1986). Proteolysis on skim milk plates could be detected after boiling a sample for 5 min, but not after 15 min. The protease was stable upon storage at 4° C for at least 9 mo.

DISCUSSION

Sequence analysis strongly suggests that the predicted LemA protein is a member of a family of transmembrane bacterial regulatory proteins (Hrabak and Willis 1992). Other members of this family, such as the VirA/VirG proteins from *Agrobacterium tumefaciens* and the BvgS/BvgA proteins from *Bordetella pertussis* (Arico *et al.* 1989; Stachel and Zambryski 1986; Stibitz and Yang 1991), are known to be involved in regulation of virulence factors. Consistent with its proposed role as a genetic regulator, the *lemA* gene is required by *P. s.* pv. *syringae* for the expression of several diverse phenotypes. The *lemA* gene of *P. s.* pv. *syringae* was previously shown to be required for formation of brown spot disease lesions on bean (Willis *et al.* 1990). Results presented here demonstrate that the *lemA* gene is also required for production of two extracellular products, protease and syringomycin, but not for motility or production of bacteriocin or pyoverdin. The fact that the *lemA* mutation did not affect motility is noteworthy, since nonmotile mutants of *P. s.* pv. *phaseolicola* and *P. s.* pv. *glycinea* are reduced in lesion formation (Hattermann and Ries 1989; Panopoulos and Schroth 1974).

In this study, we have investigated whether the syringomycin- and protease-deficient phenotypes of a *lemA* mutant of *P. s.* pv. *syringae* were related to the nonpathogenic phenotype of this mutant on bean. Additional syringomycin and protease mutants were isolated, some of which produced no detectable syringomycin or protease, respectively, by bioassay but were as pathogenic as the original wild-type strain on bean pods and leaves under laboratory assay conditions. This result suggested that neither syringomycin nor protease production were required for disease lesion formation by this pathogen, but we can not rule out the possibility that these compounds might be produced by these strains on bean plants. There is no method currently available for *in situ* detection of syringomycin or protease. However, some of the pathogenic syringomycin mutants had deletions in the *syrBCD* genes that are thought to be involved in syringomycin biosynthesis. In other bacteria where deletion of toxin biosynthetic genes has been described, antibiosis was never observed after deletion of some or all of these genes (Cundliffe 1989; Kinscherf *et al.* 1991; Mitchell 1991; Peet *et al.* 1986). Thus, the deletion mutants that we have identified would not be expected to produce syringomycin under any conditions.

The role of syringomycin in disease development has been investigated for *P. s.* pv. *syringae* isolates pathogenic either on stone fruits or on grasses. Whether syringomycin production *in vitro* and virulence were correlated varied depending upon the specific pathogenic isolate and plant host examined (Backman and DeVay 1971). For example, all Tn*5*-induced syringomycin mutants of an isolate of *P. s.* pv. *syringae* pathogenic on stone fruit were affected in virulence to some extent (Xu and Gross 1988a). On the other hand, natural pathogenic isolates from bean that do not produce syringomycin *in vitro* have been isolated (J. J. Rich, D. K. Willis, and S. S. Hirano, unpublished data). If *in vitro* syringomycin production reflects syringomycin production in the plant host, then it appears that syringomycin production by *P. s.* pv. *syringae* is necessary for disease development on some hosts, but not on others. The possibility remains that syringomycin production does contribute to the development of brown spot disease of bean, but to such a small extent that the loss of this function does not cause any observable decrease in pathogenicity. In addition, since the toxin is biocidal against many bacteria and fungi (DeVay *et al.* 1968; DeVay *et al.* 1978), syringomycin may be important for competition and epiphytic survival of the bacterium, rather than disease development. The competitive ability of syringomycin-producers compared to nonproducers on host or nonhost plants has not been studied.

In conclusion, it is likely that the *lemA* gene product is a key member of a regulatory network and that one or more genes involved in lesion formation are members of the *lemA* regulon. This work has shown that genes involved in synthesis of protease and syringomycin are also members of the *lemA* regulon. Although syringomycin and protease do not appear to play an important part in lesion formation on bean, these compounds might still have an effect on the ability of the bacteria to cause disease under field conditions. Thus, the genes within the *lemA* regulon that are directly involved in lesion formation on bean remain to be identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

P. s. pv. *syringae* strains and all plasmids used in this work are listed in Table 3. *P. s.* pv. *syringae* strains were routinely grown in King's medium B (KB) (King *et al.* 1954) at room temperature with aeration. For growth on solid medium, 1.3% agar was added and plates were incubated at 28° C. The *P. aeruginosa* strain PAO11 was cultured in Luria-Bertani (LB) broth (Sambrook *et al.* 1989)

at 37° C with aeration. Antibiotic concentrations used routinely in media were (per milliliter): tetracycline (Tet), 15 μg; kanamycin (Kan), 15 μg; rifampicin (Rif), 100 μg; ampicillin (Amp), 50 μg; spectinomycin (Spc), 100 μg; carbenicillin (Crb), 500 μg; and nalidixic acid (Nal), 200 μg. The minimal medium used to determine prototrophy was M9 (Sambrook *et al.* 1989) containing 0.8 mM $MgSO_4$-$7H_2O$ and 0.4% glucose. For motility assays, bacteria were stabbed into either LB plates solidified with 0.3% agar (Pirhonen *et al.* 1991) or 1% tryptone medium solidified with 0.4% agar (Panopoulos and Schroth 1974).

General techniques.

Restriction enzyme digestion, agarose gel electrophoresis, and Southern blot analysis were performed essentially as described (Sambrook *et al.* 1989). Random priming of linear DNA fragments was conducted according to manufacturer's directions (Promega). Isolation of chromosomal DNA (Kinscherf *et al.* 1991), triparental matings (Willis *et al.* 1988), hypersensitivity assays on tobacco (*Nicotiana tabacum* L. 'Havana 142') (Willis *et al.* 1990), and bacterial complementation assays (Hrabak and Willis 1992) were performed as previously described. For lesion assays on bean pods (*P. vulgaris* 'Bush Blue Lake 274'), stationary-phase bacterial cultures were washed with water, adjusted to $\sim10^8$ cells/ml, and 10-fold dilutions of this inoculum were injected beneath the epidermis of young bean pods (Willis *et al.* 1988). Assays for lesion formation in bean leaflets were performed by spray inoculation of plants with ~10 ml of bacterial suspension (10^6 cells/ml), followed by incubation of plants under conditions conducive to lesion formation as previously described (Willis *et al.* 1988). Pyoverdin production was detected by inoculating bacteria with a sterile toothpick into KB agar plates, a low iron medium, followed by incubation at 28° C for 3–5 days. A greenish-yellow zone was visible around colonies producing the siderophore. Pigment production was also confirmed by its fluorescence under ultraviolet light (Cody and Gross 1987). Bacteriocin production was detected with established indicator strains (kindly provided by A. Vidaver) as previously described (Vidaver *et al.* 1972).

Transposon mutagenesis.

Mutagenesis of *P. s.* pv. *syringae* isolate B728a with Tn*5* was accomplished with the transposon-containing plasmid pMO75. Donor *P. aeruginosa* PAO11 cells carrying pMO75 were incubated overnight with the recipient *P. s.* pv. *syringae* B728a on KB agar medium at 28° C. The next day the cells were resuspended, serially diluted, and spread on KB plates containing 200 μg/ml Rif and 20 μg/ml Kan. Mutants resistant to Rif and Kan were screened for sensitivity to Crb and Nal. Growth on media containing Crb indicated the presence of some or all of the suicide plasmid rather than transposition of Tn*5*. Growth on media containing Nal indicated the presence of the parent strain *P. aeruginosa* PAO11 (Whitta *et al.* 1985). Colonies were also replicated to minimal medium to check for auxotrophy. Colonies that grew on either Crb or Nal, or failed to grow on minimal medium, were discarded. Additional mutants were provided by J. J. Rich (Table 3).

Syringomycin assay.

Bacteria were transferred with a sterile toothpick into plates containing either a defined toxin production medium (SRM) (Gross 1985) or potato-dextrose agar (PDA) (Tuite 1969) and incubated at 28° C for 4 days. Toxin production was bioassayed by spraying the plates with a suspension of the toxin-sensitive yeast *Rhodotorula pilimanae* ATCC 26423 (provided by N. Iacobellis). After 1–2 days further incubation to allow for growth of *R. pilimanae,* a zone of growth inhibition of the indicator yeast around a bacterial colony was considered indicative of syringomycin production. Due to the qualitative nature of bioassays, positive (strain B728a) and negative (strain NPS3136) controls were included on each plate. A mutant reduced in syringomycin production had an inhibition zone noticeably smaller than the wild type. All strains were bioassayed at least twice.

Protease assays.

For detection of protease production from bacterial colonies on plates, cells were transferred with a sterile toothpick into plates of NYG (Daniels *et al.* 1984) supplemented with 10% skim milk (NYGM). After incubation overnight at 28° C, a clear halo around a bacterial colony was interpreted to be the result of proteolysis of proteins in the medium. Casein is generally considered to be an excellent substrate for detection of a wide variety of proteases (Fairbairn 1989).

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
P.s. pv. *syringae*		
B728a	Rif^R, causal agent of brown spot of bean	S. Hirano, Univ. of Wisconsin-Madison
BUVS1	Rif^R, Spc^R, Δ(*recA*)*51*::Ω	Hrabak and Willis 1992
NPS3136	Rif^R, Kan^R, *lemA1*::Tn*5*	Willis *et al.* 1990
NUVS1	Rif^R, Kan^R, Spc^R, Δ(*recA*)*51*::Ω, *lemA1*::Tn*5*	Hrabak and Willis 1992
KW21, KW301-302	Rif^R, Kan^R, syringomycin mutants	This work
KW304, 306, 309, 311, 314, 318, 320-323	Rif^R, Kan^R, syringomycin mutants	J. J. Rich and D. K. Willis
KW324-332	Rif^R, Kan^R, syringomycin mutants	This work
KW334	Rif^R, Kan^R, protease mutant	J. J. Rich and D. K. Willis
KW336-339	Rif^R, Kan^R, protease mutants	This work
pMO75	Kan^R, Crb^R, R91::Tn*5*	Whitta *et al.* 1985
pEMH97	Tet^R, *lemA* gene and flanking DNA	Hrabak and Willis 1992
pKW351	Tet^R, *lemA* gene and flanking DNA	Willis *et al.* 1990
pYM101	Amp^R, *syrBCD* genes cloned in pUC18	D. Gross, Univ. of Washington

For detection of extracellular proteases, cells were grown with aeration in 25 ml of NYG containing 1% skim milk for 2 days at ambient temperature. Cells were pelleted at 12,000 × *g* for 5 min at 4° C, and the supernatant was concentrated fivefold at 4° C by ultrafiltration through a YM5 membrane (Amicon), filter sterilized, and stored at 4° C. Proteolytic activity in the extracellular samples was detected by two methods. First, wells were made in NYGM plates using a 3-mm cork borer and 25 ml of sample to be tested was added. Plates were incubated upright at 28° C overnight and examined for a zone of clearing around the well. Less than 20 ng of proteinase K was detectable with the skim milk plate assay. Second, an aliquot to be tested for protease activity was adjusted to 3 ml with 10 mM sodium phosphate buffer (pH 7.0), mixed with 30 mg 100–250 mesh Azocoll (Calbiochem) and incubated at 37° C for 15 min. Azocoll is a general substrate for detecting proteolytic activity consisting of insoluble, powdered cowhide (collagen) covalently linked at many sites to a red dye. Undigested substrate was removed by centrifugation at 12,000 × *g* for 5 min and the absorbance at 520 nm of the supernatant was recorded. Proteinase K was used for standardization. About 100 ng of proteinase K was detectable with the Azocoll assays. One unit of protease activity was defined as micrograms of proteinase K equivalents per milliliter times a correction factor to adjust all samples to equal cell numbers. The correction factor was: 10^{11} cells per milliliter divided by the actual number of cfu/ml in the culture at the time of harvest.

For some experiments on the regulation of protease production, bacteria were grown at room temperature with aeration in 25 ml of one of the following media: KB, one-tenth strength KB, NYG, NYG + 1% skim milk, 1% skim milk + 2% glycerol, or 1% skim milk + 2% glycerol + 0.1% yeast extract. After 2 days of growth, aliquots were removed and filter sterilized, and 5 μl was spotted on a NYGM plate. Plates were incubated overnight at 28° C and then scored for the presence of zones of clearing.

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