Lipopeptide Phytotoxins Produced by *Pseudomonas syringae* pv. *syringae*: Comparison of the Biosurfactant and Ion Channel-Forming Activities of Syringopeptin and Syringomycin

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The phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* produces two classes of necrosis-inducing lipodepsipeptide toxins commonly referred to as the syringomycins and syringopeptins. Members of the syringomycin class are pore-forming cytotoxins that act by promoting passive transmembrane ion flux. In this study, we test the hypothesis that syringopeptin forms SP22A and SP22B likewise function as pore-forming cytotoxins and are similar in activity to syringomycin in artificial and plant membranes. Correspondingly, syringopeptin increased the conductance of black-lipid membranes in a manner indicative of ion channel formation. In tobacco protoplast assays, syringopeptin forms SP22A and SP22B were equivalent in activity causing lysis of protoplasts and measurable $^{45}\text{Ca}^{2+}$ influx at a threshold concentration of 50 ng/ml. A mixture of three forms of syringomycin did not show cytotoxic activity appreciably different from that of SP22A or SP22B in tobacco protoplast assays. Both forms of syringopeptin also displayed potent biosurfactant properties demonstrated by lowering of the interfacial tension of high-pressure liquid chromatography-grade water to 36 and 34.5 nm/m, respectively; the critical micellar concentration was 0.8 mg/ml for both forms of toxin. These results demonstrate that both classes of pore-forming lipodepsipeptides secreted by *P. syringae* pv. *syringae* are cytotoxic to plant cells at nanomolar concentrations and cause necrosis by forming ion channels that are freely permeable to divalent cations.

A distinctive characteristic of *Pseudomonas syringae* pv. *syringae* is the ability to cause necrotic infections of a broad range of monocot and dicot species (Bradbury 1986; Quigley and Gross 1994). The formation of necrotic lesions is associated with the production of two classes of lipodepsipeptide phytotoxins generically called syringomycins and syringopeptins (Ballio et al. 1991; Iacobellis et al. 1992). Both are composed of

a hydrophobic 3-hydroxy carboxylic acid tail of variable length and a cyclized peptide head that is charged (Fig. 1) (Ballio et al. 1995; Fukuchi et al. 1992; Segre et al. 1989; Vaillo et al. 1992). Members of the syringomycin class are lipodepsinona-peptides that exhibit potent antifungal activity toward *Geotrichum candidum* (Quigley and Gross 1994). In contrast, the syringopeptins contain a larger peptide moiety with 22 or 25 amino acid residues, depending on the bacterial strain, and do not inhibit *G. candidum* (Grgurina et al. 1996). For example, strain B301D of *P. syringae* pv. *syringae* produces syringopeptin forms SP22A and SP22B in addition to three forms of syringomycin (SRE, SRG, and SRA₁) with phytotoxic activity (Fig. 1) (Ballio et al. 1991; Iacobellis et al. 1992).

The activity of the lipodepsipeptide toxins of *P. syringae* pv. *syringae* is primarily targeted toward the plasma membrane of host cells (Di Giorgio et al. 1994). We recently demonstrated that syringomycin forms transmembrane pores in membrane bilayers (Hutchison et al. 1995). Syringomycin lysed erythro-

Fig. 1. Structure of syringomycin form SRE (**A**) and syringopeptin form SP22B (**B**) produced by *Pseudomonas syringae* pv. *syringae* strain B301D. The N termini of the various forms of syringopeptin and syringomycin are acylated by 3-hydroxydecanoate (SP22A, SRA₁), 3-hydroxydodecanoate (SP22B, SRE), and 3-hydroxytetradecanoate (SRG). Abbreviations: Dab = 1,4-diaminobutyric acid; Dhb = 2,3-dehydroaminobutyric acid; all other amino acids are identified by standard three-letter biochemical notation.

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cytes at a threshold concentration of 500 ng/ml, which was considerably below the critical micelle concentration (CMC) of 1.25 mg/ml at which nonspecific biosurfactant activity is expressed. In artificial planar lipid bilayers, syringomycin form SRE caused rapid increases in the conductance that fluctuated upon the opening and closing of ion channels. It was proposed (Hutchison et al. 1995) that pore formation promotes passive transmembrane ion flux that was primarily responsible for the phytotoxic activity of syringomycin. Small quantities of syringomycin cause an increase in transmembrane K^+ , H^+ , and Ca^{2+} fluxes that ultimately are lethal to cells (Bidwai and Takemoto 1987; Mott and Takemoto 1989; Takemoto 1992). A rapid and sustained cytoplasmic influx of Ca^{2+} has been documented for syringomycin-treated suspension cells of periwinkle (*Catharanthus roseus* (L.) G. Don) (Kauss et al. 1991) and red beet (*Beta vulgaris* L.) storage tissues (Takemoto et al. 1989). A massive influx of Ca^{2+} leads to activation of a cascade of events associated with cellular signaling, including induction of kinase-mediated phosphorylation of membrane proteins (Bidwai and Takemoto 1987) and incorporation of callose into plant cell walls (Kauss et al. 1991). Thus, syringomycin resembles pore-forming bacterial toxins that promote massive ion fluxes and are cytotoxic in mammalian systems (Bhakdi and Trnnum-Jensen 1987).

The discovery that *P. syringae* pv. *syringae* produces a member of the syringopeptin class of lipodepsipeptide phytotoxins (Ballio et al. 1991) suggests that toxigenesis may have a larger role in the plant-pathogen interaction than indicated from earlier studies of syringomycin. For example, syringomycin mutants resulting from inactivation of either the *syrB* or *syrC* genes involved in syringomycin biosynthesis (Zhang et al. 1995) exhibit about a 35% reduction in virulence (Quigley et al. 1993). Because abundant quantities of syringopeptin are produced by syringomycin biosynthesis mutants (Grgurina et al. 1996), the remaining virulence of the *P. syringae* pv. *syringae* mutants may be largely due to production of syringopeptin. Like syringomycin, syringopeptin causes electrolyte leakage in plant cells that leads to the development of necrotic symptoms in affected tissues (Iacobellis et al. 1992). Both toxins are similar in altering the distribution of H^+ ions across the maize plasma membrane (Di Giorgio et al. 1994). Di Giorgio et al. (1996) recently demonstrated that syringopeptin promotes stomatal closure in detached leaves of *Xanthium strumarium* L. and in epidermal strips of *Vicia faba* L., presumably as a result of a rapid K^+ efflux, as originally described for syringomycin (Mott and Takemoto 1989). However, syringopeptin was reported to be more potent than syringomycin in comparative analyses of phytotoxic activities (Di Giorgio et al. 1994, 1996; Iacobellis et al. 1992), thereby implying that syringopeptin is more important in plant pathogenesis. Nevertheless, the striking structural similarities of syringopeptin to syringomycin (Ballio et al. 1995) and other pore-forming toxins (Brodey et al. 1991; Hutchison and Johnstone 1993; Neu et al. 1990) suggest that syringopeptin likewise forms pores in plant plasma membranes and that this leads to transmembrane ion flux and cell death.

In this study, we establish that syringopeptin is a pore-forming lipodepsipeptide similar in activity to syringomycin in artificial and plant membranes. Tobacco protoplasts, which are highly sensitive to both lipodepsipeptide toxins, were used to evaluate the activities of the toxins in assays of protoplast

lysis and $^{45}Ca^{2+}$ uptake. Evidence is presented that low concentrations of syringopeptin generate a massive influx of $^{45}Ca^{2+}$ in tobacco protoplasts indicative of the formation of ion channels freely permeable to cations. In addition, the capacity of syringopeptin to lower the interfacial surface tension of water was evaluated as a measure of the biosurfactant activity of the amphipathic syringopeptin molecule. Our previous model (Hutchison et al. 1995) describing how syringomycin functions in the plant-pathogen interaction is expanded to include the parallel pore-forming activity of syringopeptin.

RESULTS

HPLC analysis of syringopeptin produced by strain B301D.

High-pressure liquid chromatography (HPLC) profiles of lipodepsipeptide toxins produced in potato dextrose broth (PDB) by strain B301D of *P. syringae* pv. *syringae* showed the presence of both syringopeptin and syringomycin at about a 1:1.4 ratio (results not shown). Syringopeptin forms SP22A and SP22B, which differ in the length of the 3-hydroxy fatty acid (Fig. 1), were produced, with SP22B being the most abundant form; SP22B composed over 65% of the syringopeptin mixture. Peaks corresponding to syringomycin activity were resolved as a mixture of SRA₁, SRE, and SRG at the same proportions reported by Hutchison et al. (1995). Typical yields of HPLC-purified syringopeptin were approximately 5 mg per liter of culture broth. The specific activities of SP22A and SP22B were both determined to be 16.6 units per μ g. Toxin preparations showed >99% purity based on reanalysis of purified peaks by HPLC conducted with a photodiode array detector. The specific activity of the syringomycin mixture was 26 units per μ g. A syringomycin mixture was used in assays because it was shown (Hutchison et al. 1995) to be comparable in biosurfactant and pore-forming activities to a pure preparation of SRE, the major form of syringomycin produced by strain B301D.

The surface-active properties of syringopeptin forms SP223A and SP22B.

Analysis of HPLC-purified syringopeptin for surface active properties by the drop weight method (Harkins and Brown 1919) demonstrated that the lipodepsipeptide toxin is a potent biosurfactant. The SP22A and SP22B forms of syringopeptin lowered the interfacial tension of HPLC-grade water, which was measured as a surface tension of 73.4 mN/m, to minimum values of 36 and 34.5 mN/m, respectively (Fig. 2). Furthermore, measurable lowering of the interfacial tension occurred at relatively low SP22A and SP22B concentrations of $\geq 10 \mu$ g/ml. The CMC, determined from a graph of log concentration versus interfacial tension, was 0.82 mg/ml for SP22A and 0.8 mg/ml for SP22B. Correspondingly, the surface tension at the CMC (γ_{CMC}) was 40.2 mN/m for SP22A and 40.7 mN/m for SP22B. The surface activity properties of syringopeptin are similar to those of syringomycin form SRE (CMC, 1.25 mg/ml; γ_{CMC} , 33 mN/m) (Hutchison et al. 1995).

Syringopeptin lyses erythrocytes and tobacco protoplasts below the CMC.

Strong hemolytic activity was observed for syringopeptin forms SP22A and SP22B, beginning at a threshold concentra-

tion of 1 $\mu\text{g/ml}$ (Fig. 3), which is slightly higher than the threshold concentration of 0.75 $\mu\text{g/ml}$ reported for SRE (Hutchison et al. 1995). A linear increase in the rate of blood cell lysis was observed at SP22A and SP22B concentrations up to about 8 $\mu\text{g/ml}$ (Fig. 3), at which point most cells lysed immediately upon addition of toxin. Although both forms of syringopeptin at a concentration of 8 $\mu\text{g/ml}$ were only 85% as effective as syringomycin (Hutchison et al. 1995), the standard errors of the rates of lysis overlapped. The rate of blood cell lysis in the presence of 5 μg of syringopeptin per ml was not affected by addition of the cations Ca^{2+} , Cs^{2+} , and Mg^{2+} at concentrations ranging up to 15 mM.

Tobacco protoplasts were highly sensitive to the lipodepsipeptide toxins (Fig. 4). At toxin concentrations as low as 100 ng/ml, roughly half of the protoplasts were lysed after a 30-min incubation, compared with protoplasts not exposed to toxin. For example, SP22A lysed about 2×10^5 protoplasts after 30 min at a concentration of 200 ng/ml (Fig. 4). Detectable lysis of tobacco protoplasts was observed at threshold toxin concentrations that were about 10-fold lower than those observed for erythrocytes (Fig. 3). Syringopeptin forms SP22A and SP22B, and a mixture of all three forms of syringomycin, were equivalent in lysing tobacco protoplasts over a range of toxin concentrations up to 1 $\mu\text{g/ml}$. At toxin concentrations of 1 $\mu\text{g/ml}$, about 30% of the protoplasts remained unlysed. A toxin concentration of 500 ng/ml during overnight incubation lysed all of the protoplasts.

Syringopeptin forms pores in planar lipid bilayers and generates an influx of $^{45}\text{Ca}^{2+}$ in tobacco protoplasts.

Syringopeptin form SP22B at a concentration of 5 $\mu\text{g/ml}$ was added to a microtiter well that also contained an artificial bilayer stretched across the end of a 1- μm -diameter patch pipette. Typically, the phosphatidylethanolamine bilayer used

had a resistance of around 10 G Ω . A holding potential of +80 mV (relative to *cis*) was applied across the membrane. Fluctuations in conductance were observed upon application of the holding potential. Addition of the toxin to the *trans* compartment increased the conductance of the bilayer, and activity characteristic of an ion channel was observed (Fig. 5). Current fluctuations were of uniform size, indicating that the effect on conductance was not caused by membrane deterioration.

Because not all tobacco protoplasts were lysed within 30 min after exposure to the phytotoxins (Fig. 4), it was possible to measure movement of ions such as Ca^{2+} across the plant plasma membrane. A rapid and sustained cytoplasmic increase in $^{45}\text{Ca}^{2+}$ was observed for protoplasts in the presence of syringopeptin, syringomycin, and mixtures of the two toxins (Fig. 6). The uptake of Ca^{2+} was measurable at toxin concentrations as low as 50 ng/ml. Syringopeptin and syringomycin were equally effective in promoting $^{45}\text{Ca}^{2+}$ uptake; accordingly, a linear relationship was observed between the rate of $^{45}\text{Ca}^{2+}$ accumulation and toxin concentrations between 50 and 800 ng/ml for SP22A, SP22B, and syringomycin. At a toxin concentration of 500 ng/ml, for example, the rate of $^{45}\text{Ca}^{2+}$ uptake was between 0.6 and 0.8 nCi $^{45}\text{Ca}^{2+}$ per min per ml. No synergistic effect on activity was observed for a 1:1 mixture of syringopeptin and syringomycin, which was as effective as individual forms of the toxins in promoting $^{45}\text{Ca}^{2+}$ uptake.

DISCUSSION

A characteristic trait of *P. syringae* pv. *syringae* is the production of two classes of lipodepsipeptide phytotoxins, syringomycin and syringopeptin, that cause cell necrosis by disrupting the integrity of the host plasma membrane. It was recently established that syringomycin acts primarily by forming transmembrane pores that are freely permeable to cations (Hutchison et al. 1995). We now extend the pore for-

Fig. 2. Effect of concentration of high-pressure liquid chromatography-purified syringopeptin SP22A (open circle) and SP22B (open triangle) on the interfacial tension of water as determined by the drop weight method. Error bars represent the standard error of 10 replicates.

Fig. 3. Effect of concentration of high-pressure liquid chromatography-purified syringopeptin SP22A (open circle) and SP22B (open triangle) on the rate of lysis of heparinated horse erythrocytes. Error bars indicate the $\pm\text{SD}$ of two trials, six replicates per trial.

mation model to syringopeptin based on its ability (i) to increase the conductance of artificial planar lipid bilayers, (ii) to lyse both tobacco protoplasts and erythrocytes, and (iii) to generate a rapid and sustained cytoplasmic increase of $^{45}\text{Ca}^{2+}$ in tobacco protoplasts. Syringopeptin rapidly lyses tobacco protoplasts at nanomolar concentrations, which substantiates its pore-forming activity in lipid bilayers.

Tobacco protoplasts were highly sensitive to the toxic effects of syringopeptin and syringomycin, permitting direct comparisons of the phytotoxicity of the two lipodepsipeptides. Syringopeptin forms SP22A and SP22B, which differ in the length of the hydrophobic 3-hydroxy fatty acid tail (Ballio et al. 1991), displayed equivalent activity in tobacco protoplast assays, causing lysis and measurable $^{45}\text{Ca}^{2+}$ influx at a threshold concentration of 50 ng/ml. The similarity of syringopeptin to syringomycin in apparent pore-forming activity was strik-

ing in tobacco protoplast assays. These results differ from those of Iacobellis et al. (1992), who reported that syringopeptin was several times more active than syringomycin in causing necrosis in tobacco leaves and in causing electrolyte leakage of carrot disks. However, the assays used to compare phytotoxicity did not appear to be highly sensitive because neither toxin showed activity below a concentration of 0.4 μM ($\approx 0.5 \mu\text{g/ml}$). In the study by Di Giorgio et al. (1996), stomatal conductance (g_s) in *X. strumarium* leaves was affected at threshold concentrations of 10 μM ($\approx 21 \mu\text{g/ml}$) for syringopeptin form SP22A and 200 μM ($\approx 245 \mu\text{g/ml}$) for syringomycin form SRE. These concentrations are more than 1,000-fold higher than the minimum toxin concentrations required for measurable activity in assays of tobacco protoplasts (Figs. 5 and 6). Experiments by Di Giorgio et al. (1996) were conducted by immersing the petioles of whole leaves in solutions of toxin. Consequently, stomates were not directly exposed to the toxin solution, and the results reflect toxin transport through the vascular system of leaves to stomatal guard cells. Because of dilution effects resulting from leaf transport, the local threshold toxin concentrations affecting stomatal conductance are expected to be significantly lower than the values reported by Di Giorgio et al. (1996). In contrast, Mott and Takemoto (1989) observed reductions in stomatal apertures in *V. faba* after direct exposure of isolated epidermes to a threshold syringomycin concentration of only 120 ng/ml (effective

Fig. 4. Effect of the concentration of lipodepsipeptide phytotoxins produced by *Pseudomonas syringae* pv. *syringae* on lysis of tobacco protoplasts. Protoplasts ($\approx 3.5 \times 10^5$) were incubated for 30 min with syringopeptin SP22A (open circle), syringopeptin SP22B (open triangle), and syringomycin (a mixture of forms SRA₁, SRE, and SRG) (open square). The number of unlysed protoplasts was counted directly by light microscopy with a hemacytometer. Error bars indicate the $\pm$ SD of two separate trials, three replicates per trial.

Fig. 5. Ion channel formation by syringopeptin form SP22B in an artificial lipid bilayer. Bilayers were formed across the end of a patch pipette that separated solutions of 30 mM KCl (*cis*) and 10 mM KCl (*trans*). Addition of the phytotoxin (approximately 5 $\mu\text{g/ml}$) was to the *trans* side of the bilayer. Current leakage was recorded *trans* with respect to *cis*, and both closed (C) and open (O) states of ion channels were recorded. Horizontal bar corresponds to a 5-s interval; vertical bar corresponds to 5×10^{-12} Amps.

Fig. 6. Effect of syringopeptin and syringomycin concentrations on the rate of cytoplasmic uptake of $^{45}\text{Ca}^{2+}$ by tobacco protoplasts. Treatments were syringopeptin SP22A (open circle), syringopeptin SP22B (open triangle), syringomycin (a mixture of forms SRA₁, SRE, and SRG) (open square), and a 1:1 (wt/wt) mixture of high-pressure liquid chromatography-purified syringomycin (all three forms) and syringopeptin (SP22A and SP22B) (open hexagon). Results are corrected to compensate for solvent quenching. Error bars indicate the $\pm$ SD of two separate trials, three replicates per trial.

concentration as corrected by Di Giorgio et al. [1996]), a concentration that is near the minimum dose required for measurable Ca^{2+} influx in tobacco protoplasts. Correspondingly, Kauss et al. (1991) found syringomycin induced leakage of K^{+} and Ca^{2+} in suspension cells of *C. roseus* at concentrations as low as 200 ng/ml. Therefore, close inspection of comparative data on effective phytotoxic dosages indicates that both toxins are roughly equivalent in potency toward plant cells.

We predict that syringopeptin at nearly double the size of syringomycin would form functional pores composed of fewer monomers than syringomycin, and in this sense would be the more potent phytotoxin. But it also is important to consider the relative levels of the two toxins produced by a given strain of *P. syringae* pv. *syringae*. For example, strain B301D produces 1.4 to 5 times more syringomycin than syringopeptin, depending on the medium (Grgurina et al. 1996). Although the relative amounts produced in situ are unknown, higher levels of syringomycin may be produced during phytopathogenesis to effectively balance differences in phytotoxic activity of the two classes of lipodepsipeptides. Evidence that both toxins are produced in high quantities in vitro by highly virulent strains of *P. syringae* pv. *syringae* under the same set of environmental conditions (Grgurina et al. 1996; Quigley and Gross 1994; Vassilev et al. 1996) supports fundamentally important roles for both toxins in the plant-pathogen interaction.

Syringopeptin is capable of forming ion channels in simple artificial bilayers composed of 1-acyl-palmitoyl-2-acyl-oleoyl-phosphatidylethanolamine. Demonstration of pore-forming activity in a pure black-lipid membrane is significant because it shows that activity does not arise from syringopeptin-mediated opening of native ion channels found in membranes patched from cells. In addition, syringopeptin formed channels in artificial bilayers in the absence of membrane sterols. Sterols have been reported to induce formation of oligomeric toxin structures for certain cytotoxins (Duncan and Schlegel 1975; Maget-Dana and Peypoux 1994). Furthermore, Julmanop et al. (1993) and Taguchi et al. (1994) suggested that sterols, such as ergosterol, facilitated binding of syringomycin to cells. Although the presence of sterols may enhance formation of channels by syringopeptin and syringomycin in artificial membranes, sterols are not necessary for activity. In addition to our results with syringopeptin form SP22B in artificial membranes (Fig. 4), recent studies by Feigin et al. (1996) demonstrated that syringomycin form SRE at low concentrations formed ion channels in artificial lipid bilayers lacking sterol.

It was not surprising to find that syringopeptin displays hemolytic activity. As a lipopeptide with a net positive charge at low pH, syringopeptin resembles a wide range of lipopeptides from *Pseudomonas* that have been shown or are likely to form pores in erythrocyte membranes (Ballio et al. 1996; Hutchison and Johnstone 1993; Neu et al. 1990). Accordingly, syringopeptin displayed levels of hemolytic activity similar to those of syringomycin. Lysis also occurred well below the CMC of ≈ 0.8 mg/ml for syringopeptin forms SP22A and SP22B at which nonspecific biosurfactant activity is expressed. We predict that erythrocytes are lysed by a colloid osmotic mechanism (Knowles and Ellar 1987) triggered by a massive cytoplasmic influx of ions and, as a consequence, the osmotic movement of water. Nevertheless, nearly 10-fold higher concentrations of syringopeptin were required for de-

tectable lysis of erythrocytes, compared with those required with tobacco protoplasts. This difference in sensitivity is attributed to the sedimentation of erythrocytes lowering attenuation during assays at low syringopeptin concentrations rather than innate differences in the sensitivity of cells to the toxin. Direct counts of surviving tobacco protoplasts gave a more definitive estimate of toxin activity. Although the presence of divalent cations has been observed to reduce or eliminate the hemolytic activity of some lipopeptides (Rainey et al. 1991), the rate of lysis at a syringopeptin concentration of 5 $\mu\text{g/ml}$ was unaffected by Ca^{2+} , Cs^{2+} , or Mg^{2+} ; addition of these cations also did not cause an appreciable change in pH. Consequently, the pores formed by syringopeptin are predicted to be highly permeable to cations such as Ca^{2+} . This was demonstrated subsequently by a rapid influx of $^{45}\text{Ca}^{2+}$ into tobacco protoplasts at syringopeptin concentrations as low as 50 ng/ml (Fig. 6).

Analysis of the surface-active properties of syringopeptin forms SP22A and SP22B demonstrated that the lipopeptide toxin is a powerful surfactant capable of lowering the interfacial tension of water to approximately 35 mN/m. The biosurfactant activity of syringopeptin is comparable to that of several lipopeptides produced by fluorescent pseudomonads, including viscosin by *P. fluorescens* (Neu et al. 1990) and tolaasin by *P. tolaasii* (Hutchison and Johnstone 1993). Compared with the biosurfactant properties of syringomycin form SRE (Hutchison et al. 1995), syringopeptin has a lower CMC (SP22B, 0.8 mg/ml; SRE, 1.25 mg/ml) but a higher γ_{CMC} (SP22B, 40.7 mN/m; SRE, 33.0 mN/m). Plots of interfacial tension versus phytotoxin concentration showed significant differences in the curves for syringopeptin, compared with those for syringomycin. The gradient of the syringomycin curve is initially steeper and more linear than the corresponding plot for syringopeptin. This may signify a stable submicellar aggregate of syringopeptin monomers at low concentrations. Alternatively, the larger charged peptide head of syringopeptin may impede intermolecular associations at low concentrations of the toxin. Because the concentration of syringopeptin required for micelle formation is considerably higher than the concentration eliciting phytotoxic effects in tobacco protoplasts and pore formation in artificial bilayers, the role for biosurfactant activity of syringopeptin in the plant-pathogen interaction is uncertain. Nevertheless, it is important to recognize that syringopeptin at relatively low concentrations of 10 $\mu\text{g/ml}$ effectively lowers the surface tension of pure water (Fig. 2). Thus, both syringopeptin and syringomycin may facilitate spread of the bacterium on plant surfaces by reducing the contact angle of water. Such a role has been proposed for the surface-active properties of tolaasin in the spread of *P. tolaasii* on mushroom caps (Hutchison and Johnstone 1993).

In comparing the cytotoxic activities of syringopeptin to those of syringomycin, it is evident that the toxins have fundamentally similar functions in plant pathogenesis. The amphipathic lipopeptide structures of the toxins facilitate their insertion into lipid bilayers to form ion channels that allow passive transmembrane ion fluxes. Therefore, very similar modes of action centered around pore formation are proposed for the two toxins. As depicted in Figure 7, pores formed by either syringopeptin or syringomycin are composed of monomers that form aggregates in lipid bilayers. Feigin et al.

(1996) estimated that at least six monomers of syringomycin are needed to form an ion channel. Because syringopeptin has a larger charged head than syringomycin, we believe that either the pores formed by syringopeptin will be larger than those formed by syringomycin, or that fewer molecules of syringopeptin will be required for a functional pore. Although we did not see a synergistic interaction between syringomycin and syringopeptin in tobacco protoplast assays, it is foreseeable to have pores formed that are composed of a chimera of syringopeptin and syringomycin given the overall structural similarities of the lipopeptides. Once functional pores are formed, passive nonspecific ion fluxes would endanger cell viability. The pH of the cytoplasm would be lowered due to an influx of H^+ that collapses the pH gradient across the plasma membrane (Di Giorgio et al. 1994); this would be accompanied by an efflux of K^+ (Di Giorgio et al. 1996; Mott and Takemoto 1989). Furthermore, the formation of functional pores would lead to a rapid and sustained influx of Ca^{2+} that in turn activates a cascade of events associated with cellular signaling (Kauss 1990; Takemoto et al. 1989). The generation of a free Ca^{2+} signal in response to exposure to syringomycin is known to induce intracellular responses such as kinase-mediated phosphorylation of membrane proteins (Bidwai and Takemoto 1987) and incorporation of callose into plant cell walls (Kauss et al. 1991). The generation of a massive influx of $^{45}Ca^{2+}$ in tobacco protoplasts at nanomolar concentrations of syringopeptin would trigger the same series of cellular signaling events as similar concentrations of syringomycin. As a result, the bacterial pathogen is nourished by the systematic release from the cytoplasm of small cellular metabolites, such as simple sugars, amino acids, and inorganic ions.

The selective advantage conferred to *P. syringae* pv. *syringae* by producing two cytotoxic lipopeptides is less clear with evidence that they exhibit comparable phytotoxic activities centered around formation of transmembrane pores. Syringopeptin and syringomycin display different spectra of anti-

microbial activities (Ballio et al. 1996; Grgurina et al. 1996) and perhaps this is important to survival of the bacterium as an epiphyte. In recent studies (M. L. Hutchison and D. C. Gross, *unpublished*), a genetic approach was used to evaluate the relative contribution of syringopeptin and syringomycin to bacterial virulence. Mutants of strain B301D that fail to produce one of the lipopeptide toxins are reduced in virulence between 35 and 50%, while double mutants that produce neither toxin are substantially reduced in virulence (Gross et al., in press). The two phytotoxins were concluded to be the major virulence determinants released by *P. syringae* pv. *syringae* in the plant-pathogen interaction. In addition, a relatively large portion of the bacterial genome is dedicated to the synthesis of the two lipopeptide toxins based on mapping of the *syr* and *syp* gene clusters to a DNA region extending over 100 kb in size (Caponero et al., in press; Gross et al., in press). Thus, *P. syringae* pv. *syringae* expends considerable resources toward producing the pore-forming toxins. Future work will, it is hoped, elucidate the differential roles of syringopeptin and syringomycin in survival and growth of *P. syringae* pv. *syringae* in the plant environment.

MATERIALS AND METHODS

HPLC purification of syringopeptin and syringomycin.

Syringopeptin was isolated from cultures of the syringomycin nonproducing mutant strain BR334 (Quigley et al. 1993) grown in PDB under the same conditions normally used to produce syringomycin by wild-type strain B301D (Hutchison et al. 1995). After removal of cells by centrifugation ($5,000 \times g$, $4^\circ C$, 10 min), the supernatant was adjusted to pH 2.0 with 5 M acetic acid. An equal volume of acetone was added and the precipitate formed at $4^\circ C$ was removed by centrifugation as performed above. Following concentration of the toxin preparation by rotary flash evaporation, the toxin was partitioned into *n*-butanol and processed as described by Gross and DeVay (1977) to yield an aqueous extract suitable for preparative chromatography. Syringopeptin was partially purified by Dowex 1 (Sigma Chemical, St. Louis, MO) anionic exchange chromatography (Hutchison and Johnstone 1993). Pure syringopeptin was obtained after separation in a Waters HPLC system (Waters Corp., Milford, MA) equipped with a semi-preparative C-18 reversed-phase column (Altex Ultrasphere-ODS, 1×25 cm; Beckman Instruments, Fullerton, CA). A linear acetonitrile gradient of 20 to 80% (vol/vol) in 0.1% (vol/vol) trifluoroacetic acid was used to separate syringopeptin forms SP22A and SP22B; a flow rate of 2.5 ml per min was used during the course of a 30-min run. Syringomycin was extracted from cultures of strain B301D and purified as reported previously (Hutchison et al. 1995). Peaks observed at A_{214} were collected and tested for biological activity with *Bacillus megaterium* strain Km and *Geotrichum candidum* strain F-260 for detection of syringopeptin and syringomycin, respectively (Grgurina et al. 1996). A unit of syringopeptin activity is defined as the minimum amount of toxin contained within a 10- μ l droplet that completely inhibits growth of *B. megaterium* within the area of application. Units of syringomycin activity are defined similarly, with *G. candidum* as the bioassay test organism (Quigley and Gross 1994). All bioactive phytotoxin fractions were pooled before lyophilization and stored at $-20^\circ C$. Relative concentrations of the major

Fig. 7. Model depicting pores formed by the lipopeptide toxins of *Pseudomonas syringae* pv. *syringae* in a plant plasma membrane. Plant plasma membrane phospholipids are shown as a bilayer between the extracellular space and the cytoplasm. Amphipathic molecules of syringopeptin (large ovals, shaded) and syringomycin (small ovals, white) insert into the lipid bilayer to form pores that are freely permeable to cations. A rapid and sustained net influx of Ca^{2+} (indicated by arrows) activates events associated with cellular signaling.

forms of phytotoxin were calculated from peak area with the Waters 990 chromatogram analysis integration software module (Millipore, Bedford, MA).

Measurement of the surface activity of syringopeptin.

The surface activity of HPLC-purified syringopeptin forms SP22A and SP22B was determined by the drop-weight method of Harkins and Brown (1919). An "Agla" type syringe (Wellcome Research Laboratories, Beckenham, England) was used as described by Hutchison et al. (1995) to measure the surface activities of HPLC-grade water adjusted to a final syringopeptin concentration ranging from 1 μg to 5 mg per ml. Each data point represented the mean surface tension value determined from 10 replicate droplets. A plot of interfacial surface tension versus log [syringopeptin] was used to determine the CMC and γ_{CMC} (Sheppard and Mulligan 1987).

Erythrocyte assays for hemolytic activity.

The assay used to test syringopeptin and mixtures of syringopeptin and syringomycin for ion channel-forming activity was described by Hutchison et al. (1995). Assays were conducted with heparinated horse erythrocytes (0.2 ml) washed in PBS (10 mM sodium phosphate, 0.9% NaCl [pH 7.0]) prior to suspension in 100 ml of PBS. The concentration of blood cells (1 to 2 μl) was adjusted with PBS to an OD₆₀₀ between 0.09 and 0.1 in a final volume of 1 ml. Syringopeptin forms SP22A and SP22B were prepared as 1 mg/ml stock solutions. Toxin was added to blood cells to a final concentration of 0.5 to 8 $\mu\text{g}/\text{ml}$. After mixing for 1 to 2 s, the rate of lysis was monitored as ΔOD_{600} per minute by a spectrophotometer equipped with a photodiode array detector (model 8452A, Hewlett Packard, Palo Alto, CA). Assays for erythrocyte lysis were performed at 22°C. Each data point was the result of at least six individual replicates performed as two separate trials. Effects of divalent metal ions on hemolysis were determined as described previously (Hutchison et al. 1995).

Artificial bilayer conductance assay.

Artificial lipid bilayers were generated across the end of a patch pipette by double immersion in an aqueous solution of lipid. The lipid was prepared by addition of 5 μl of PO-phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL), which was dissolved in hexane, to 200 μl of a solution of KCl (10 mM) and CaCl_2 (500 μM). All solutions were filtered with a 0.2- μm nitrocellulose membrane (Millipore). The bilayer separated solutions of ≈ 200 μl (*cis*) of KCl (30 mM) internal to the patch pipette, and 300 μl (*trans*) of KCl (10 mM) contained in a well of a microtiter plate. Solutions were buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (4 mM) to pH 7.0 and contained CaCl_2 at a concentration of 500 μM . Experiments were performed at 25°C. Currents were monitored under voltage-clamp conditions with a Axopatch 200A integrating patch clamp (Axon Instruments, Foster City, CA) connected to the bilayer chambers by Ag/AgCl electrodes and 3 M KCl/1% agar salt bridges. Data were stored on video tape and displayed after low-pass filtering at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Darmstadt, Germany). Membrane potentials were recorded *trans* with respect to *cis*, the former being the side to which phytotoxin was added. Syringopeptin form SP22B was tested for activity in artificial bilayers at a concentration of approximately 5 $\mu\text{g}/\text{ml}$.

Preparation of tobacco protoplasts, and assays of toxins on protoplast lysis and cytoplasmic $^{45}\text{Ca}^{2+}$ accumulation.

The method of Beier and Bruening (1975) as modified by Wyatt and Wilkinson (1984) was used to prepare protoplasts from tobacco (*Nicotiana tabacum* cv. *xanthi*) leaves grown under standard greenhouse conditions. After an overnight incubation in the dark, tobacco leaves (≈ 15 cm in length) were brushed with Carborundum (640 mesh), and rinsed in water to remove most of the abrasive. Leaves were sectioned with a razor blade and then incubated for 4 h at 25°C with agitation (100 rpm; Psychrotherm, New Brunswick Sci. Co., Edison, NJ) in 0.6 M mannitol containing bovine serum albumin (0.2% [wt/vol]) (Sigma), pectin lyase (0.5% [wt/vol]) (Sigma) and cellulase (1% [wt/vol]) (Sigma). Protoplasts were filtered through Miracloth (Calbiochem-Novabiochem, La Jolla, CA), harvested by centrifugation (500 $\times g$, 2 min), and washed three times in 0.3 M NaCl or 0.2 M CaCl_2 . The quality of protoplast preparations was checked under a microscope.

Protoplasts were used at an OD₆₀₀ of 0.2 ($\approx 3 \times 10^5$ cells per ml) to test the effects of lipopeptide toxins on protoplast lysis and the rate of cytoplasmic influx of $^{45}\text{Ca}^{2+}$. An aliquot (500 μl) of the protoplast suspension in isotonic buffer (5 mM HEPES [pH 7.0], 0.2 M CaCl_2) was incubated with a range of phytotoxin concentrations added in a constant volume of 10 μl . Syringopeptin forms SP22A and SP22B were prepared as 1 mg/ml stock solutions; syringomycin was used as a mixture of all three forms (SRA₁, SRE, and SRG) prepared as a 1 mg/ml stock. The toxins were added to blood cells at final concentrations ranging from 0.01 to 1 $\mu\text{g}/\text{ml}$. The incubations were for 30 min on a rocking platform (Bellco Biotechnology, Vineland, NJ), and then lysis was measured by direct counts of intact protoplasts with a hemacytometer. In experiments measuring the rate of cytoplasmic influx of $^{45}\text{Ca}^{2+}$, 2 μCi of $^{45}\text{CaCl}_2$ (DuPont, Wilmington, DE) was added to the protoplast suspension prepared as described above. An aliquot (100 μl) of buffer (0.1 M CaCl_2 , 5 mM HEPES [pH 7.0], 0.3 M mannitol) was added to prevent further Ca^{2+} movement. Protoplasts were pelleted (15,000 $\times g$, 2 min) and washed twice in 0.2 M CaCl_2 . Radioactive $^{45}\text{Ca}^{2+}$ that had accumulated in the plant cell cytoplasm was quantified by suspending the protoplasts in 1 ml of deionized water, vortex mixing the suspension with 5 ml of ScintiVerse universal LS cocktail (Fisher Scientific, Pittsburgh, PA), and counting in a Minaxi Tri-Carb 4000 scintillation counter (Packard Instrument, Downers Grove, IL). Readings were converted to nCi with correction for solvent quenching.

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