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Role of Biosurfactant and Ion Channel-Forming Activities of Syringomycin in Transmembrane Ion Flux: A Model for the Mechanism of Action in the Plant-Pathogen Interaction

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Syringomycin is a necrosis-inducing lipopeptide toxin synthesized and secreted by the phytopathogen, *Pseudomonas syringae* pv. *syringae*. Although small quantities of syringomycin are known to activate a cascade of physiological events in plasma membranes, the mechanism of action of the phytotoxin has never been fully characterized. The objective of this study was to test the hypothesis that the primary mode of action of syringomycin is to form transmembrane pores that are permeable to cations. Accordingly, direct measurement of ion fluxes were performed using artificial bilayers. The hemolytic properties and surface activity of HPLC-purified syringomycin were quantified by use of an erythrocyte lysis assay and by the drop weight method. Assays were performed using syringomycin form SRE alone or a mixture containing all forms of the phytotoxin. At a threshold concentration of 500 ng/ml, syringomycin induced hemolysis by forming ion channels in membranes. Osmotic protection studies indicated a channel radius of between 0.6 and 1 nm. The ion channel-forming activity was insensitive and permeable to both monovalent and divalent cations, suggesting that syringomycin causes lysis of erythrocytes by colloid osmotic lysis. In addition, syringomycin, like other lipopeptide antibiotics, is a potent biosurfactant capable of lowering the interfacial tension of water to 31 mN/m. The critical micellar concentration of syringomycin was calculated to be 1.25 mg/ml and the γ_{CMC} was 33 mN/m. A model is presented depicting the mechanism of action of syringomycin in the plant-pathogen interaction. The model integrates known effects of the toxin on ion flux in plasma membranes with formation of ion channels and the consequential cascade of effects associated with cellular signalling.

Additional keywords: biosurfactant, colloid osmotic lysis, interfacial tension, lipodepsipeptide toxin.

Syringomycin is a potent necrosis-inducing phytotoxin produced by most strains of *Pseudomonas syringae* pv. *syringae*, a plant pathogen that causes necrotic lesions on a broad range of monocot and dicot species (Bradbury 1986; Quigley and Gross 1994). Based on quantitative evaluations of syringomycin-minus strains in pathogenicity tests, production of syringomycin is an important virulence determinant of *P. s.* pv. *syringae* (Mo and Gross 1991a; Quigley *et al.* 1993). It is common for highly virulent strains, such as B301D, to produce large quantities (i.e., 10 to 80 μg/ml) of syringomycin in vitro (Gross and DeVay 1977; Quigley and Gross 1994). In the plant environment, specific plant metabolites can modulate syringomycin production (Mo and Gross 1991b). Recently, it was observed that over 90% of a sampling of toxigenic strains of *P. s.* pv. *syringae* produced larger quantities of toxin in the presence of plant signal molecules, with some strains producing toxin only in the presence of metabolites with signal activity (Quigley and Gross 1994). Cherry (*Prunus avium* L.) leaves, for example, contain two classes of plant metabolites with signal activity, namely phenolic glycosides, such as dihydrowogonin 7-glucoside, and sugars, such as D-fructose and sucrose (Mo et al. 1995). This indicates that the bacterium is attuned to a dynamic plant environment, having a sensory mechanism for plant metabolites that signals activation of genes required for syringomycin biosynthesis.

Syringomycin is a cyclic lipodepsinonapeptide composed of a polar peptide head and a hydrophobic 3-hydroxy fatty acid tail, containing either a decanoic (SRA_1), dodecanoic (SRE), or tetradecanoic (SRG) acid derivative (Fig. 1) (Segre et al. 1989; Fukuchi et al. 1992). Distinctive structural features of the peptide moiety include the presence of two D-amino acid residues, and 4-chlorothreonine at the C terminus linked to the N-terminal serine residue by an ester linkage to form a macrocyclic lactone ring. The nonapeptide portions of SRA_1, SRE, and SRG are identical (Segre et al. 1989; Vaillo et al. 1992). Consequently, it is not surprising that both SRE and SRG have been reported to exhibit a specific activity of 52.2 units/μg (Iacobellis et al. 1992).

It was established several years ago that the plasma membrane of host cells is the primary target of the phytotoxic activity of syringomycin (Backman and DeVay 1971; Paynter

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and Alconero 1979). The lipopeptide structure of syringomycin facilitates its insertion into the lipid bilayers of membranes, which leads to rapid and prolonged effects on ion transport across the plant plasma membrane (Takemoto 1992). Both plant and yeast cells respond similarly to the toxic activity of syringomycin (Reidl and Takemoto 1987). Small quantities of syringomycin activate a cascade of physiological events in plasma membranes, including rapid efflux of K^+ accompanied by H^+ and Ca^{2+} influx, that causes change in membrane potential. For example, Kauss et al. (1991) used suspension cells of periwinkle (*Catharanthus roseus*) to demonstrate that low concentrations of syringomycin (e.g., 200 ng/ml) increased cytoplasmic Ca^{2+} uptake, which in turn induced incorporation of 1,3-β-callose into the plant cell wall. Consequently, syringomycin affects Ca^{2+} transport processes commonly involved in cellular signalling (Kauss 1990). Bidwai and Takemoto (1987) reported that syringomycin-induced phosphorylation of several proteins in the plasma membrane of red beet (*Beta vulgaris* L.) storage tissues, an effect associated with Ca^{2+}-mediated activation of a plant signal cascade. Furthermore, leaf stomata of *Vicia faba* were closed by minute quantities of syringomycin (i.e., 12 ng/ml), an event initiated by an efflux of K^+ from stomatal guard cells (Mott and Takemoto 1989). Finally, syringomycin was reported to both inhibit (Che et al. 1992) and stimulate (Bidwai et al. 1987) membrane-bound ATPase activity. Although the sequences of these responses to syringomycin are unknown, Takemoto (1992) postulated that the primary effect of syringomycin was to open Ca^{2+} channels. By an unproven mechanism the resultant influx of Ca^{2+} would trigger a net influx of H^+ and efflux of K^+ leading to ionic imbalance that is lethal to cells.

The amphipathic structure of syringomycin suggests that the toxin exerts a surface-active effect (i.e., biosurfactant activity [Zajic and Seffens 1984]) on the plasma membrane. Indeed, Backman and DeVay (1971) observed that a partially purified preparation of syringomycin exhibits detergentlike action by lowering the surface tension of water and by causing hemolysis of erythrocytes. Furthermore, Che et al. (1992) suggested that a detergentlike action of syringomycin is largely responsible for inhibition of H^+-ATPase activity in mung bean (*Vigna radiata* L.) cells. The potential biosurfactant activity of syringomycin, however, has not been characterized, although it is known that biosurfactants are produced by several species of *Pseudomonas* (Bunster et al. 1989; Neu et al. 1990; Hutchison and Johnstone 1993; Persson et al. 1988). For example, *P. tolaasii* produces a lipodepsipeptide called tolaasin with strong biosurfactant properties which facilitate spread of the pathogen on mushroom caps by lowering the surface tension of water. In addition, tolaasin at concentrations as low as 200 ng/ml forms Zn^{2+}-sensitive voltage-gated ion channels in membranes (Brodey et al. 1991; Rainey et al. 1991). Consequently, tolaasin, at threshold concentrations of 500 ng/ml (Hutchison and Johnstone 1993), induces lysis of erythrocytes by a colloid osmotic mechanism, whereby a massive cytoplasmic influx of ions results in osmotic movement of water into cells effecting lysis (Knowles and Ellar 1987).

The capacity of syringomycin to form ion channels in membranes is suggested from analysis of partially purified preparations of syringotoxin, an amino acid analog of syringomycin (Gross 1991). Ziegler and Pavlovkin (1985) observed that syringotoxin formed voltage-gated ion channels in lipid bilayers, and concluded that polymeric complexes of syringotoxin are required for activity. Unfortunately, the results were confounded by use of an impure sample of syringotoxin; the preparation was shown by Ballio et al. (1991) to be contaminated with syringopeptin, a second class of lipodepsipeptide phytotoxins produced by *P. s.* pv. *syringae* (Iacobellis et al. 1992).

In this study, we reexamine the mechanism by which syringomycin kills host cells. We hypothesized that the primary mode of action of syringomycin is to form pores in plasma membranes that are freely permeable to ions. Formation of ion channels would in turn promote ion fluxes across the membrane that activate events associated with cellular signalling. Evidence is presented that syringomycin forms pores in artificial bilayers, and lyses heparinated horse erythrocytes by the formation of ion channels that are insensitive to both mono- and divalent cations. In addition, we quantify the surface active properties of syringomycin, and demonstrate that syringomycin is a highly effective biosurfactant. A model is presented that describes how syringomycin functions in the plant-pathogen interaction. In particular, the model integrates known physiological effects of the toxin on membranes with the formation of ion channels.

RESULTS

HPLC analysis of syringomycin purified from strain B301D.

HPLC analysis (results not shown) of a syringomycin preparation from strain B301D of *P. s.* pv. *syringae* showed the presence of the three 3-hydroxy fatty acid forms of syringomycin, SRA_1, SRE, and SRG, described originally by Ballio et al. (1988). SRE and SRG were the most abundant, composing 39.2 and 20.8% of the syringomycin mixture, respectively. In addition to the three biologically active forms of syringomycin, several minor peaks were observed in the HPLC profile. The retention times of the minor peaks correspond to those of inactive or weakly active forms of syringomycin that appear to be nonchlorinated derivatives or degradation products (Ballio et al. 1988; Grgurina et al. 1994). Typical yields of a purified mixture of the various forms of syringomycin were 10 to 15 mg/liter of culture broth. Because *P. s.* pv. *syringae* is predicted to secrete a mixture of the different forms of syringomycin in planta, both pooled fractions of syringomycin containing all of the different forms, and a separately purified fraction containing only SRE were used in tests for biosurfactant and pore-forming activities. The use of a syringomycin mixture in these assays was sub-

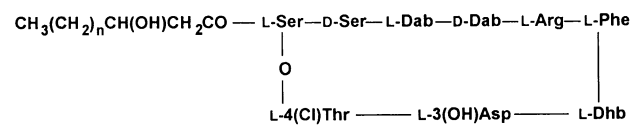

Fig. 1. Structure of syringomycin produced by *Pseudomonas syringae* pv. *syringae* strain B301D. Syringomycin forms SRA_1, SRE, and SRG differ in the 3-hydroxy fatty acid moiety with n = 6, 8, and 10, respectively. Abbreviations are Dab, 1,4-diaminobutyric acid, and Dhb, 2,3-dehydroaminobutyric acid; the amino acids are identified using standard three-letter biochemical notation.

stantiated by comparison to the corresponding activity of a pure preparation of SRE. The specific activities of the syringomycin mixture and SRE, were 26 and 18 units/μg, respectively.

The surface-active properties of syringomycin.

The HPLC-grade water control was found to have a surface tension of 73.4 mN/m. Analysis of the surface active properties of HPLC-purified syringomycin by the drop weight method demonstrated that the phytotoxin is a potent biosurfactant (Fig. 2). Pure preparations of syringomycin were determined to have a CMC of 1.25 (SRE) and 1.18 mg/ml (mixture). Furthermore, SRE lowered the interfacial surface tension of water to a minimum value of 30.9 mN/m; the surface tension at the CMC (γ_{CMC}) for SRE was 33.0 mN/m. Relatively low SRE concentrations of ≥10 μg/ml exhibited measurable lowering of interfacial tension. The CMC and γ_{CMC} values for syringomycin are of similar magnitude as those of other pseudomonad biosurfactants, including viscosin (CMC, 0.15 mg/ml; γ_{CMC}, 34 mN/m) (Neu et al. 1990), tolaasin (CMC, 0.46 mg/ml; γ_{CMC}, 38 mN/m) (Hutchison and Johnstone 1993), and the white-line inducing principle (WLIP) of *P. reactans* (CMC, 1.26 mg/ml; γ_{CMC}, 33 mN/m) (M. L. Hutchison and K. Johnstone, unpublished).

Syringomycin lyses erythrocytes below the CMC.

The syringomycin mixture exhibited strong hemolytic activity, based on lysis of erythrocytes at a threshold toxin concentration of 500 ng/ml; SRE induced lysis at a threshold concentration of 750 ng/ml (Fig. 3). At lower toxin concentrations, the rate of lysis was too slow to measure accurately because of sedimentation of erythrocytes. The rate of blood cell lysis was linear between syringomycin concentrations of 1 and 5 μg/ml. At syringomycin concentrations greater than 8 μg/ml, lysis of erythrocytes was very rapid with a large proportion of cells lysing immediately following exposure to the toxin. Micelle formation, as measured by the ability of solutions of surfactants to scatter light at A_{600} (Rosenberg 1986), was not observed at syringomycin concentrations between 0.5 and 8 μg/ml (data not shown). In addition, because a syringomycin stock solution of 1 mg/ml was used for all lysis assays, diluted solutions were checked at A_{600} for micelle formation. Although the equilibrium constant for micelle formation by syringomycin is unknown, no measurable micelle formation was observed 15 s postdilution.

Association of syringomycin with membranes was determined to be reversible, based on the recovery of biologically active toxin from suspensions of erythrocytes pretreated with syringomycin. Controls using inert glass and latex polystyrene beads in place of the primary erythrocyte incubation gave no detectable lysis when compared with a buffer control. After pretreatment of lysed erythrocytes with intermediate levels of syringomycin, the residual solution contained sufficient quantities of syringomycin to lyse fresh erythrocytes. Using an initial syringomycin concentration of 10 μg/ml, the rate of lysis at 10 s post addition of the second aliquot of erythrocytes was similar to that observed for 3 μg/ml syringomycin in our standard assay. The results indicate that the amphipathic syringomycin molecules, despite a strong affinity for lipid bilayers, are not irreversibly bound and can dissociate from the plasma membrane of erythrocytes. The ability of syringomycin to reversibly insert into the erythrocyte plasma membrane indicates that the colloid osmotic lysis mechanism is most likely responsible for hemolysis at syringomycin concentrations below the CMC (Rainey et al. 1991; Knowles and Ellar 1987).

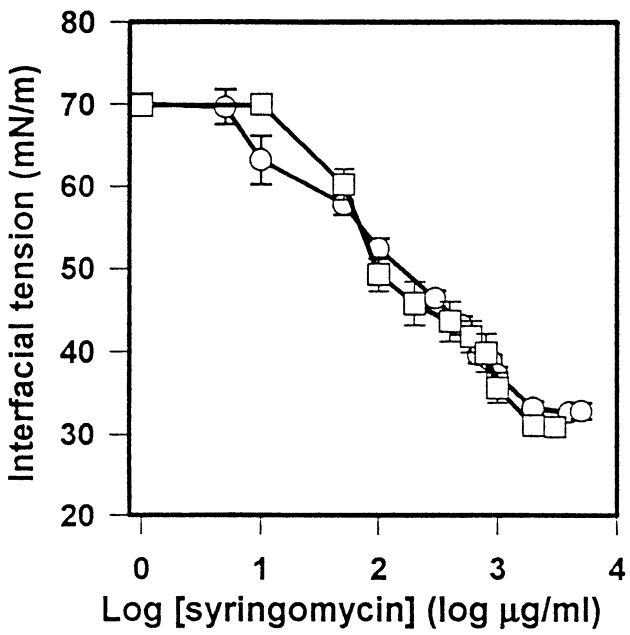

Fig. 2. Effect of syringomycin form SRE (□) and the syringomycin mixture (○) concentration on the interfacial surface tension of high-performance liquid chromatagraphy grade water, as determined by the drop weight method. Results are shown for the purified mixture containing the various forms of syringomycin identified in Figure 1. Error bars indicate the ± SD of 10 replicates.

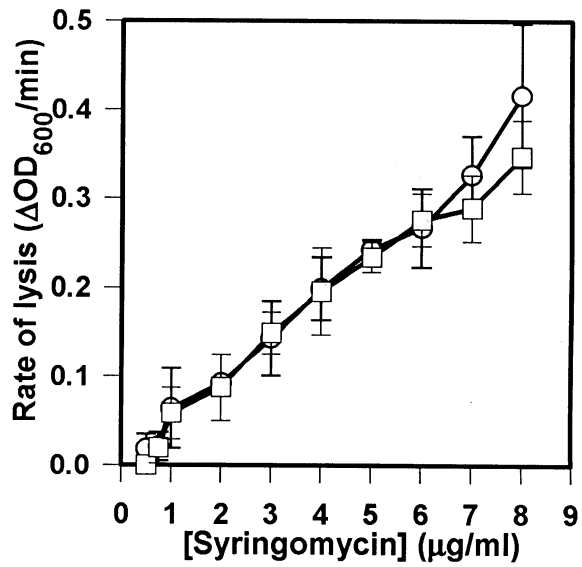

Fig. 3. Effect of syringomycin E (□) and the syringomycin mixture (○) concentration on the rate of lysis of heparinated horse erythrocytes. Results are shown for the purified mixture containing the various forms of syringomycin identified in Figure 1. Error bars indicate the ± SD of three individual trials, three replicates per trial.

Syringomycin forms cation-specific pores in membranes permeable to ions.

When syringomycin (form SRE) was added to artificial planar lipid bilayers at concentrations of 0.5 to 2.0 μg, rapid increases in both conductance and capacitance were observed. The conductance was cation-selective, and could occasionally be seen to show changes similar to those occurring upon opening and closing of ion channels (Fig. 4). Most usually, however, only a gradual and/or stepwise increase in conductance (Fig. 4) was observed.

Colloid osmotic lysis of plasma membranes can be prevented by molecules that physically block the pores, obstructing the flux of ions that leads to cell lysis (Rainey et al. 1991). Accordingly, osmotic protection assays were performed on erythrocytes using a range of sugars and polyethylene glycols to obtain further evidence that syringomycin is an ion channel-forming peptide and to estimate the pore size. Protection of erythrocytes against syringomycin-induced lysis was a function of the molecular size of the compound tested (Fig. 5). Additions of polyethylene glycol with viscometric radii ≥1.0 nm fully protected erythrocytes from the lytic effects of syringomycin. In contrast, sugars with a viscometric radius of 0.5 nm or less (i.e., arabinose, glucose, and sucrose) had much smaller effects on the rate of lysis. Erythrocytes exposed to sugars (i.e., lactose and raffinose) with a viscometric radius between 0.55 and 0.6 nm had lower rates of lysis, indicating partial blockage of the pores. Consequently, the mean channel radius of pores formed in plasma membranes by syringomycin was estimated to be between 0.6 and 1.0 nm.

Although divalent cations can inhibit the activity of pore-forming toxins such as tolaasin (Rainey et al. 1991), the pore-forming activity of syringomycin was insensitive to monovalent and divalent cations. For example, Zn^{2+}, Ca^{2+}, and Mg^{2+} tested at concentrations ranging up to 10 mM had no significant effect on the rate of erythrocyte lysis caused by syringomycin (Fig. 6). These results are consistent with the formation of nonspecific pores with a radius between 0.6 and 1.0 nm, which are freely permeable to all of the monovalent and divalent cations tested.

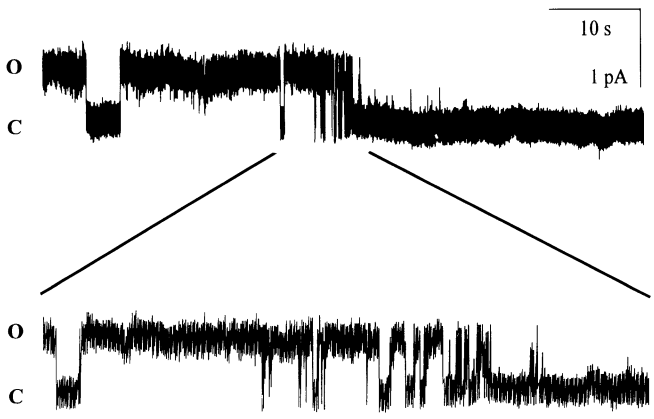

Fig. 4. Current fluctuations seen in a planar lipid bilayer upon addition of 1.3 μg/ml syringomycin (form SRE) to the *cis* side of the bilayer (trace A). A holding potential of +80 mV was used with 30 mM KCl *cis* and 10 mM KCl *trans*. **C** and **O** represent closed and open states, respectively. Trace B is a sixfold time-expanded plot of the central portion of the upper trace. Bars in trace A represent 10 s and 10^{-12} Amps (pA).

DISCUSSION

Transmembrane pore formation in plant-pathogen interactions.

Syringomycin production by *P. s.* pv. *syringae* is an integral part of the plant-pathogen interaction in which the integrity of the host plasma membrane is compromised by the lipopeptide toxin. In this study, syringomycin was found to form functional pores readily in membrane bilayers. Hence, we propose that pore formation by syringomycin constitutes the primary mechanism responsible for phytotoxic activity by promoting passive transmembrane ion flux, which is ultimately lethal to cells. In mammalian systems, bacterial pathogens commonly produce pore-forming proteins or peptides that cause cytolysis as a result of massive ion fluxes (Bhakdi and Tranum-Jensen 1987). Medically important examples of pore-forming cytolysins include hemolysin of *Escherichia coli* and α-toxin of *Staphylococcus aureus* (Braun and Focareta 1991; Welch 1991). In contrast, the involvement of pore-forming peptides and proteins in plant-microbe interactions represents a new and emerging concept. This is exemplified by the recent discovery in *Rhizobium leguminosarum* bv. *viciae* that the nodulation signalling protein, NodO, shares homology with the hemolysin A protein of *E. coli* (de Maagd et al. 1989) and forms ion channels in the plasma membranes of legumes (Sutton et al. 1994). Pore formation by NodO appears to be an important determinant of host-specific nodulation of legumes (Sutton et al. 1994). Tolaasin exemplifies a pore-forming lipopeptide toxin that reproduces brown blotch disease symptoms in mushrooms, resulting from infection by *P. tolaasii* (Hutchison and Johnstone 1993). Therefore, the discovery that syringomycin is a pore-forming peptide phytotoxin substantiates the concept of host plasma membranes as an important interface of plant-microbe interactions.

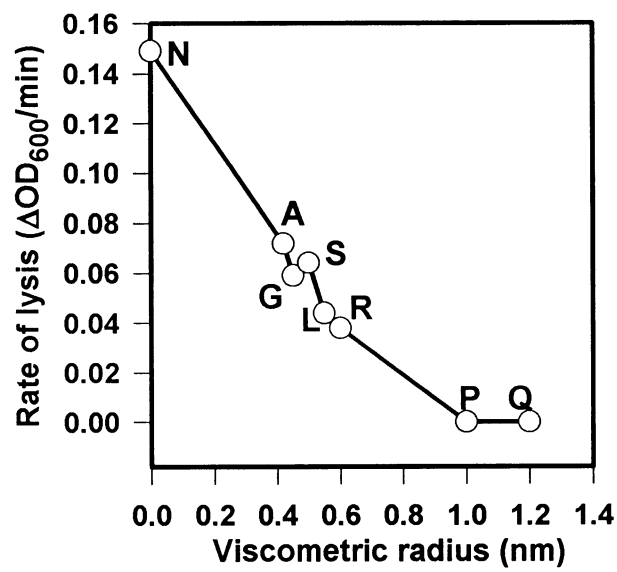

Fig. 5. Effect of preincubation with colloid osmotic protectants on the rate of lysis of heparinated horse erythrocytes treated with syringomycin. A purified mixture containing the various forms of syringomycin was used at a final concentration of 3 μg/ml. The following osmotic protectants were tested: A, arabinose; G, glucose; S, sucrose; L, lactose; R, raffinose; P, polyethylene glycol (mol. wt., 1,000); and Q, polyethylene glycol (mol. wt., 1,450). N is a negative control containing only syringomycin. Data are the mean of at least three individual trials.

Pore formation by syringomycin appears to be a highly efficient process in lipid bilayers, requiring only nanomolar quantities of toxin for measurable activity. Assays of erythrocyte lysis are a classical means of measuring pore-forming activity (Bhakdi and Tranum-Jensen 1987), and syringomycin was observed to lyse erythrocytes at a threshold concentration of 500 ng/ml. Thus, erythrocyte lysis occurs at toxin concentrations more than three orders of magnitude below the CMC of 1.18 mg/ml where nonspecific biosurfactant activity is expressed. Moreover, the lytic activity of syringomycin in erythrocyte assays is consistent with reported values of threshold activity in plant assays. Syringomycin (form SRE) at a concentration of 4 μM (~5 μg/ml) was reported to cause electrolyte leakage of carrot tissues (Iacobellis et al. 1992). At syringomycin concentrations much lower than that required for measurable cell lysis, however, significant levels of apparent pore-forming activity appear to be expressed in plant systems. For example, Kauss et al. (1991) reported that syringomycin concentrations as low as 200 and 500 ng/ml, respectively, induced leakage of K^+ and uptake of Ca^{2+} in suspension cells of *Catharanthus roseus*. Furthermore, approximately 50% of the cellular K^+ was lost by suspension cells exposed to 6 μg/ml quantities of the toxin. In a study by Mott and Takemoto (1989), the stomatal apertures in leaves of *Vicia faba* were reduced by exposure to only 10 nM of syringomycin; this was attributed to activation of a K^+ efflux in stomatal guard cells. Accordingly, we surmise that the bacterium produces sufficient quantities of syringomycin during the plant-pathogen interaction to cause extensive pore formation in host plasma membranes, which ultimately is visualized as necrosis. This is supported by evidence that highly virulent strains of *P. s.* pv. *syringae* characteristically produce between 10 and 80 μg/ml of syringomycin in vitro (Gross and DeVay 1977; Quigley and Gross 1994), and that syringomycin biosynthesis is modulated by specific plant signal molecules (Mo and Gross 1991b; Mo et al. 1995). Hence, a typical strain of *P. s.* pv. *syringae* has the capacity to produce copious quantities of toxin in the plant environment (Quigley and Gross 1994).

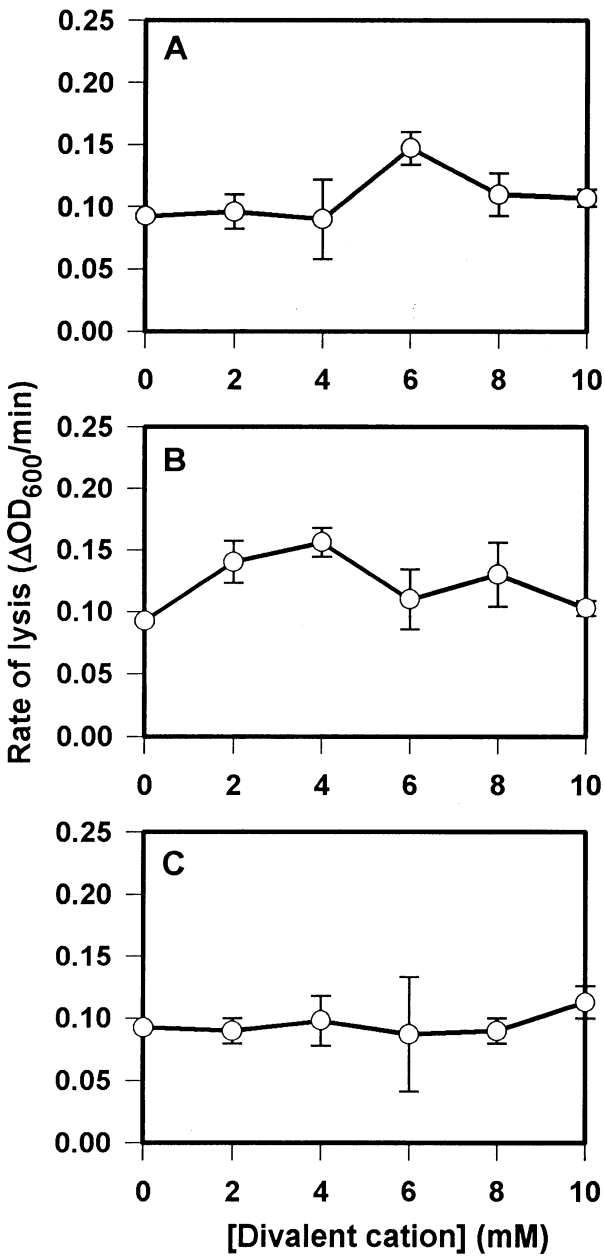

Fig. 6. Effect of divalent metal cations on the rate of lysis of heparinated horse erythrocytes treated with syringomycin. Ions were added to a final concentration of up to 10 mM. A purified mixture containing the various forms of syringomycin was used at a final concentration of 3 μg/ml. Results are shown for the following cations: **A,** Zn^{2+}; **B,** Ca^{2+}; and **C,** Mg^{2+}. Error bars indicate the ±SD of three individual trials.

Syringomycin forms transmembrane pores with a relatively small channel radius between 0.6 and 1 nm. Similar-sized pores are formed in lipid bilayers by tolaasin, (channel radius, 0.6 to 1.0 nm) (Rainey et al. 1991), and α–hemolysin (channel radius, 0.75 to 1.5 nm) (Bhakdi et al. 1986). In contrast to tolaasin (Hutchison and Johnstone 1993; Rainey et al. 1991), however, the pore-forming activity of syringomycin is not inhibited by Zn^{2+} and Ca^{2+}. Our findings in both the erythrocyte lysis assay and using direct measurements in bilayers show that syringomycin instead forms pores that are highly permeable to these cations. This is substantiated by the ability of syringomycin to cause a massive cytoplasmic influx of Ca^{2+} in artificial, plant (Kauss et al. 1991), and yeast (Takemoto et al. 1991) membranes. Although Takemoto (1992) speculated that syringomycin activity was responsible primarily for opening Ca^{2+} channels in plasma membranes, the fact that syringomycin itself forms ion channels would account for the observed transmembrane ion flux. Furthermore, the cytoplasmic influx of Ca^{2+} would be expected to stimulate the Ca^{2+}-sensitive plant cytoplasmic protein kinase. This would in turn phosphorylate and activate the plant plasma membrane-bound NAD(P)H oxidase, resulting in the catalysis of O_2 from atmospheric oxygen (Mehdy 1994). Syringomycin has been observed to evoke the production of active oxygen from cells (J. Y. Takemoto, Utah State University, personal communication).

The ability of syringomycin to lower the cytoplasmic pH of cultured cells of mung bean (Che et al. 1992) can largely be attributed to its capacity to form pores. Once pores are formed in the plasma membrane, the pH gradient that exists between the cytoplasm and the surrounding environment would collapse very rapidly. For example, Che et al. (1992) observed that the cytoplasmic pH of mung bean cells dropped from 7.4

to 6.8 within 8 min of exposure to large amounts of toxin. Although the ATPase likely would function as a proton pump to oppose an influx of H^+, syringomycin was shown by Che et al. (1992) to weakly inhibit the plasma membrane ATPase in mung bean cells. In a similar manner, pore formation by syringomycin will collapse the concentration gradient of any molecule of suitable size across a bilayer. The effects reported by Takemoto et al. (1991) include an increase in transmembrane K^+, H^+, and Ca^{2+} fluxes, and the associated change in membrane potential. The size of a Ca^{2+} ion (0.99 Å; Hille 1992) is considerably smaller than the pore diameter of a syringomycin channel. In a medium that is hypotonic to the cell cytoplasm, such as that employed by Takemoto et al. (1991), a net influx of Ca^{2+} is observed because pore formation is uninhibited by the presence of Ca^{2+}. Depolarization of a typical plant cell would further enhance K^+ efflux by the opening of outward rectifying K^+ channels (Tester 1990).

Aggregation and synergy of pore formation by syringomycin.

Due to the small size of the syringomycin molecule, the pores formed by the lipopeptide are predicted to be oligomeric structures. Aggregation of monomers in pore formation also is indicated by the dependency of erythrocyte lysis on syringomycin concentration, beginning at a toxin threshold of 500 ng/ml. Ziegler et al. (1984) determined that individual syringotoxin molecules cannot form discrete pores, based on the uniform conductivity of ion channels formed by the toxin in reconstituted lipid bilayers. Pore formation by tolaasin, which is nearly twice the size of syringomycin, also is dependent on aggregation of monomers (Rainey et al. 1991).

Ion channel formation by syringomycin may be facilitated by the characteristically low pH of the micro-environment adjacent to the plant cell membrane. The free amino groups associated with the diaminobutyric acid and arginine residues of the syringomycin molecule (Fig. 1) would be in the protonated NH_3 form. Consequently, insertion of the syringomycin molecule into the plant cell membrane may be enhanced by the inside negative potential difference of the cytoplasm and the charge on the protonated phytotoxin. Insertion may be further improved by the presence of membrane sterols such as ergosterol (Taguchi et al. 1994). Similar observations have been described previously for some types of pore-forming toxins (Bhakdi and Tranum-Jensen 1987; Latoud et al. 1990). For example, streptolysin-O, produced by β-hemolytic group A streptococci, attacks only membranes containing cholesterol; the requirement for cholesterol reflects its ability to induce formation of oligomeric toxin structures (Alouf 1980; Duncan and Schlegel 1975). Correspondingly, cholesterol added to culture media at concentrations of 10 μM fully protected yeast cells against the cytotoxicity of syringomycin at 3 μg/ml (Julmanop et al. 1993). The protective effect was reduced significantly if cells were preincubated with the toxin before exposure to cholesterol, which we interpret as evidence that the cells were killed before the addition of external supplies of cholesterol. We propose that pore formation occurs rapidly in the plasma membrane, and that the presence of sterols, such as cholesterol, in the membrane makes the insertion of syringomycin into lipid bilayers energetically more favorable.

A role for the biosurfactant properties of syringomycin.

Although syringomycin is a potent biosurfactant that can lower the interfacial tension of water to 31 mN/m, the biological significance of biosurfactant activity is unclear because concentrated supplies of the toxin would be necessary to achieve micelle formation at 1.25 mg/ml in nature. Nevertheless, the surfactant properties of the molecule are apparent at concentrations as low as 10 μg/ml (Fig. 2), a concentration which may be significant on plant surfaces. The thin aqueous films on plant surfaces may be conducive to concentrating the toxin as water evaporates, thereby enhancing detergent activity. Similarly, it appears that sufficient quantities of tolaasin (CMC, 400 μg/ml) are produced by *P. tolaasii* on mushroom caps for expression of biosurfactant activity, which in turn reduces the contact angle of water to facilitate spread of the toxin-laden water film across the mushroom surface (Hutchison and Johnstone 1993). Consequently, the bacterium is spread to new areas of the host surface where nutrients tend to partition and concentrate at solvent interfaces (Rosenberg 1986). Toxin production ceases when environmental conditions become unfavorable to the bacterium, and this allows departitioning of the cell from the interface (Rosenberg 1986). Whether such a scenario describes how the biosurfactant activity of syringomycin functions in the plant-pathogen interaction will require further investigation. Nevertheless, micelle formation may have a critical role in pore formation itself. Because of the propensity of amphipathic syringomycin molecules to partition into the host plasma membrane, 'reverse' micelle formation promoted by calcium ions may occur spontaneously once the concentration of toxin in the lipid bilayer becomes sufficient for the formation of functional pores (Wolman and Wiener 1963).

Interactions of syringopeptin and syringomycin.

The ability of *P. s.* pv. *syringae* to concurrently produce syringopeptin, a second lipodepsipeptide with phytotoxic activity (Ballio et al. 1991), and syringomycin suggests similar, if not interrelated, roles in the plant-pathogen interaction. Although little is known about the mechanism of action of syringopeptin, Iacobellis et al. (1992) demonstrated that it causes electrolyte leakage in plant cells and necrotic symptoms in tobacco leaves. Furthermore, a recent report by Di Georgio et al. (1994) shows that both toxins have indistinguishable effects on plasma-membrane H^+-transport in maize. We propose the amphipathic syringopeptin structure promotes insertion into the host plasma membrane and leads to formation of pores that are freely permeable to cations. Because the syringopeptin molecule is considerably larger than syringomycin, the higher phytotoxic activity of syringopeptin (Di Georgio et al. 1994) may in part reflect an ability to form functional pores composed of fewer monomers of syringopeptin as compared to pores formed by syringomycin. Iacobellis et al. (1992) reported a synergism between the two lipopeptide toxins, and this suggests formation of membrane pores composed of a chimera of syringopeptin and syringomycin. Similarly, strains of *Bacillus subtilis* produce both surfactin and iturn A simultaneously. Interactions between the two lipopeptides reveal a synergistic effect on biological activity against *Saccharomyces* (Thimon et al. 1992) and on hemolytic activity of iturin A (Maget-Dana et al. 1992).

A model depicting the mechanism of action of syringomycin in planta.

The seminal studies of Takemoto and associates (e.g., Bidwai and Takemoto 1987; Kauss et al. 1991; Mott and Takemoto 1989; Takemoto 1992) documented multiple effects of syringomycin on plasma membranes, including formation of ion fluxes of H^+, K^+, and Ca^{2+} and induction of events associated with cellular signaling. Nevertheless, it remained unknown as to how syringomycin initiated these events that ultimately are lethal to cells. The discovery that syringomycin forms pores in plasma membranes allows us to redefine a mechanism of phytotoxic action centered around the formation of ion channels that allows passive transmembrane ion flux. Therefore, a model is presented in Figure 7 that depicts the cascade of events occurring in host membranes in response to syringomycin. The model also helps explain a number of anomalies and conflicting observations about the mode of action of syringomycin, such as inhibition (Che et al. 1992) and stimulation (Bidwai et al. 1987; Zhang and Takemoto 1987) of ATPase.

Following invasion of susceptible host tissues, *P. s.* pv. *syringae* secretes small quantities of syringomycin that are rapidly partitioned into plant plasma membranes. Monomers of syringomycin form aggregates in the lipid bilayer leading to formation of functional pores. Only a few nanomoles of toxin would be sufficient to induce pore formation, based on evidence for activation of a K^+ efflux in stomatal guard cells (Mott and Takemoto 1989). We hypothesize that the pore

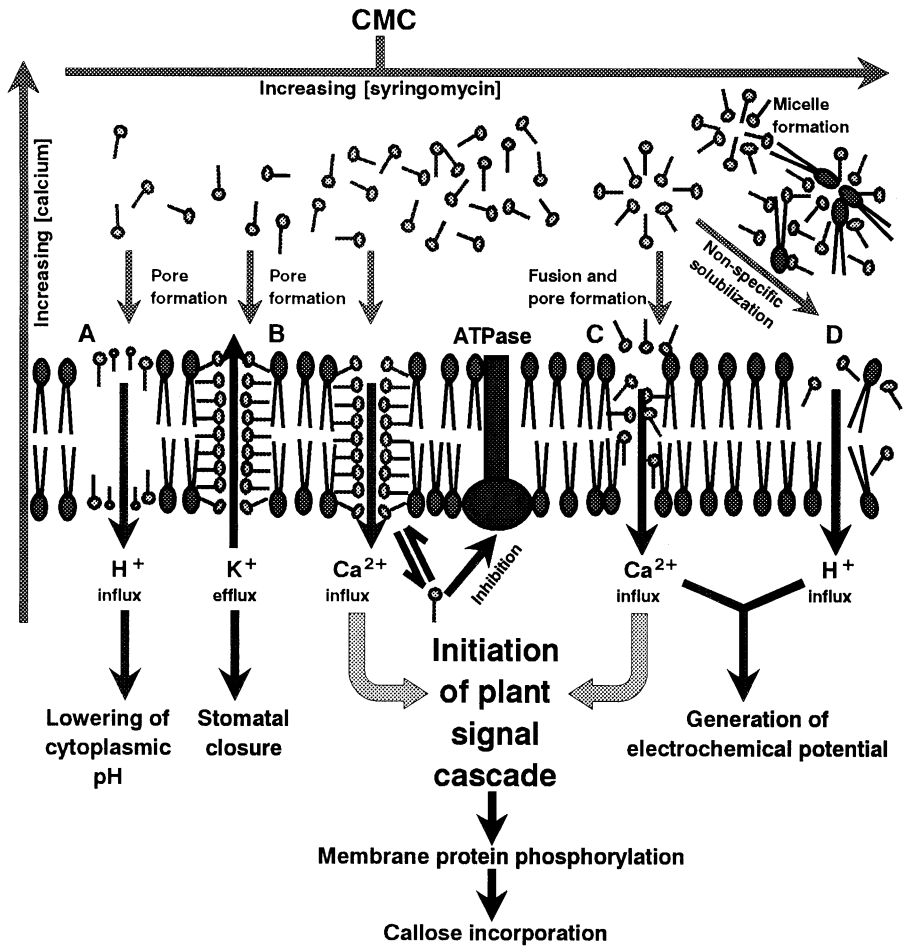

Fig. 7. Proposed model for the mechanism of phytotoxicity of syringomycin in the plant-pathogen interaction, as described in the Discussion. Individual syringomycin molecules are shown as , and plant plasma membrane phospholipids are shown as . The lower half of the diagram represents the cytoplasm of the plant cell. The concentration gradient of syringomycin increases from left to right, and the critical micellar concentration (CMC) of the syringomycin mixture is 1.18 mg/ml. Below the CMC (A, B), syringomycin exists in solution as single molecules or as small non-micellar aggregations. Due to the amphipathic nature of the phytotoxin, it tends to reversibly insert and be concentrated in the plant cell membrane. Eventually, pore formation occurs when a threshold level of syringomycin is attained to promote reverse micelle formation in the membrane. The pore structure depicted in (B) would occur predominantly during the plant-pathogen interaction. Although the formation of pores with the structure shown in (A) is postulated to occur at very low concentrations of the toxin, such structures would be unstable and may have a poor ability to allow free movement of ions. At concentrations very close to the CMC (C), the predominant form of the phytotoxin is micellar. Membrane fusion occurs, leading to either pore formation or incorporation of the micelle into the plasma membrane. When the [syringomycin] greatly exceeds the CMC (D), the large number of micelles that are formed fuse with the plant plasma membrane, leading to nonspecific membrane solublization. All of the mechanisms of pore formation described by this model permit the free movement of species of less than 1 nm viscometric radius down their concentration gradients, generating a net influx of Ca^{2+} and H^+, and an efflux of K^+. An H^+ influx would lead to a lowering of the cytoplasmic pH, and a K^+ efflux would induce closure of stomates. In addition, syringomycin would either directly or indirectly inhibit ATPase activity. The resultant flux of ions across the membrane would activate events associated with cellular signaling, which include phosphorylation of membrane proteins and incorporation of 1,3-β-callose into the plant cell wall.

structure can be one of two types (Fig. 7A and B), and favor structure B with a hydrophilic core extending across the plasma membrane. An association with sterols, such as cholesterol and ergosterol (Julmanop et al. 1993; Taguchi et al. 1994), may enhance formation of oligomeric toxin structures as has been observed for the lipopeptide iturins (Maget-Dana and Peypoux 1994). The rapid insertion of substantial quantities of the toxin into membranes would promote reverse micelle formation, which may be an integral step in pore formation. In contrast, aggregates of syringomycin are present only on one side of the membrane in structure A, consequently, it is uncertain how the toxin would be flipped to the cytoplasmic face. Because the three-dimensional structure of syringomycin is unknown, the structure of pores formed by syringomycin is highly speculative.

If the concentration of syringomycin rises above the CMC in the plant environment, biosurfactant activity then would have a dominant role in attacking the plasma membrane (Fig. 7). At concentrations near the CMC of syringomycin, a small number of micelles will form. The micelles fuse with the plant plasma membrane, and functional pores eventually are formed (structure C). At very high syringomycin concentrations, the toxin exists predominantly as micelles and can directly solublize the plasma membrane (structure D). Although both of these nonspecific modes of lysis are well documented to occur at high biosurfactant concentrations (Attwood and Florence 1983), the CMC of 1.25 mg/ml for syringomycin appears to be too high for significant levels of micelle formation in situ.

Pore formation, regardless of the mechanism, leads to passive nonspecific ion fluxes that threaten cell viability (Fig. 7). An inward movement of H^+ is accompanied by an efflux of K^+ which generates an electrochemical gradient and collapses the pH gradient across the plasma membrane, leading to acidification of the cytoplasm (Reidl et al. 1989; Reidl and Takemoto 1987; Zhang and Takemoto 1987). A concurrent influx of Ca^{2+} occurs which in turn leads to activation of a cascade of events associated with cellular signalling (Kauss et al. 1991; Takemoto et al. 1989, 1991). Accordingly, syringomycin is known to induce kinase-mediated phosphorylation of membrane proteins (Bidwai and Takemoto 1987) and incorporation of callose into plant cell walls (Kauss et al. 1991), both of which are modulated by intracellular responses to a free Ca^{2+} signal (Kauss 1987, 1990). In particular, the activation of protein kinases by syringomycin appears to be responsible for phosphorylation of the proton pump ATPase (Bidwai and Takemoto 1987). The plasma membrane ATPase will be stimulated in the short term (Bidwai et al. 1987) as it hydrolyzes ATP in an attempt to pump H^+ and Ca^{2+} back out into the extracellular space; however, such action will be relatively ineffective against the collapse of the cation gradients of the cell. Because insertion of syringomycin into the membrane is reversible, small quantities of the toxin may enter the cytoplasm of the plant cell and inhibit the plasma membrane ATPase as reported by Che et al. (1992). The mechanism responsible for weak inhibition of ATPase activity is unknown, but does appear to be a direct effect of the toxin on the enzyme (Che et al. 1992).

Pore formation benefits the bacterium by systematically releasing cellular metabolites into the intercellular spaces of host tissues, thus providing an environment more advantageous to bacterial growth. Growth will be enhanced both by alkalization of the intercellular fluids, and by a rapid efflux of sugars, amino acids and inorganic ions from the plant cytoplasm. In particular, the transmembrane pores will be readily permeable to sucrose and fructose which, together with phenolic signal molecules, modulate syringomycin synthesis in *P. s.* pv. *syringae* (Mo and Gross 1991b; Quigley and Gross 1994). A rapid efflux of sucrose is known to accompany a K^+/H^+ exchange in both susceptible and hypersensitive responses of plants to bacteria (Atkinson and Baker 1987), and a comparable stimulation of sucrose efflux would likely result from exposure to syringomycin. The resultant amplification of toxin production will in turn result in high levels of pore formation within host cells and thereby intensify disease severity. Consequently, phytotoxin production and pore formation coexist in a delicate balance, reflecting the dynamic interaction occurring in nature between *P. s.* pv. *syringae* and the plant host.

MATERIALS AND METHODS

HPLC purification of syringomycin.

Syringomycin was extracted and partially purified from potato-dextrose broth according to the method of Fukuchi et al. (1992). Briefly, *Pseudomonas syringae* pv. *syringae* strain B301D (Xu and Gross 1988) was grown in still culture (100 ml) for 5 days at 25°C (Gross 1985). Cells were removed from the broth by centrifugation (5,000 × *g*, 4°C, 10 min), and the supernatant acidified to pH 2.0 with 5 M acetic acid. The supernatant was passed through an XAD-2 column (Sigma Chemical Co., St. Louis, Mo.) prior to further purification on XAD-7 (Sigma) and Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, New Jersey) columns. The crudely purified syringomycin was freeze dried and resuspended in HPLC grade water at a concentration of 1 mg/ml prior to further purification by HPLC.

Pure syringomycin was obtained after separation in a Waters HPLC system (Millipore Corp., Milford, MA) equipped with a semipreparative C-18 reversed-phase column (Altex Ultrasphere-ODS, 1 × 25 cm; Beckman Instr., Fullerton, Calif.). The toxin was separated by a linear acetonitrile gradient of 20 to 50% (v/v) in 0.1% (v/v) trifluoroacetic acid (TFA); the flow rate for a 30-min run was 1.5 ml/min. Peaks observed at A_{214} were collected and tested for biological activity using the indicator organism, *Geotrichum candidum* strain F-260 (Quigley and Gross 1994); a unit of syringomycin is defined as previously described (Quigley and Gross 1994). All bioactive syringomycin fractions were pooled before lyophilization and stored at –20°C. Relative concentrations of the two major forms of syringomycin (SRE and SRG) were calculated from peak area using the Waters 990 chromatogram analysis integration software module (Millipore). Preparative runs containing a mixture of all three active forms of syringomycin were performed using a 10-min linear acetonitrile gradient of 15 to 65% (v/v) in 0.1% (v/v) TFA. Syringomycin eluted as a single peak due to the increased speed of the gradient. The syringomycin mixture was free of syringopeptin forms A and B, which eluted in preparative runs as a separate single peak at about 8.5 min. In addition to the preparation containing a mixture of all forms of syringomycin, a pure preparation of SRE, the major form of syringo-

mycin produced by strain B301D, was prepared for assays of biosurfactant and pore-forming activities.

Measurement of surface activity by the drop weight method.

The surface activity of syringomycin was measured by the drop-weight method (Harkins and Brown 1919) using an "Agla" type syringe (Wellcome Research Laboratories, Beckenham, England) fabricated with a tip radius of 0.35 cm. The plunger of the syringe was operated by use of a micrometer. Solutions of HPLC-purified syringomycin (SRE or a mixture of all forms of syringomycin) were dissolved in HPLC grade water (1 μg to 5 mg/ml). Droplets were released from the syringe tip and weighed on an analytical balance. Ten replications were prepared for each data point. A high relative humidity inside the weighing chamber was maintained with wet paper towels. The method of Harkins and Brown (1919) was used to convert the weights of the drops to surface tension values. The critical micellar concentration and γ_{CMC} were determined from a plot of interfacial surface tension versus log [syringomycin] as described by Sheppard and Mulligan (1987).

Erythrocyte assays.

Except for a few modifications, the assay used to test syringomycin for ion channel-forming activity was described by Hutchison and Johnstone (1993). Heparinated horse erythrocytes (0.2 ml) were added to four volumes of PBS (10 mM sodium phosphate, 0.9% NaCl [pH 7.0]), mixed by vortexing for 10 s, and centrifuged (14,000 × *g*, 4°C, 30 s). Five subsequent washings were performed prior to final suspension of the erythrocytes in 100 ml of PBS. All assays were performed at 22° C. Blood cells (1 to 2 μl) were mixed with PBS to give a final volume of 1 ml and a final OD_{600} between 0.09 and 0.1. Syringomycin (SRE or a mixture of all forms of syringomycin), prepared as a 1 mg/ml stock solution, was added to the blood cells to give final concentrations of 0.5 to 8 μg/ml and mixed for 1 to 2 s. The rate of lysis, expressed as ΔOD_{600} per minute, was monitored by a spectrophotometer equipped with a photodiode array detector (Hewlett Packard, Palo Alto, Calif., model 8452A). Assays, performed on three different days, were standardized using a known amount of syringomycin (3 μg/ml). Each data point was the result of three individual trials.

The effect of monovalent and divalent cations on the activity of syringomycin was determined by adding chlorine salts of test cations ($ZnCl_2$, $MgCl_2$, $CaCl_2$, KCl, CsCl) to erythrocytes suspended in PBS; the final concentration of the salts ranged from 2 to 10 mM. Assays for lysis of erythrocytes by syringomycin (3 μg/ml) were performed as described above. No significant change in pH occurred upon addition of the ions.

To determine if syringomycin can insert reversibly into erythrocyte membranes, blood cells, prepared as described above, were suspended in PBS (pH 7.0) and preincubated (15 min at 22°C) with syringomycin (4 and 10 μg/ml). Cellular debris were recovered by centrifugation (12,000 × *g*, 3 min), and washed before resuspension in 1 ml of PBS (pH 7.0). After incubation (37° C, 15 min), the debris was again pelleted, and the supernatant added to a fresh sample of erythrocytes (final OD_{600}, 0.1). Controls using only PBS and substituting inert glass beads (Sigma; mean diameter 170 μm) and latex polystyrene (Sigma; mean diameter 0.8 μm) for the primary erythrocyte incubation were performed. Samples were quantitatively analyzed for hemolytic activity.

Osmotic protection experiments.

Osmotic protection assays for erythrocytes treated with syringomycin were performed as described by Knowles and Ellar (1987). Briefly, erythrocytes were suspended in PBS (pH 7.0) as described above. Blood cells (1 to 2 μl) were mixed with PBS containing 10 mM of a sugar or polyethylene glycol (PEG) to give a final volume of 1 ml and a final OD_{600} between 0.09 and 0.1. The osmotic protectants used were arabinose, glucose, sucrose, lactose, raffinose, and PEG with molecular weights of 1,000 and 1,450; all were purchased from Sigma. The cells were incubated for 5 min at 22° C before the addition of 3 μg of HPLC-purified syringomycin. The rate of lysis was measured as described above. Each data point was the result of three individual trials. Pore size was estimated from a curve of the rate of lysis versus viscometric radius of the sugar or PEG (Knowles and Ellar 1987).

Artificial bilayer conductance assay.

Planar lipid bilayers were painted across a 0.2-mm-diameter hole in the wall of a styrene copolymer cup (Brodey et al. 1991). Lipids from a decane (Sigma) dispersion of 1-palmitoyl-2-oleoyl (PO) phospholipids (13.1 mM) (Avanti Polar Lipids, Birmingham, Ala.) as PO-phosphatidylethanolamine (8.6 mM), PO-phosphatidylserine (3.2 mM), PO-phosphatidylcholine (1.3 mM), and cholesterol (25 mM) (Sigma) were used. The bilayer separated solutions of 600 μl (*cis*) and 5 ml (*trans*) buffered with 5 mM *N*-tris-(hydroxymethyl)methyl-2-amino ethanesulfonic acid/Tris (pH 7.0). All solutions were filtered using a 0.2-μm nitrocellulose membrane (Millipore). Experiments were performed at 25° C. Currents were monitored under voltage-clamp conditions using a List EPC-7 amplifier (Haverhill, Mass.) connected to the bilayer chambers by Ag/AgCl electrodes and 3 M KCl/1% agar salt bridges. Data were stored on digital audio tape and displayed after low-pass filtering at 100 Hz using an eight-pole bessel filter (Frequency Devices, Darmstadt, Germany). Membrane potentials were recorded *trans* with respect to *cis*, the latter being the side to which syringomycin was added.

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