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Permeation of Bacterial Cells, Permeation of Cytoplasmic and Artificial Membrane Vesicles, and Channel Formation on Lipid Bilayers by Peptide Antibiotic AS-48

ANTONIO GÁLVEZ,* MERCEDES MAQUEDA, MANUEL MARTÍNEZ-BUENO, AND EVA VALDIVIA

Departamento de Microbiologia, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain

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Peptide AS-48 induces ion permeation, which is accompanied by the collapse of the cytoplasmic membrane potential, in sensitive bacteria. Active transport by cytoplasmic membrane vesicles is also impaired by AS-48. At low concentrations, this peptide also causes permeability of liposomes to low-molecular-weight compounds without a requirement for a membrane potential. Higher antibiotic concentrations induce severe disorganization, which is visualized under electron microscopy as aggregation and formation of multilamellar structures. Electrical measurements suggest that AS-48 can form channels in lipid bilayers.

The production of bacteriocins and other antibacterial substances by group D enterococci is well documented (2, 5, 11, 17), although broad-spectrum inhibitors are rarely found. In addition, the mode of action of most of the antibacterial substances in this group has not been studied extensively. An enterococcal strain isolated in our laboratory (*Enterococcus faecalis* subsp. *liquefaciens* S-48) produces two inhibitory substances: bacteriocin Bc-48, which has a molecular mass of 77 kDa and is described elsewhere (14a), and antibiotic AS-48. This peptide has been purified from culture supernatants of strain S-48 and its nonbacteriocinogenic mutant A-48-32. Reversed-phase liquid chromatography analysis revealed a single peptide with a molecular mass of 7.4 kDa, as estimated from its amino acid composition (4). AS-48 is rich in basic and neutral amino acids and has an isoelectric point close to 10.5. It resembles other antimicrobial peptides, such as nisin (8), Pep-5 (23), and the small bacteriocin C3603 (9). AS-48 is encoded by the 56-kb conjugative plasmid pMB2, which was transferred to plasmid-free *E. faecalis* OG1X. This plasmid carries the genes for immunity and for the production of AS-48 (15).

Peptide AS-48 has a broad inhibitory spectrum against gram-positive and gram-negative bacteria. Its bactericidal effect is mediated through the inhibition of amino acid uptake, O_2 consumption, and the alteration of the cytoplasmic content of K^+ and Na^+ ions (6). AS-48 also induces bacteriolysis in several gram-positive bacteria after prolonged incubation, an effect that is considered to be secondary.

This paper describes the effects of AS-48 on the permeability of whole cells and cytoplasmic membrane vesicles derived from sensitive bacteria. Its action on artificial membranes (liposomes) is also discussed with regard to changes in permeability and morphological alterations observed under electron microscopy. The formation of channels in lipid bilayers is also suggested.

MATERIALS AND METHODS

Bacterial strains and growth media. *E. faecalis* S-47 was isolated in our laboratory and described elsewhere (5). Other bacteria used were purchased from culture collections or belonged to our laboratory collection. *E. faecalis* S-47 was grown in brain heart infusion broth (Becton Dickinson and Co., Paramus, N.J.). Other bacterial strains were grown in Luria broth at 37°C under agitation.

Chemicals. All radioactive materials were purchased from DuPont, NEN Research Products, Boston, Mass. *N,N'*-dicyclohexylcarbodiimide (DCCD), valinomycin, lysozyme, RNase, DNase, and L-α-phosphatidylcholine were purchased from Sigma Chemical Co., St. Louis, Mo. AS-48 was purified in our laboratory as described elsewhere (4).

Preparation of rubidium-loaded cells and rubidium uptake. Cultures growing in brain heart infusion broth were harvested by centrifugation at the mid-logarithmic phase (optical density at 620 nm, 0.1 to 0.2) and washed and resuspended in 25 mM Tris-maleate buffer (pH 7.4) containing 1% glucose, 5 μM potassium phosphate, and 5 mM $MgSO_4$. Portions of the cell suspensions were incubated with $^{86}Rb^+$ (2.72 mCi/mg; 6 μCi/ml) at 37°C for 30 min. Loaded cells were centrifuged, resuspended in fresh buffer without rubidium, and incubated with AS-48. For Rb^+ uptake experiments, the cell suspensions were preincubated for 2 min at 37°C with AS-48 or buffer before $^{86}Rb^+$ (6 μCi/ml) was added.

Aliquots (100 μl) were removed at desired intervals and filtered through Whatman GF/C filters. The filters were washed twice with 2 ml of 25 mM Tris-maleate buffer (pH 7.4). Once dry, they were placed in vials, to which scintillation fluid was added. The radioactivity retained in the filters was measured in a Beckman scintillation counter.

Measurement of the membrane potential. Cells were grown in brain heart infusion broth to an optical density at 620 nm of 0.7, centrifuged, and washed twice and resuspended in 25 mM Tris-maleate buffer (pH 7.4) containing 10 mM $MgSO_4$. The suspension was used either immediatedly or after 1 h of incubation at 37°C. For experiments with energized cells, 10 mM glucose was added. [^{3}H]tetraphenylphosphonium ([^{3}H]TPP^+) (39.5 Ci/mmol) was added to a concentration of 6 μCi/ml. AS-48 was added to a final concentration of 15 μg/ml. Controls were treated with 2 μM DCCD. Duplicate portions (100 μl each) were removed periodically, filtered through Whatman GF/C filters, and washed twice with 3 ml of buffer. Samples were corrected for nonspecific binding of [^{3}H]TPP^+ by subtraction of the counts of 10% butanol-treated aliquots. The membrane potential ($\Delta\psi$) was calcu-

* Corresponding author.

lated from the Nernst equation, $\Delta\psi = 2.3 \times R \times (T/F) \times \log(TPP^+ \text{ in}/TPP^+ \text{ out})$. Internal water space and cell protein were determined as described by Kobayashi and Unemoto (13) and Sheffield et al. (25), respectively.

Preparation of membrane vesicles. *Escherichia coli* membrane vesicles were prepared as described by Bisschop and Konings (1), except that 40 mM potassium phosphate buffer (pH 7.2) was used throughout. The vesicles obtained were finally resuspended in this buffer to a concentration of 1.4 mg/ml.

E. faecalis vesicles were prepared by resuspension of the cells from overnight growth to 0.1 g (wet weight) per ml in 10 ml of 50 mM Tris-maleate buffer (pH 7.4) containing 25% sucrose and 2 mM $MgSO_4$ and further incubation with lysozyme (10 mg) for 90 min at 37°C. The resulting protoplasts were collected by centrifugation (5,000 × *g* for 20 min) and resuspended in 5 ml of 50 mM Tris-maleate buffer (pH 7.4) containing 100 mM KCl and 5 mM $MgSO_4$. After the addition of DNase and RNase (50 μg/ml each), the suspension was manually homogenized with a Potter-Elvehjem homogenizer while being kept in ice and diluted to 150 ml with buffer. The mixture was centrifuged at 4,000 × *g* for 15 min to remove debris. The vesicles were collected by centrifugation (48,000 × *g* for 40 min) and resuspended in the same buffer at 4 mg of protein per ml.

The vesicles were kept in Eppendorf tubes and frozen in liquid nitrogen. The preparations were stored at −75°C and thawed in a water bath at 40°C before use. The vesicles were never refrozen for further use.

For efflux experiments, *E. coli* vesicles were diluted 1:100 in the original buffer and incubated at 25°C under aeration in the presence of L-[2,3,4,5-^{3}H]proline (112 Ci/mmol) (final concentration, 3 μCi/ml). After 5 min of preincubation, active transport was started by the addition of ascorbate-phenazine methosulfate (Asc/PMS) (20 mM and 100 μM, respectively).

The uptake of leucine mediated by valinomycin-induced potassium efflux in *E. faecalis* vesicles was measured as described by Otto et al. (18); the vesicles were diluted 100-fold in 50 mM Tris-maleate buffer (pH 7.4) containing 100 mM choline chloride and 1 μM valinomycin. L-[4,5-^{3}H]leucine (120 Ci/mmol) was added to a final concentration of 3 μCi/ml, and the mixture was incubated at 25°C.

At desired times of incubation, AS-48 was added to *E. faecalis* and *E. coli* vesicles to a final concentration of 4 μg/ml. Aliquots (100 μl) were removed periodically, filtered through Millipore GSTF02500 filters (0.22-μm pore size), and washed twice with 2 ml of ice-cold 100 mM lithium chloride containing 5 mM unlabeled amino acid. Once dry, the filters were counted for retained radioactivity.

Preparation of freeze-thaw vesicles. Asolectin vesicles were prepared with L-α-phosphatidylcholine from soybeans (type IV-S; Sigma) without further purification. Asolectin (40 mg) was homogenized in 2 ml of buffer (20 mM sodium phosphate [pH 7.2]) by sonication in a bath-type sonicator (Bransonic 12), and the labeled precursors $^{86}Rb^+$ (2.72 mCi/mg), [5,6-^{3}H]uridine (48 Ci/mmol), L-[U-^{14}C]aspartic acid (224 mCi/mmol), and [carboxyl-^{14}C]dextran (0.6 mCi/g; M_w, 50,000 to 70,000) were added to final concentrations of 0.5, 3, 3, and 1 μCi/ml, respectively. The mixture was sonicated until it became opalescent, frozen in liquid nitrogen, and thawed rapidly in a water bath at 45°C. The suspension was clarified by brief sonication (10 to 30 s) and chromatographed on Sephadex G-25 or Sephacryl S-200 (for dextran-loaded liposomes) columns (0.5 by 10 cm; flow rate, ca. 0.5 ml/min) with sodium phosphate buffer to remove the untrapped radioactivity. Vesicles were diluted 100-fold in this buffer before use. To impose a K^+ diffusion potential, we prepared vesicles in sodium phosphate buffer containing 100 mM KCl and diluted them 100-fold in buffer containing 100 mM choline chloride. A potential of ca. 120 mV (negative inside) was generated by the addition of 3 μM valinomycin.

For efflux experiments, portions (100 μl) of the vesicle suspensions were removed periodically, filtered through Millipore GSTF02500 filters, and washed with 2 ml of 100 mM lithium chloride. The radioactivity retained in the filters was measured.

Electron microscopy of vesicles. Samples of lipid vesicles obtained by sonication as described above were applied to Formvar-coated copper grids at a concentration of 2.5 mg/ml, washed with 20 mM sodium phosphate buffer (pH 7.2), and stained with 2% uranyl acetate for 30 s for observation under a Zeiss 902 electron microscope.

Conductance measurements. Planar phospholipid bilayer membranes (ca. 1-mm^2 area) were formed by the brush technique (16) across a hole in a sheet of Teflon separating two compartments (3 ml) of a Teflon chamber containing buffered solutions. The lipid used was asolectin (type IV-S; Sigma) from which neutral lipids had been extracted with acetone (10). Symmetrical solutions of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) containing 100 mM KCl, 3 mM $CaCl_2$, 3 mM $MgCl_2$, and 0.1 mM EDTA (pH 7.0) in Millipore Milli-Q water bathed the membranes.

Electrical measurements were made at room temperature under voltage clamp conditions with a single pair of calomel electrodes contacting the solutions through saturated KCl junctions. After formation of a membrane and determination of its high resistance ($10^8\ \Omega \cdot cm^2$), an aliquot of AS-48 (3 μg) was added to one compartment (*cis* side). The voltage of this side was set to zero. The conductance of the membrane (*g*) in symmetrical salt solutions is defined as current divided by voltage; in the absence of AS-48, *g* is about 10 pS.

RESULTS

Effect of AS-48 on rubidium retention and uptake. Previous experiments showed that AS-48 causes an inhibition of amino acid uptake in *E. faecalis*. Determinations by atomic absorption spectrophotometry of the cell contents of K^+ and Na^+ ions also indicated significant differences between controls and cells treated with AS-48 (6). Therefore, we studied the capacities of several types of bacterial cells to maintain internal Rb^+ or to accumulate external Rb^+ as an analog of K^+ in the presence of AS-48, since this method leads to more accurate determinations in a shorter time. The addition of AS-48 to *E. faecalis* cells loaded with rubidium induced a rapid efflux of this ion. Loaded cells lost more than 95% of the accumulated $^{86}Rb^+$ after 5 min of incubation with AS-48 (10 μg/ml), while the controls maintained most of the initial concentration during the entire assay (30 min) (Fig. 1A). Other bacteria, such as *Corynebacterium glutamicum* CECT78 or *Bacillus subtilis*, lost 92 and 83% of the accumulated rubidium, respectively, after 5 min of incubation with the same concentration of AS-48 (data not shown).

Some gram-negative bacteria which are sensitive to AS-48 require higher concentrations of this antibiotic for inhibition but also exhibit alterations similar to those of gram-positive bacteria upon incubation with AS-48 (inhibition of synthesis, amino acid uptake, and respiration) (7). In our experiments, the amount of radioactivity retained by cells loaded with

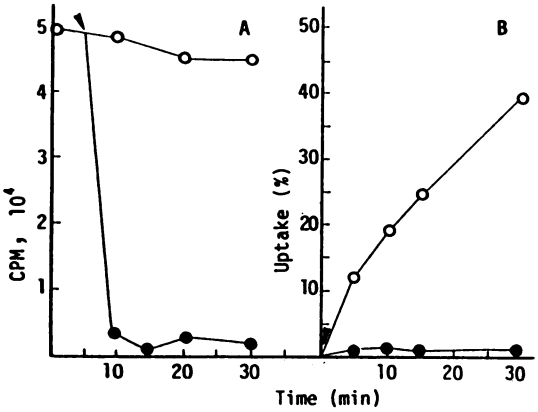

FIG. 1. Effect of AS-48 on $^{86}Rb^+$ retention (A) and uptake (B) in *E. faecalis* S-47. (A) The cells were loaded with $^{86}Rb^+$ and washed and resuspended in 25 mM Tris-maleate buffer (pH 7.4) containing 1% glucose. The suspension was incubated at 37°C with AS-48 (arrowhead). (B) The cells were washed and resuspended in the same buffer, and the suspension was divided. AS-48 was added to one portion (arrowhead) and $^{86}Rb^+$ was added to both. The percent uptake represents the percentage of added label taken up. Symbols: ○, controls; ●, effect of AS-48 (10 μg/ml).

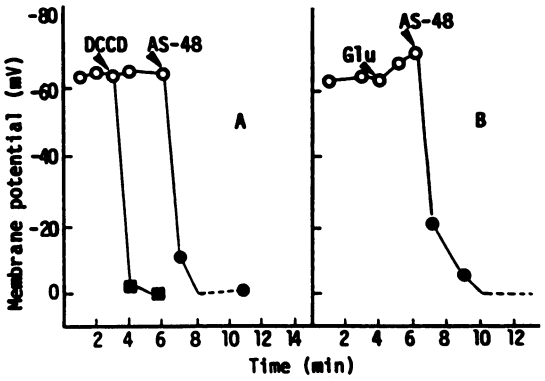

FIG. 2. Effect of AS-48 on the membrane potential of *E. faecalis* S-47. Δψ was calculated from the [^{3}H]TPP$^+$ distribution as described in the text. (A) Resting cells. (B) Cells energized with 1% glucose (Glu). Symbols: ○, controls; ■, effect of DCCD (2 μM); ●, effect of AS-48 (15 μg/ml).

rubidium and incubated with AS-48 was investigated. After 10 min of incubation with this antibiotic (70 μg/ml), *E. coli* U-9, *Salmonella typhimurium* LT2, and *Klebsiella pneumoniae* CECT141 lost significant percentages (75, 76, and 49%, respectively) of the radioactivity incorporated. These results suggest that AS-48 inhibits gram-negative bacteria through the same membrane-damaging mechanism as that responsible for the inhibition of *E. faecalis*.

In separate experiments, the effect of AS-48 on the accumulation of external $^{86}Rb^+$ by *E. faecalis* S-47 was tested. As expected, *E. faecalis* cells preincubated with AS-48 for a short time (2 min) failed to incorporate external rubidium, while a steady accumulation was observed in the controls (Fig. 1B).

Influence of AS-48 on the cytoplasmic membrane potential of whole cells. All of the above-described results on the biological effects of peptide AS-48 suggest that the cytoplasmic membrane is the primary target for its action. A failure to incorporate rubidium (as an analog of potassium) and to retain cytoplasmic rubidium or potassium must have severe effects on the membrane potential of sensitive microorganisms. The effect of AS-48 on this parameter was monitored by measuring the distribution of the lipophilic cation TPP$^+$ in *E. faecalis* S-47, in cells both at rest and energized with glucose. The estimated membrane potential ranged from −65 to −72 mV, values which correspond to those obtained by other authors (12). The addition of AS-48 led to a rapid decrease in the membrane potential, comparable to the effect of the ATPase inhibitor DCCD (Fig. 2A).

No differences were found in the action of AS-48 on cells at rest or energized with glucose, although the membrane potential was slightly higher in the latter (Fig. 2B). Nevertheless, the incorporation of TPP$^+$ by cells starved after incubation at 37°C for 1 h was too low to allow significant measurements. Abolition of the membrane potential by AS-48 would rapidly impair the transport of amino acids and other substrates, resulting in the inhibition of biosynthetic pathways and cell replication. The free diffusion of ions across the membrane would also alter the pH gradient and the cytoplasmic pH, although no measurements of these parameters have been made.

Effect of AS-48 on cytoplasmic membrane vesicles. Since the cytoplasmic membrane seems to be the primary target for AS-48, we decided to determine the effects of this peptide on membrane vesicles from sensitive microorganisms (e.g., gram-negative bacteria) in which the different degrees of sensitivity to AS-48 could be related to the presence of an outer membrane. Asc/PMS-energized vesicles from *E. coli* U-9, which is more resistant than is *E. faecalis*, proved highly sensitive to a low concentration of AS-48 (4 μg/ml), which brought about a rapid impairment of proline uptake and a loss of preaccumulated amino acid (Fig. 3A).

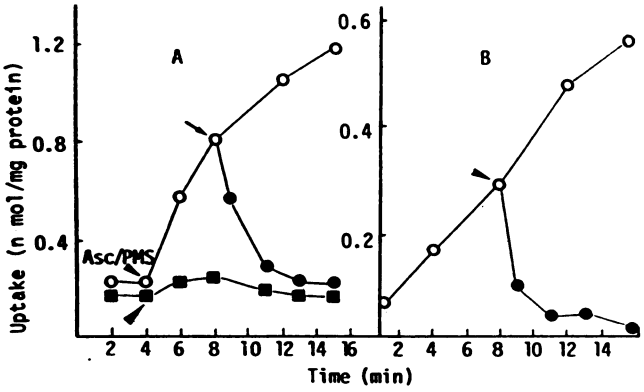

FIG. 3. (A) Accumulation (○) of L-[2,3,4,5-^{3}H]proline by *E. coli* cytoplasmic membrane vesicles and efflux (●) of the amino acid upon the addition of AS-48 (arrow). Vesicles were prepared as described in the text and diluted 1:100 in buffer. After the addition of labeled proline and incubation for 5 min, transport was started by the addition of Asc/PMS. In separate experiments, vesicles were incubated with AS-48 for 3 min before the addition of Asc/PMS (■). (B) *E. faecalis* vesicles were diluted 1:100 in 50 mM Tris-maleate buffer (pH 7.4) containing 100 mM choline chloride, 1 μM valinomycin, and L-[4,5^{3}H]leucine. The accumulation of labeled leucine was measured in controls (○) and in vesicles incubated with AS-48 (arrowhead) (●). The concentration of AS-48 was 4 μg/ml for all experiments. Portions (100 μl) of each vesicle preparation were filtered through Millipore GSTF02500 filters and washed twice with buffer. Amino acid transport is expressed as nanomoles per milligram of protein.

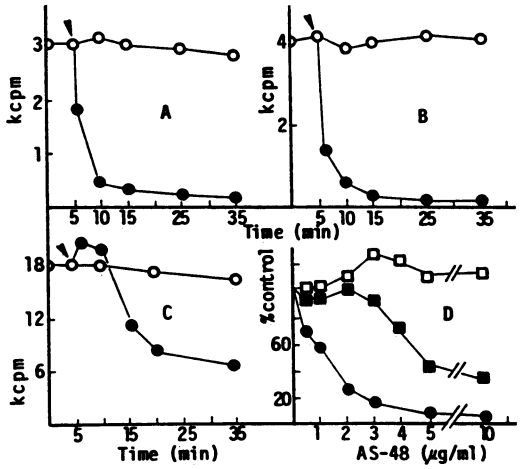

FIG. 4. Efflux of $^{86}Rb^+$ (A), [5,6-^{3}H]uridine (B), and [carboxyl-^{14}C]dextran (C) from L-α-phosphatidylcholine (asolectin) liposomes mediated by the addition of AS-48 (5 μg/ml) (●). ○, Controls. (D) Liposomes loaded with uridine (●) were incubated for 5 min with increasing antibiotic concentrations. Liposomes loaded with [carboxyl-^{14}C]dextran were incubated with increasing antibiotic concentrations for 5 min (□) or 15 min (■). Liposomes were prepared in 20 mM potassium phosphate buffer (pH 7.2) as described in the text and diluted 1:100 in this buffer before use. Aliquots (100 μl) from controls and from portions treated with AS-48 were collected on Millipore GSTF02500 filters.

In separate experiments, cells were preincubated with labeled proline and AS-48 for 3 min. The addition of Asc/PMS resulted in a negligible increase in the amount of radioactivity retained in the filters (Fig. 3A), indicating that the capacity of the vesicles to carry out active transport upon energization was impaired by previous incubation with AS-48 without a requirement for a preexisting membrane potential.

The uptake of labeled leucine promoted by valinomycin-induced potassium efflux in *E. faecalis* vesicles was equally impaired by a low concentration of AS-48 (4 μg/ml). Preaccumulated leucine was also lost upon treatment with the antibiotic (Fig. 3B). Thus, the removal of the cell envelopes did not diminish the sensitivity of *E. faecalis* cells. *E. coli* vesicles were more sensitive to AS-48 at 4 μg/ml than were intact cells, in which the effects of AS-48 were observed at concentrations 10 to 15 times higher (7).

Effect of AS-48 on artificial asolectin vesicles. To determine the capacity of peptide AS-48 to interact with artificial membranes, we prepared liposomes and loaded them with labeled rubidium, uridine, or aspartic acid. The addition of AS-48 (5 μg/ml) rapidly impaired the capacity of the liposomes to retain labeled rubidium or uridine, even when no artificial membrane potential was imposed (Fig. 4A and B). The efflux of labeled uridine proceeded as fast as the rubidium loss, and the radioactivity retained by the liposomes dropped to basal (nonspecific) levels within 10 min following the addition of AS-48 (Fig. 4B). Similarly, the radioactivity of liposomes loaded with aspartic acid dropped rapidly after 5 min of incubation with AS-48 (data not shown). These results also suggest a nonspecific interaction of AS-48 with membranes, leading to the formation of nonselective pores and the free diffusion of low-molecular-weight solutes.

The loss of uridine by potassium-loaded liposomes in which an artificial diffusion potential (negative inside) was imposed by the addition of valinomycin was similar to that shown in Fig. 4B, suggesting that the effect of AS-48 is independent of the membrane potential. Clumping of liposomes was observed when higher concentrations of AS-48 were used.

In separate experiments, the effect of AS-48 on the retention of labeled dextran (M_w, 50,000 to 70,000) was investigated. The addition of AS-48 (5 μg/ml) to dextran-loaded liposomes resulted in an increase in the counts retained in the filters during the first minute following AS-48 addition. Within the next 5 min, the counts of treated liposomes remained higher than those of the controls; they dropped gradually to about 35% of control counts by 20 min. Further incubation did not reduce the levels of retained radioactivity below these values (Fig. 4C). No binding of labeled dextran was observed when labeled dextran was filtered alone or mixed AS-48.

The relationship between antibiotic concentration and liposome permeation was also investigated. Liposomes loaded with labeled uridine or dextran were incubated for 5 min with increasing concentrations of antibiotic and tested for retention of the labeled markers. The loss of labeled uridine was directly proportional to increasing antibiotic concentrations up to 3 μg/ml (Fig. 4D). Liposomes treated with any antibiotic concentration higher than this retained less than 20% of the initial uridine after 5 min of incubation.

On the other hand, when liposomes loaded with labeled dextran were incubated with different concentrations of AS-48 (between 0 and 10 μg/ml) for 5 min, no loss of radioactive material was observed. On the contrary, the level of radioactivity retained in the filters was about 25% higher than the control level for antibiotic concentrations between 2 and 5 μg/ml. Since our liposome preparations may have contained untrapped labeled dextran, we attribute this increase in radioactivity to the fusion of liposomes upon incubation with AS-48. This phenomenon has been visualized by electron microscopy and will be discussed below. A large visual change in the turbidity of the liposome suspension, which became opaque when concentrations of AS-48 higher than 5 μg/ml were used, was also observed.

Increasing the time of incubation with AS-48 from 5 to 15 min caused a concentration-dependent loss of labeled dextran. Nevertheless, higher concentrations of antibiotic (above 3 μg/ml) were required to reduce the amount of radioactivity retained to about 40% of that retained by controls (Fig. 4D).

These results suggest that the primary effect of AS-48 is the formation of pores that allow the free diffusion of low-molecular-weight compounds, while those of a higher mass, such as dextran, are retained or diffuse much more slowly. Prolonged incubation and an increase in the antibiotic concentration may bring about secondary effects such as fusion of membranes and, probably, more chaotic disorganization.

Liposomes prepared by sonication were seen by electron microscopy as unilamellar structures (Fig. 5A) of variable size (from 1.3 to 4.5 μm in diameter). When treated with AS-48 for 15 min, they appeared as multilamellar aggregates (Fig. 5C). Intermediate stages in which very small vesicles or blebs seemed to protrude from or to adhere to larger liposome structures were also observed (Fig. 5B). These structures were always smaller (less than 0.55 μm in diameter) than control liposomes. Fusion of liposomes and formation of multilamellar structures would explain the entrapment of dextran seen in earlier experiments.

Electrical measurements. The effect of AS-48 on planar

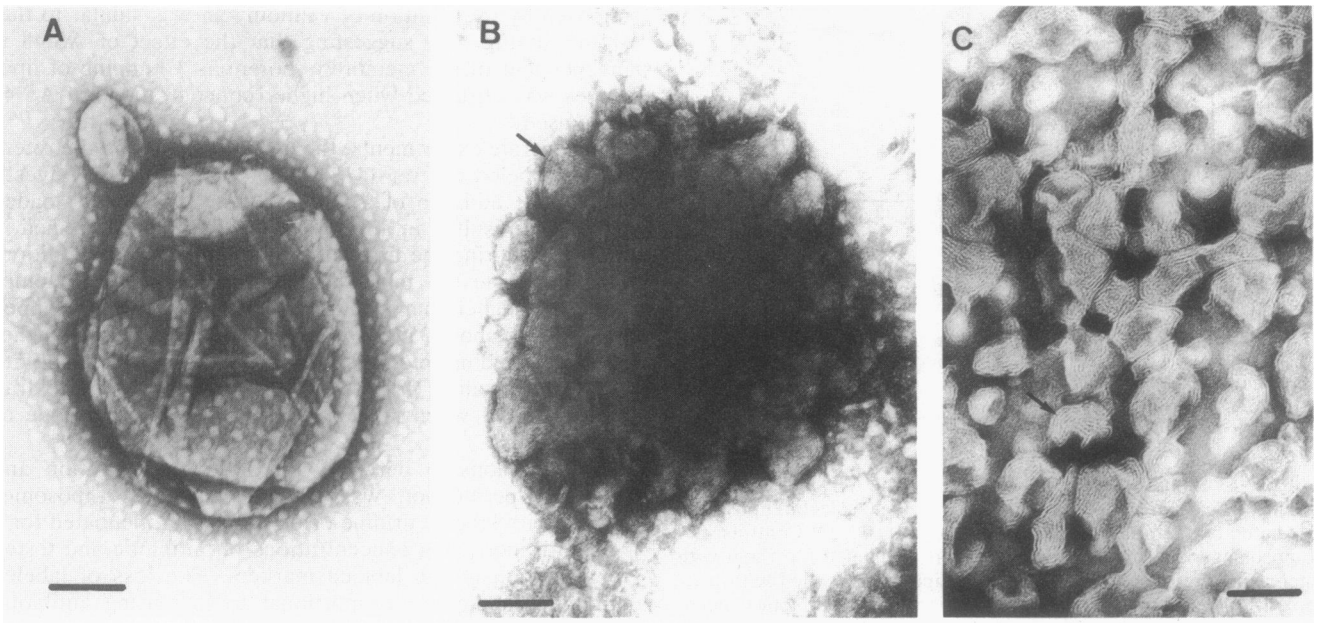

FIG. 5. Electron micrographs of asolectin vesicles (liposomes) stained with uranyl acetate as described in Materials and Methods. (A) Controls (magnification, ×63,000). (B and C) Liposomes treated with AS-48 for 5 and 15 min, respectively (magnification, ×63,000 each). The concentration of AS-48 was 5 μg/ml. Bar, 0.1 μM. The arrows in B and C indicate multilamellar structures.

phospholipid bilayer membranes was also tested, because of the suspicion that AS-48 might be able to form channels. Bilayers were formed from asolectin as described in Materials and Methods. After it was determined that the bilayers had a high resistance, AS-48 was added to the *cis* compartment. After a potential of −50 mV was applied to the *trans* compartment, a stepwise increase in conductivity occurred and led to a rapid loss of membrane resistance (in about 7 s) (Fig. 6A). During this short period of time, channels with conductances of 12 and 18 pS were observed (Fig. 6B). Some of these channels closed rapidly, while others remained open for a longer period of time or never closed. The application of lower potentials (−20 to −40 mV) resulted in long silent periods after antibiotic addition and then a final outburst of channels, and the recordings obtained were very similar to those shown in Fig. 6A. The increase in the conductivity of the bilayers may be attributed to an increase in the number of simultaneously open channels or to an opening of pores. The rapid course of the accumulation of electrical events and the short life of the bilayers once the process began (about 7 to 10 s) made it difficult to characterize this phenomenon more closely. Nevertheless, a rough estimate of the sizes of the pores formed by AS-48 can be made with the formula $\Lambda = \sigma \times \pi \times (r^2/l)$. Assuming that a cylindrical pore with a length (l) of 6 nm (corresponding to the membrane thickness) is filled with a solution of the same specific conductance (σ) as the external solution, the average pore diameter ($d = 2r$) can be estimated. For a pore conductivity (Λ) of 18 pS, the pore diameter would be 0.7 nm.

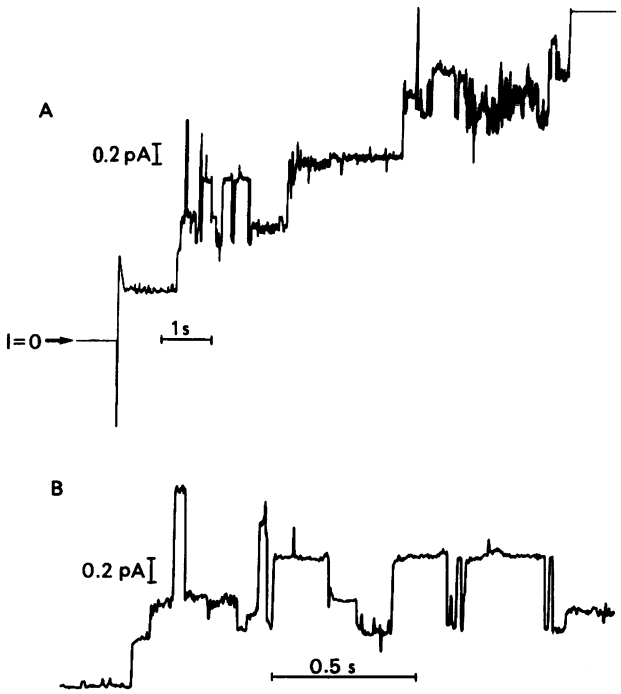

FIG. 6. Current (conductance) fluctuations induced by AS-48 in a phospholipid bilayer. The large transient marks the time when the voltage was switched from 0 to −50 mV. Immediately afterwards, a sudden burst of electrical events occurred (A) and resulted in the disruption of the bilayer in about 7 s. The initial part of the recording is shown in an expanded scale in panel B. During this interval, channels with conductances of 12 and 18 pS could be seen. Membranes were bathed with salt solutions of 10 mM HEPES containing 100 mM KCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.1 mM EDTA (pH 7.0). AS-48 was added to a final concentration of 1 μg/ml.

DISCUSSION

The data presented in this paper strongly suggest that peptide AS-48 forms channels or pores in bacterial as well as artificial membranes. Its capacity to render bacterial membranes permeable to ions and low-molecular-weight compounds and to bring about the collapse of the membrane

potential satisfactorily explains its bactericidal action on sensitive microorganisms. These effects are also observed in gram-positive bacteria with other antibacterial low-molecular-weight basic peptides, such as nisin (21) and Pep-5 (22). As evidence of its broad spectrum, peptide AS-48 also inhibits several gram-negative bacteria, although they are somewhat less sensitive, probably because of their different cell envelope, since the outer membrane must be considered a barrier that must be overcome before the cytoplasmic membrane is reached. Higher concentrations of AS-48 may induce a more pronounced membrane disorganization, a useful mechanism for permeation of the outer membrane. It is worth noting that most antibacterial substances produced by gram-positive bacteria fail to inhibit gram-negative microorganisms.

The capacity to permeate membrane vesicles derived from *E. faecalis* as well as those derived from *E. coli* suggests that AS-48 can interact directly with the cytoplasmic membrane without the mediation of surface receptors. This idea is supported by the effects of AS-48 on artificial lipid systems, such as liposomes or bilayers. Like colicins I_a and E1 (26), AS-48 can interact with artificial membrane vesicles and induce permeability to low-molecular-weight solutes when it is used at low concentrations. This property is not shared by the peptides nisin (21) and Pep-5 (22). AS-48 does not require a potential for membrane interaction, as the above-mentioned peptides and certain colicins do. In addition, higher concentrations of AS-48 can also induce disorganization of lipid structures and rearrangement into multilamellar aggregates. Fusion of phospholipid vesicles has also been described for several colicins (19).

Finally, the formation of ion channels or pores of low specificity by the insertion of AS-48 molecules into the cytoplasmic membrane provides an efficient mechanism for the induction of cell depolarization. The formation of transmembrane pores by AS-48 (with an estimated diameter of about 0.7 nm) would allow the diffusion of low-molecular-weight solutes from the cells (mainly ions and amino acids), dissipating the membrane potential and rendering the cells nonviable. The formation of voltage-dependent pores is the mechanism by which peptide Pep-5 (14) and colicins A (20), E_1 (3), K (24), I_a (26), and I_b (27) exert their lethal actions. The ionophoric activity of these colicins has been localized in C-terminal fragments of about 20 kDa.

Despite the fact that AS-48 resembles the peptides nisin and Pep-5 in its mode of action, its broader inhibitory spectrum and lack of lanthionine are two main differences. Further elucidation of the sequence and spatial configuration of AS-48 could be very helpful in determining its interaction with phospholipid membranes. Presumed amphipathic helixes present in AS-48 could be essential for maximal interactions with phospholipids. Attempts at sequencing carried out recently in our laboratory with Edman degradation as well as digestion with carboxypeptidases have failed, suggesting that the molecule has cyclic structure or is blocked at both ends.

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