ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1989, p. 641–645 Vol. 33, No. 5
0066-4804/89/050641-05$02.00/0
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# Effect of Peptide AS-48 on *Enterococcus faecalis* subsp. *liquefaciens* S-47

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Received 7 October 1988/Accepted 1 February 1989

**The enterococcal peptide AS-48 exerts a concentration-dependent bactericidal effect on *Enterococcus faecalis* subsp. *liquefaciens* S-47; cell rescue by cardiolipin and trypsin can be effected only in the first few minutes after antibiotic addition. Gramicidin-exposed cells are protected from killing by AS-48. Long-term and pulse incorporation of radiolabeled substrates into trichloroacetic acid-precipitable material, $O_2$ consumption, and the ability to maintain intracellular potassium levels are impaired shortly after addition of AS-48.**

Bacteria that produce antimicrobial substances showing a potential for broad-spectrum inhibition are restricted to two main groups: the sporeforming bacilli of the genus *Bacillus* and actinomycetes belonging mainly to the genus *Streptomyces*. For the genus *Enterococcus*, the peptide nisin has been the only antibiotic described for many years (8), although bacteriocin production by members of most species belonging to this genus has been thoroughly documented (3, 9, 10).

In earlier studies (6) we reported the production of a new antibacterial substance by a particular strain of group D enterococci; we called this substance antibiotic AS-48. This inhibitor is a low-molecular-weight peptide produced by *Enterococcus faecalis* subsp. *liquefaciens* S-48. Purified preparations of this compound show inhibitory activity against most gram-positive bacteria tested and also against many gram-negative species. Addition of AS-48 to exponential-phase cultures of *Enterococcus* spp. produces a significant reduction of the cell population within a short period (6). Bacteriolysis is also observed in many cases, although a prolonged incubation with antibiotic is usually required, and therefore this has been considered as a secondary effect.

In the present work we report that AS-48 interferes with most of the metabolic functions that are dependent on the cytoplasmic membrane, thus making it a target candidate for the primary biological effects of AS-48. The kinetics of AS-48 interaction with susceptible cells are also discussed.

## MATERIALS AND METHODS

**Susceptible strain and growth media.** *E. faecalis* subsp. *liquefaciens* S-47 (a clinical isolate) was selected as the indicator strain. Brain heart infusion (Becton Dickinson and Co., Paramus, N.J.) buffered with 0.15 M sodium phosphate (pH 7.2) was used as the growth medium (BHI broth). For radioactive labeling, this medium was diluted 1/15 in the same phosphate buffer and supplemented with glucose (1%) (BHI-DG). When solid, BHI was supplemented with 1.5% agar (BHA). KTY2XH (7) was used for growth in the presence of gramicidin D.

**Antibiotic preparation.** Purified preparations of antibiotic AS-48 (6) were used (specific activity, 3 arbitrary units [AU]/μg of protein). The arbitrary units were determined by the agar well diffusion method (6, 18). The final concentration was 8 AU/ml in all experiments, unless otherwise specified.

**Bactericidal assays.** When exponential-phase cultures growing in BHI broth or BHI-DG at 37°C reached an $A_{620}$ of 0.1 to 0.2, phosphate buffer or antibiotic (5%, vol/vol) was added. At desired intervals, samples were removed and serially diluted into ice-cold saline solution. The appropriate dilutions were plated on triplicate BHA petri dishes, and the average number of colonies obtained was used to construct growth and survival curves. Growth and lysis were monitored turbidimetrically at 620 nm with a Spectronic-20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

**Antagonism by cardiolipin, trypsin, and gramicidin D.** The capacity of bovine heart cardiolipin (Sigma Chemical Co., St. Louis, Mo.) (and also trypsin), which neutralizes AS-48 rapidly to rescue *Enterococcus* strain S-47 from the lethal action of AS-48, was investigated. Cardiolopin (12.5 μg/ml) and trypsin (0.4 mg/ml) were added separately to the cultures at different time intervals after AS-48 addition, and the cultures were monitored for optical density and viable cells as previously described.

At 30 min before AS-48 addition, gramicidin D (final concentration, 10 mM) was added to exponential-phase cultures of S-47 growing in KTY2XH broth.

**Measurement of macromolecular synthesis and uptake of precursors of macromolecular synthesis.** Exponential-phase cultures of S-47 growing in BHI-DG were incubated until the $A_{620}$ reached 0.1. At that point, duplicate portions (4 ml) were removed and incubated at 37°C with the following radioactive precursors: [6-$^3$H]thymidine, [5,6-$^3$H]uridine, and L-[4,5-$^3$H]leucine (2.5 μCi/ml each). After 30 min of incubation, each pair of tubes received 0.2 ml of 0.075 M phosphate buffer (pH 7.2) or antibiotic solution. At desired intervals, duplicate samples (0.3 ml) were removed from each tube and diluted in 4 ml of cold 6% trichloroacetic acid (TCA). Diluted samples were kept in ice for 60 min and then filtered through glass fiber filters (Whatman GF/C; Whatman Ltd., Maidstone, England); the filters and tubes were washed four times with 5 ml of cold TCA. The filters were oven dried and placed in vials to which scintillation liquid was added. Radioactivity was measured in a scintillation counter (no. LS70500; Beckman Instruments, Inc., Fullerton, Calif.). All radioactive material was supplied by Amersham Intl. Plc., Amersham, England.

In pulse-labeling experiments, when cultures growing in BHI-DG reached an $A_{620}$ of 0.15, phosphate buffer or AS-48 was added. At desired intervals, duplicate 0.3-ml portions were removed and incubated for 30 s in tubes previously warmed at 37°C and containing [6-$^3$H]thymidine (4.8 μCi/

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ml), [5,6-$^3$H]uridine (2.66 μCi/ml), or L-[4,5-$^3$H]leucine (4.8 μCi/ml). Following incubation, 4 ml of cold TCA was added to each tube, and the tubes were kept in ice for 60 min. Precipitated material was collected on filters and measured for radioactivity as described above.

Cell wall synthesis was monitored by measuring the incorporation of [1-$^{14}$C]acetate into *N*-acetyl groups of amino sugars (5). The cultures ($A_{620}$ = 0.1) were incubated in BHI-DG with [1-$^{14}$C]acetate (sodium salt; 8 μCi/ml), and phosphate buffer or antibiotic was added 30 min later. At desired intervals, samples (0.4 ml) were removed and placed in cold TCA. The samples were filtered through glass fiber filters and further subjected to trypsin digestion as described previously (2). The filters were finally counted for radioactivity.

**$O_2$ uptake.** An oxygen probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) was used to monitor the oxygen content of exponential-phase S-47 cultures growing in BHI broth. The temperature of the cultures was held constant in a water bath at 37°C, and the solution was stirred constantly to ensure an even distribution of $O_2$. The oxygen content was recorded with an Omni Scribe Recorder (Houston Instruments). After 30 s of incubation, when a steady decline in the oxygen concentration of the sample was established, antibiotic (8 AU/ml) or phosphate buffer was added with a hypodermic needle, and consumption was further recorded.

**Measurement of $Na^+$ and $K^+$ content.** The cellular $Na^+$ and $K^+$ content was determined as follows. Portions (6 ml) of cultures growing in buffered BHI broth (0.15 M sodium phosphate [pH 7.2]) were taken periodically and filtered immediately through membrane filters (pore size, 0.45 μm; Millipore Corp.). The cells were washed with ice-cold 0.25 M sucrose solution and suspended in 6 ml of double-distilled water. A 3-ml portion of this suspension was withdrawn for dry-weight determination. The remaining 3 ml was heated at 100°C with 1 M sulfuric acid, cooled, and then centrifuged at 13,000 × *g* for 15 min (20). The supernatant content of $Na^+$ and $K^+$ was determined by using an atomic absorption spectrophotometer (SP-1900; PYE-Unicam).

## RESULTS

**Time course of killing by antibiotic.** When cultures of *Enterococcus* strain S-47 (1.0 × $10^7$ CFU/ml) were incubated with increasing antibiotic concentrations (5, 10, and 15 AU/ml) for different times, a decrease in the number of viable cells was observed as the time of incubation or the antibiotic concentration increased (Fig. 1A). However, for antibiotic concentrations higher than 10 AU/ml, the number of viable cells decreased rapidly after a very short period of incubation with AS-48.

In separate experiments, exponential-phase cultures (1.4 × $10^8$ CFU/ml) were incubated with increasing antibiotic concentrations for 5 min and the number of viable cells was determined. As the antibiotic concentration increased above 5 AU/ml, a progressive decrease in the logarithm of the surviving fraction was observed (Fig. 1B). However, the proportionality between the antibiotic dose and the number of nonviable cells changed for antibiotic concentrations lower than 5 AU/ml. Plotting of all the values obtained resulted in a multihit kinetics curve (Fig. 1B).

**Cell rescue.** Bovine heart cardiolipin is very effective in neutralizing AS-48 solutions. It is also a physiological inhibitor of cell wall autolysins in *Enterococcus* spp. (4). Consequently, the possibility of using cardiolipin to rescue cells which had been in contact with AS-48 for different periods was investigated.

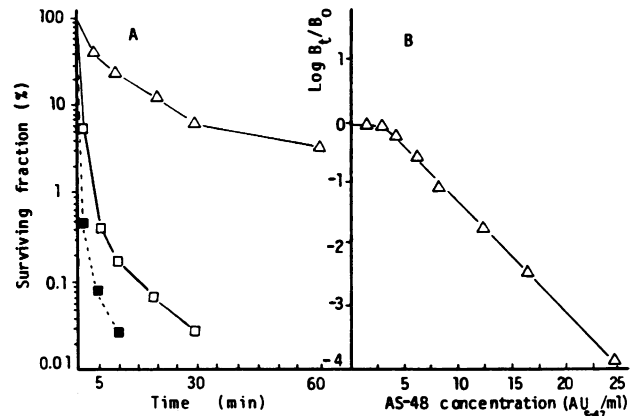


FIG. 1. Time course of AS-48-mediated killing of *Enterococcus* strain S-47 growing in buffered BHI broth. (A) Exponential-phase cultures (1.0 × $10^7$ CFU/ml) were incubated for different periods with the following increasing antibiotic concentrations (arbitrary units per milliliter): 5 (△), 10 (□), and 15 (■). (B) Cultures (1.4 × $10^8$ CFU/ml) were incubated for 5 min with increasing antibiotic concentrations. $B_o$ is the number of viable cells at time zero. $B_t$ is the number of viable cells after 5 min of incubation with antibiotic.

The capacity of cardiolipin to rescue S-47 cells depended upon the period of preincubation with AS-48; an efficient rescue (as estimated from viable count and optical density) was observed only when cardiolipin was added within 5 min of incubation with the antibiotic, but not after 15 min or more (Fig. 2A and B). Similar results were obtained when trypsin was used as the inactivating agent. Addition of cardiolipin to cultures incubated with AS-48 for longer periods (between 20 and 120 min) was also effective in preventing lysis, most probably as a result of its regulatory effect on the cell wall autolysins (4) (Fig. 2B).

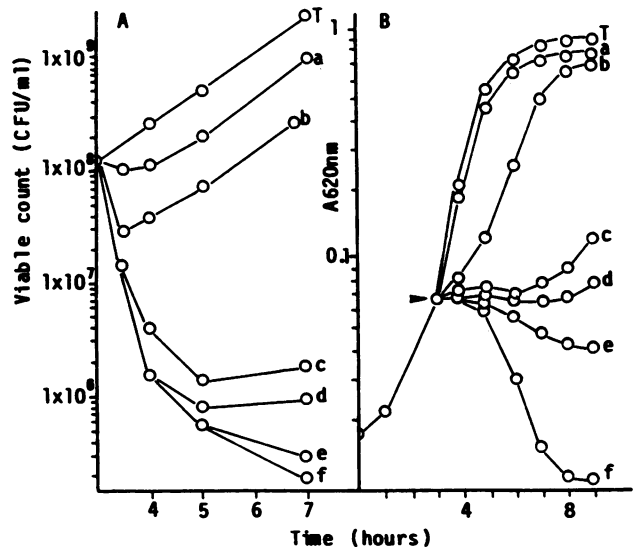


FIG. 2. Cell rescue of *Enterococcus* strain S-47 by cardiolipin as determined from viable counts (A) and optical density (B). Cardiolipin (12.5 μg/ml) was added to exponential-phase cultures growing in buffered BHI broth either 10 min before AS-48 addition (curve a) or 5, 20, 60, or 120 min after AS-48 addition (curves b to e, respectively). Other labels: T, control; f, effect of AS-48 (8 AU/ml) alone.

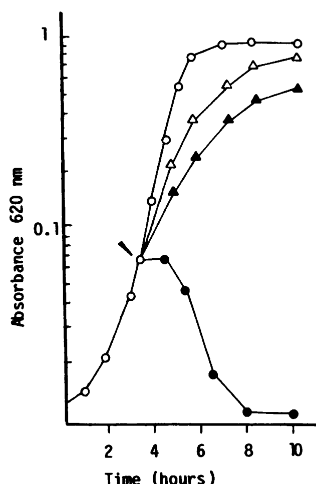


FIG. 3. Effect of AS-48 (8 AU/ml) on *Enterococcus* strain S-47 growing in KTY2XH broth with (▲) or without (●) gramicidin D (final concentration, 10 μM). Gramicidin D was added 30 min before AS-48. The effect of gramicidin alone (△) and the behavior of control cultures without gramicidin (○) are also shown.

**Antagonism by gramicidin D.** Cell growth in the absence of membrane potential has been described for both *Enterococcus* spp. (7) and *Escherichia coli* (11), provided that adequate culture conditions are present. Addition of gramicidin D (10 μM) to cultures of S-47 cells growing in BHI broth had a remarkable inhibitory effect on growth (data not shown). Nevertheless, exponential growth continued after the addition of 10 μM gramicidin D if incubation was carried out in KTY2XH broth (Fig. 3). Still lower concentrations of gramicidin D were effective in depleting both the membrane potential and pH gradient in *Enterococcus* cells growing in KTY2XH (7). Therefore, the effect of AS-48 on *Enterococcus* strain S-47 growing under these defined conditions was investigated. When cultures growing in KTY2XH reached an $A_{620}$ of 0.08 gramicidin D was added (final concentration, 10 μM). After another 30 min of incubation ($A_{620}$ = 0.1), AS-48 (8 AU/ml) was added. The results show that AS-48 had very little effect on S-47 growing in KTY2XH plus gramicidin D (Fig. 3). A bactericidal and bacteriolytic action was observed when AS-48 was tested on cultures growing in KTY2XH without gramicidin (Fig. 3).

**Effect of AS-48 on incorporation of labeled precursors.** The incorporation of radioactive precursors into acid-precipitable material was taken as a measure of macromolecular synthesis. Treatment of S-47 cultures with AS-48 resulted in a total cessation of the incorporation of L-[4,5-$^3$H]leucine, [5,6-$^3$H]uridine, [6-$^3$H]thymidine, and [1-$^{14}$C]acetate into protein, RNA, DNA, and cell wall, respectively, following antibiotic addition (Fig. 4). In addition, a partial loss of the radioactive material previously incorporated into RNA and cell wall was observed after 30 min of incubation with AS-48.

Pulse uptake of labeled precursors of macromolecular synthesis was also examined. Exponential-phase cells incubated with AS-48 for different periods (10 s and 1, 3, and 6 min) were tested for incorporation of radioactive precursors during 30-s pulses. Uptake was stopped by addition of ice-cold TCA. The radioactivity incorporated was expressed as a percentage of the amount incorporated by controls at the moment of antibiotic addition. The results are shown in Fig. 5. Incorporation of all precursors dropped below 40% of

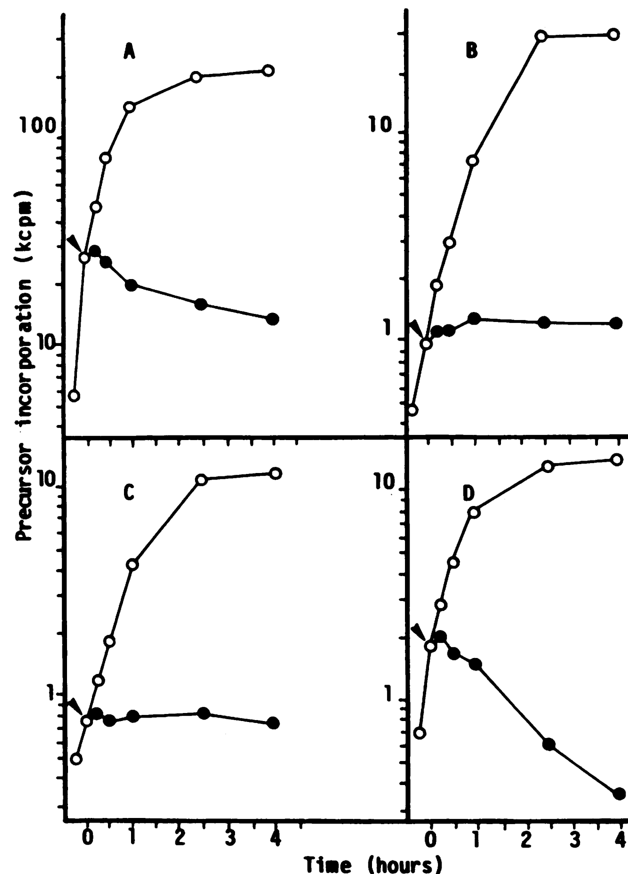


FIG. 4. Effect of AS-48 on the incorporation by *Enterococcus* strain S-47 of labeled precursors into RNA (A), DNA (B), protein (C), and cell wall (D); incubation took place in BHI-DG; after 30 min of preincubation with the labeled precursors, either AS-48 (8 AU/ml) (●) or sodium phosphate buffer (○) was added (arrowhead).

the initial incorporation within 1 min after antibiotic addition, and it was significantly reduced (below 10%) after 5 min of incubation. These values represent both transport and incorporation into macromolecules. A net transport assay was hampered by the high resistance of *Enterococcus* strain S-47 to protein synthesis inhibitors such as chloramphenicol or erythromycin.

**Effect of AS-48 on $O_2$ consumption.** When the $O_2$ content of exponential cultures of S-47 was monitored, a rapid decrease was observed. Addition of AS-48 markedly inhibited the capacity for $O_2$ uptake within 30 s (Fig. 6). Control cultures continued active consumption over the entire period monitored.

**Effect on the intracellular sodium and potassium content.** The effect of AS-48 on the intracellular $Na^+$ and $K^+$ content in cultures growing in sodium phosphate-buffered BHI broth was also examined. The capacity to maintain the cytoplasmic levels of $K^+$ was completely lost 5 min after antibiotic addition (Fig. 7B). During this interval, an increase in the cellular $Na^+$ content was also observed (Fig. 7A). These results clearly indicate a misfunctioning of the cytoplasmic membrane following addition of AS-48, with disruption of the permeability barrier for these ions at least.

## DISCUSSION

The results presented above concerning the kinetics of the interaction of peptide AS-48 with sensitive *Enterococcus*

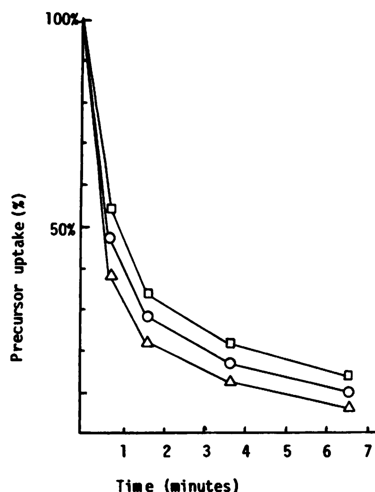


FIG. 5. Effect of AS-48 on pulse uptake of labeled leucine (○), thymidine (□), and uridine (△) by *Enterococcus* strain S-47. Samples were withdrawn from exponential-phase cultures in BHI-DG at different times after antibiotic addition (final concentration, 8 AU/ml) and incubated for 30 s with the labeled precursors. Uptake was stopped by addition of 4 ml of ice-cold TCA as described in the text. Uptake is expressed as a percentage of the amount of radioactivity incorporated by the controls at the moment of antibiotic addition.

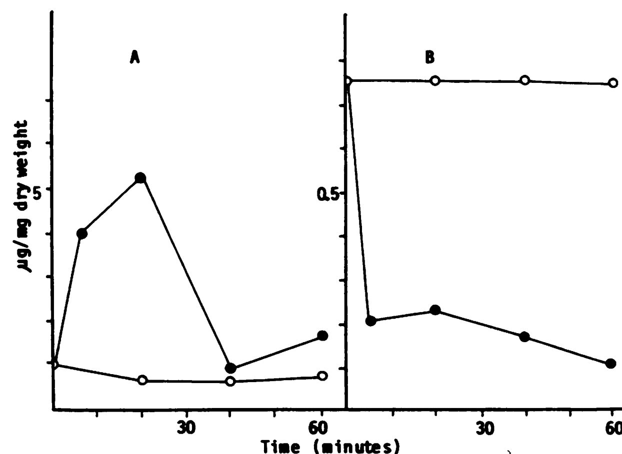


FIG. 7. Evolution of the cell content of $Na^+$ (A) and $K^+$ (B) in *Enterococcus* strain S-47 cultures growing in 0.15 M sodium phosphate (pH 7.2)-buffered BHI broth following addition of the same phosphate buffer (○) or AS-48 (8 AU/ml) (●). After the desired periods of incubation with AS-48, samples were removed and processed for ion determination as described in the text.

cells indicate that adsorption and killing take place very rapidly. Shortly after antibiotic addition, bactericidal action can be neutralized by cardiolipin and trypsin, but this is not possible after prolonged incubation. These observations are consistent with the short interval of time between antibiotic addition and the detection of biological effects (e.g., alteration of ion permeability, precursor uptake, and biosynthesis). These early effects would lead to a rapid loss of cell viability, thus making cell rescue unlikely after prolonged incubation. The fact that lysis (but not killing) can be prevented by cardiolipin during the later stages of incubation suggests that this is a secondary effect of the bactericidal action of AS-48 and that autolytic enzymes become involved, probably as a consequence of primary membrane lesions.

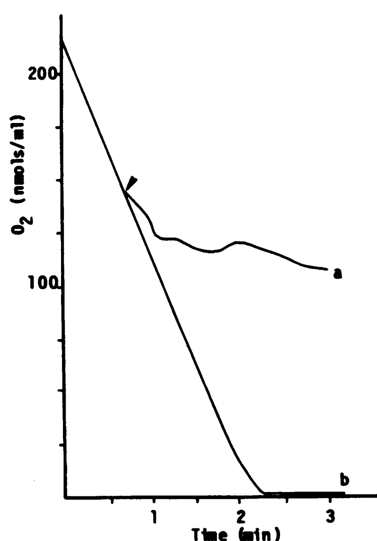


FIG. 6. Effect of AS-48 (8 AU/ml) addition on oxygen uptake by *Enterococcus* strain S-47 cultures growing in buffered BHI broth. The $O_2$ content was monitored with an oxygen electrode. The arrowhead represents the moment of antibiotic addition for curve a; curve b represents $O_2$ uptake by controls.

The impairment of the capacity to accumulate labeled substrates and to incorporate them into macromolecules, together with the loss of the capacity to maintain the cytoplasmic levels of sodium and potassium following antibiotic addition, suggests that the cytoplasmic membrane becomes damaged. In addition, membrane damage could account for other effects observed, such as cessation of oxygen uptake; synthesis of protein, DNA, RNA, and cell wall; and autolysis. Furthermore, recent experiments indicate that AS-48 increases membrane conductance in whole mammalian cells.

The high resistance to AS-48 shown by cultures growing in the absence of a membrane potential and a pH gradient strongly suggests a role for these parameters in the bactericidal effect of AS-48 on *Enterococcus* strain S-47. It is tempting to consider that either membrane potential or pH gradient or both may constitute a decisive factor in the interaction (direct or indirect) of AS-48 with the cytoplasmic membrane, as is the case for many bacteriocins such as colicins E1, Ia, A, and K (12), bacteriocin C3603 (19), and other antibacterial agents such as nisin (13) or Pep-5 (14, 16, 17) which act by forming voltage-dependent channels in planar membranes. However, additional studies must be carried out to elucidate this point.

The biological effects exerted by AS-48 on *Enterococcus* cells are similar to the effects (mainly impairment of active amino acid transport, leakage of low-molecular-weight compounds, and a total cessation of biosynthetic processes) reported for other membrane-damaging inhibitors, such as nisin and Pep-5, which also resemble AS-48 in their basic nature and molecular size. In addition, these peptides show the capacity to induce autolysis in susceptible bacteria (1, 6). The results obtained so far with AS-48 also support the idea of a general mechanism of antibiosis, by means of perturbation of the cytoplasmic membranes of microorganisms, that is mediated by strongly cationic peptides, as has been previously proposed (15).

## ACKNOWLEDGMENTS

This work was supported by a scholarship from the Ministerio de Educacion y Ciencia and a grant from the Junta de Andalucia.

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