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Cellular effects of monohydrochloride of L-arginine, N$^{\alpha}$-lauroyl ethylester (LAE) on exposure to *Salmonella typhimurium* and *Staphylococcus aureus*

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ABSTRACT

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Aims: Here we study the effect of monohydrochloride of L-arginine, N$^{\alpha}$-lauroyl ethylester (LAE), a cationic preservative derived from lauric acid and arginine, on the cell envelopes of *Salmonella typhimurium* and *Staphylococcus aureus* at sub-lethal concentration such as their respective minimal inhibitory concentrations, 32 and 8 μg ml^{-1}, respectively.

Methods and Results: Bacterial populations were studied by using transmission electron and fluorescence microscopy (TEM and FM), flow cytometry (FC) and ion-flux across the cellular membrane. Cell integrity was altered mainly in the outer membrane of *S. typhimurium*, but there was no significant change in the cytoplasm. However, in *Staph. aureus,* clear zones, abnormal septation and mesosome-like structures were observed in the cytoplasm. Bacterial populations were double-stained with propidium iodide (PI) and SYTO-13 for FC analysis. In *S. typhimurium* the proportion of damaged cells after 24 h was 97% and in *Staph. aureus* 56·3%. LAE induced transmembrane ion flux, the increase of potassium leakage after 30 min of contact was 7·7 and 3·34 μg ml^{-1} for *Staph. aureus* and *S. typhimurium*, respectively. Membrane disruption was detected by measuring the proton flow across the membrane.

Conclusions: Disturbance in membrane potential and structural changes was caused by LAE, although cells were not disrupted.

Significance and Impact of the Study: This is the first time the cellular effects of LAE on bacterial cells were studied.

Keywords: antimicrobial effect, electron microscopy, flow cytometry, surfactants, transmembrane ion flux.

INTRODUCTION

The antimicrobial activity of surfactants is a direct consequence of their chemical properties. These properties include reduction of surface tension and the formation of ionic aggregates, which in turn lead to changes in conductivity and solubility of solutions. These surfactants may degrade or solubilize cell membranes at concentrations even below critical micellar concentration, leading to loses of membrane potential, altering cell permeability and leaking of ions and cell constituents. The results of these alterations are metabolic inhibition, growth arrest or cell lysis (Kanazawa *et al.* 1995).

Monohydrochloride of L-arginine, N$^{\alpha}$-lauroyl ethylester (LAE) is a cationic preservative (Infante *et al.* 1984). It is derived from lauric acid and arginine. The preparation and application of this product is described in Spanish patent application ES 512643 A1 (García Domínguez *et al.* 1983) and European Patent applications (Urgell and Seguer 2003a,b,c). This compound inhibits the proliferation of several micro-organisms, such as bacteria, fungi

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and yeasts (Infante *et al.* 1984). Its chemical and physico-chemical properties, i.e. solubility in water, surface tension and critical micellar concentration, have been described (Infante *et al.* 1985), but, no studies on its interaction with the bacterial cell have appeared to date.

Different approaches have been developed to understand the mode of action of cationic compounds: potential membrane disturbance, alteration of the efflux pumps, leakage of cytoplasm constituents or structural changes (Paulsen *et al.* 1996; Suller and Russel 2000; Tattawasart *et al.* 2000). Flow cytometry (FC) is increasingly used to assess membrane damage, depolarization, bacterial integrity and cell viability (Davey and Kell 1996; Suller and Lloyd 1999). Antimicrobials may have dramatic effects upon the integrity of bacterial envelopes causing lysis or massive leakage of cell constituents. Consequently, assessment of potassium leakage and of proton-gradient disturbance is a way of assessing the effects of antimicrobials (Denyer and Hugo 1991). Structural changes in the cell envelopes can be observed through transmission electron microscopy (TEM), which has been used to analyse the effect of various antimicrobial agents on bacteria (Friederich *et al.* 2000; Tattawasart *et al.* 2000).

The aim of this paper is to study the effect of LAE on the cell envelopes of *S. typhimurium* and *Staph. aureus* by using electron and fluorescence microscopy (FM) observations, FC and ion-flux across the cell membrane.

MATERIAL AND METHODS

Antibacterial product and chemicals

LAE was supplied by LAMIRSA, SA (Terrassa, Spain). Stock solutions of powdered LAE were prepared in bi-distilled water (Millipore, MA, USA). Molecular dyes were supplied by Molecular Probes Europe BV, Leiden, the Netherlands. Microbiological products were supplied by ADSA (Barcelona, Spain), Pronadisa (Barcelona, Spain) and Scharlau (Barcelona, Spain). All other chemicals and reagents were of analytical grade supplied by Panreac (Barcelona, Spain) or Sigma Chemicals Co (MA, USA) and used as purchased.

Micro-organisms

The *S. typhimurium* ATCC 14028 and *Staph. aureus* ATCC 6538 used in this study were obtained from the ATCC (Manassas, VA, USA) and sub-cultured weekly on trypticase soya agar (TSA; Pronadisa, Barcelona, Spain). Strains were maintained frozen in cryovials (AES Laboratoire, Combourg, France) at −80 °C.

Minimal inhibitory concentration (MIC)

The MICs of LAE were determined by using a broth micro-dilution assay (Woods and Washington 1995). Serial dilutions of LAE, between 256 and 0·5 μg ml^{-1} final concentration, in Muller–Hinton Broth (Oxoid Ltd, Basingstoke, UK) were dispensed in the corresponding wells of a 96-well polypropylene microtitre plate (Costar; Corning Incorporated, Corning, NY, USA). The corresponding dilutions were inoculated with a suspension of the test organism on Muller–Hinton Broth to a final concentration of *ca* 10^4 CFU ml^{-1}. The MIC was defined as the lowest concentration of antibacterial agent that inhibited development of visible growth after 24 h of incubation at 37°C. Experiments were conducted in triplicate.

Exposure to LAE

Suspensions of the micro-organisms were obtained by growing the bacteria overnight at 30°C in TSA and then resuspending them in 10 ml Ringer's 1/4 solution filtered through 0·22 μm pore-size membrane (Millipore). Each bacterial suspension was pelleted by centrifuging at 8000 *g* for 20 min, washed twice in sterile filtered 1/4 Ringer's solution, and finally resuspended to obtain a concentration of 10^7–10^8 CFU ml^{-1}. An appropriate volume (250 μl) of the respective cell suspensions was used to inoculate flasks containing 24 ml of buffered peptone water (ADSA) to obtain a cell density of *ca* 10^5–10^6 CFU ml^{-1}. LAE stock solutions (1 ml) was added to flasks containing 24 ml of the respective bacterial suspensions in order to reach a final concentration of LAE corresponding to the MIC, 32 and 8 μg ml^{-1} for *S. typhimurium* and *Staph. aureus*, respectively, in a final volume of 25 ml of peptone water. The inoculated flasks were kept in darkness at room temperature.

For TEM observations the contact time was 3 h; 12·5 ml samples were taken and centrifuged at 8000 *g* for 30 min. The sediment was resuspended in 2 ml of Ringer's 1/4 solution filtered through 0·22 μm pore-size membrane. The contact times established for FC were 30 min, in 3, 6 and 24-h experiments. At each time point, 5 ml samples were centrifuged at 8000 *g* for 30 min and washed as described above, to eliminate LAE.

In all cases, control experiments were carried out in parallel; cells were incubated in LAE-free buffer solution and treated under the same conditions.

Staining protocols

All dyes were made up as stock solutions at 500 μM for SYTO-13 in dimethyl sulphoxide and 1 mg ml^{-1} for propidium iodide (PI) in distilled water.

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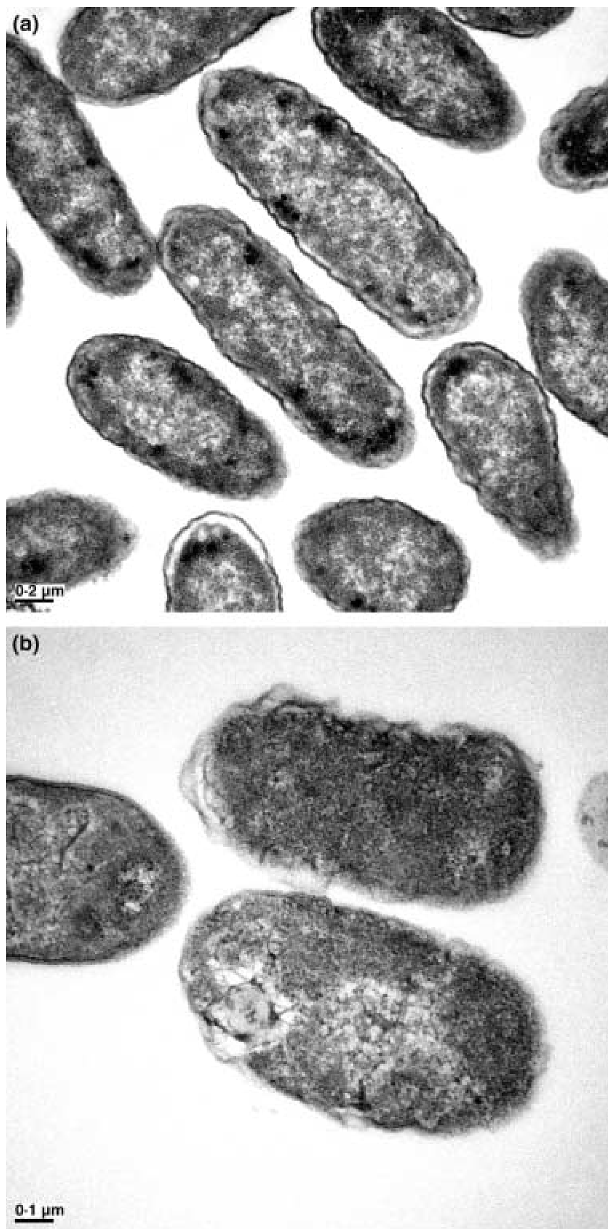

Fig. 1 Electron transmission microscopy of *Salmonella typhimurium* ATCC 14028. (a) Control cells, bar: 0·2 μm; (b) cells treated with L-arginine, N^{α}-lauroyl ethylester (32 μg ml^{-1}) 3 h, bar: 0·1 μm

Dyes and staining protocols for FM were as follows: 2 μl of the probe stock solution of SYTO-13 and 20 μl of the probe stock solution of PI were added to aliquots (1 ml) of bacterial suspensions in filtered Ringer's 1/4 solution. The mixtures were incubated at room temperature for 30 and 2 min for *S. typhimurium* and *Staph. aureus*, respectively.

Staining protocols for FC observations were as follows: 1 μl of a stock solution of SYTO-13 and 10 μl of a stock solution of PI were added to 500 μl of the bacterial suspension in filtered Ringer's 1/4 solution. The bacteria were allowed to incubate with the dyes for 30 and 2 min for *S. typhimurium* and *Staph. aureus*, respectively, in both cases at room temperature, prior to FC analysis. A second 500μl aliquot was treated identically, except that the LAE was not added. Cells killed by heat exposure were used as controls for PI staining.

Transmission electron microscopy

After treatment of cell suspensions with LAE for 3 h at the minimal inhibitory concentrations (MIC) for each micro-organism, the bacterial pellets were rinsed in a 0·1 M phosphate buffer (pH 7·4), washed three times and fixed with 2·5% buffered glutaraldehyde for 1 h at 4°C. The cells were then postfixed in 1% buffered osmium tetroxide for 1 h, stained with 1% uranyl acetate, dehydrated in a graded series of ethanol, and embedded in L.R. White (London Resin Co. Ltd, London, UK) white resin. Ultra-thin sections were prepared and stained with 1% uranyl acetate and sodium citrate. Microscopy was performed with a Philips EM 30 (Eindhoven, Germany) microscope under an acceleration of 60 kV.

Fluorescence microscopy

After being dyed, the bacterial suspensions were filtered using 0·2 μm and 13 mm diameter black polycarbonate Isopore^{TM} membrane filters (Millipore; Cat. number GTBP01300) specific for epifluorescence. The membrane filters together with the marker-stained cells were placed on a glass slide, fixed with a slide cover and observed with a Leica DMRB microscope fitted with a 100-W mercury arc lamp, Leica L4.513810 filter (excitation, 450–490 nm; emission 515–560 nm) for SYTO-13 and Leica N2.1.513812 filter (excitation, 515–560 nm; emission 580 nm) for PI and a ×63 and ×100 oil immersion lens. Photomicrographs were obtained with a Cooled CCD Micromax RTE 782–4 camera (Digital imaging Systems, Fairfield, IA, USA) and photographs and analysis of images were treated with METAMORPH (Universal Imaging Corp., Wetchester, PA, USA).

Bacterial counts

Viable counts were calculated from the colony forming unit (CFU ml^{-1}) obtained on TSA. After an appropriate dilution in Ringer 1/4, the sample was inoculated on plates and incubated at 37°C for 24–48 h. Rapid separation of bacteria from LAE was achieved by centrifugation at 5000 *g* in a bench centrifuge for 10 min and subsequent dilution on Ringer 1/4 prior plating. Cell counting was performed per triplicate.

Flow cytometry

From the centrifuged bacterial suspensions prepared as described above, the pellet was resuspended in 5 ml of Ringer's 1/4 solution (ADSA), filtered through 0·22 μm pore-size membrane and stained with SYTO-13 as described above. Nucleic acid SYTO-13 dye penetrates in bacterial cells, whether they are alive or dead, and produces green fluorescence. Excitation was carried out at 488 nm, green fluorescence was detected at the 525 nm zone and background noise remained nonfluorescent. PI is a fluorochrome that is used as a nucleic acid intercalator and produces red fluorescence.

Flow cytometry studies were performed with a Coulter Epics Elite flow cytometer (Coulter Corp. Miami, FL, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser (for SYTO-13 and PI excitation) and was set up with the standard configuration. Fluorescent beads (1 μm Fluoresbrite carboxylate micro spheres; Polysciences, Warrington, PA, USA) were used as an internal standard for scatter and fluorescence. The green emission from SYTO-13 was collected through a 525 nm band-pass filter. The red emission from PI was collected with a 675 nm band-pass filter. Forward- and side-scatter and fluorescence signals were collected in a logarithmic scale. Bacteria were always detected by their SYTO-13 or PI fluorescence; thus, a double discrimination on green and red fluorescence was used rather than scatter, so obtaining a better resolution and decreasing the background. Data were analysed with Elitesoft version 4.1 (Coulter Corp.) and WinMDI version 2.8 software (Windows Multiple Document Interface. Build #05 03-09-1999© 1993–1998, Joseph Trotter, The Scripps Research Institute).

Ion efflux

Potassium leakage was determined as described by Denyer and Hugo (1991). Briefly, the method uses cells grown on TSA at 37°C for 12 h. Cells were then harvested in 10 ml of 0·9% NaCl, washed three times with 0·9% NaCl by centrifugation at 5000 *g* for 30 min at 15°C and resuspended in 25 ml of 1 mM glycil–glycine buffer solution pH 6·8, to obtain a cell density of $8{\cdot}6 \times 10^7$ and $7{\cdot}5 \times 10^7$ CFU ml^{-1} for *S. typhimurium* and *Staph. aureus*, respectively. At time intervals of 0, 30, 90 and 180 min, 5 ml of cell suspension were removed and centrifuged in a bench centrifuged at 5000 *g* for 10 min to separate cells from LAE. As control experiments, cells were incubated in LAE-free buffer solution in the same conditions. The potassium concentration in the supernatant was measured using an atomic absorption Philips PU9200X spectrophotometer (Philips, Cambridge, UK). Absorbance values were converted into potassium ion concentration (ppm) by reference to a curve pattern previously established using standard potassium ion solutions of 0, 0·05, 0·1, 0·2, 0·3 and 0·5 ppm concentrations. Experiments were conducted in triplicate.

To determine proton extrusion, cultures were grown on the surface of a nutrient agar plate at 37°C for 12 h. Cells were then harvested in 10 ml of 0·9% NaCl (Panreac) washed three times by centrifugation 5000 *g* for 30 min at 15°C and finally resuspended in 10 ml of 1 mM glycil–glycine pH 6·8 buffer solution (Sigma) buffer solution pH 6·8 to obtain a cell density of $2{\cdot}86 \times 10^9$ and $4{\cdot}57 \times 10^9$ CFU ml^{-1} for *S. typhimurium* and *Staph. aureus*, respectively. Stock solution (400 μl) of LAE was added to 10 ml of the respective bacterial suspensions to obtain LAE concentration of 32 μg ml^{-1} for *S. typhimurium* and 8 μg ml^{-1} for *Staph. aureus*. Cell suspension control was also run in parallel. Experiments were conducted in triplicate. The suspensions with LAE were allowed to equilibrate for 5 min and then a sufficient quantity of HCl (10 mmol l^{-1}) was added to reduce the pH of the cell suspensions to 4·6–5·0. The pH was then measured at 1-min intervals for 5 min in a Crisson micropH 2000 (Crisson, Alella, Spain). The pH initially decreased (typically by 0·5 U pH), but reverted because of the flow of protons through the membrane to the interior cytoplasm. The proportion of proton accumulation was calculated from the increase in pH obtained. The change in pH was used to calculate the rate of proton accumulation, expressed in nanomoles H^+ per minute per $2{\cdot}86 \times 10^9$ CFU ml^{-1}, for both control samples and treated samples of *S. typhimurium* and expressed in millimoles H^+ per minute per $4{\cdot}57 \times 10^9$ CFU ml^{-1} in the case of *Staph. aureus*.

RESULTS

The MIC of LAE against *S. typhimurium* and *Staph. aureus* were found to be 32 and 8 μg ml^{-1} respectively. In this study, these sub-lethal concentrations were used to observe the bacterial population with different levels of cell damage.

Electron microscopy

In order to observe structural modifications of bacterial cells because of LAE treatment, the ultra-thin sections of bacteria were examined under TEM. Control population of *S. typhimurium* at 3 h of cultivation is shown in Fig. 1a. Swelling of the outer membrane and membrane-enclosed vacuoles because of the effect of LAE on *S. typhimurium* are clearly seen (Fig. 1b). However, cell integrity was evident and the cytoplasm does not appear significantly altered. This finding is consistent with the epifluorescent electron microscopy micrographs (Fig. 2), which showed that most of the population, after 3 h of contact with LAE, was stained with PI. A TEM micrograph of the control

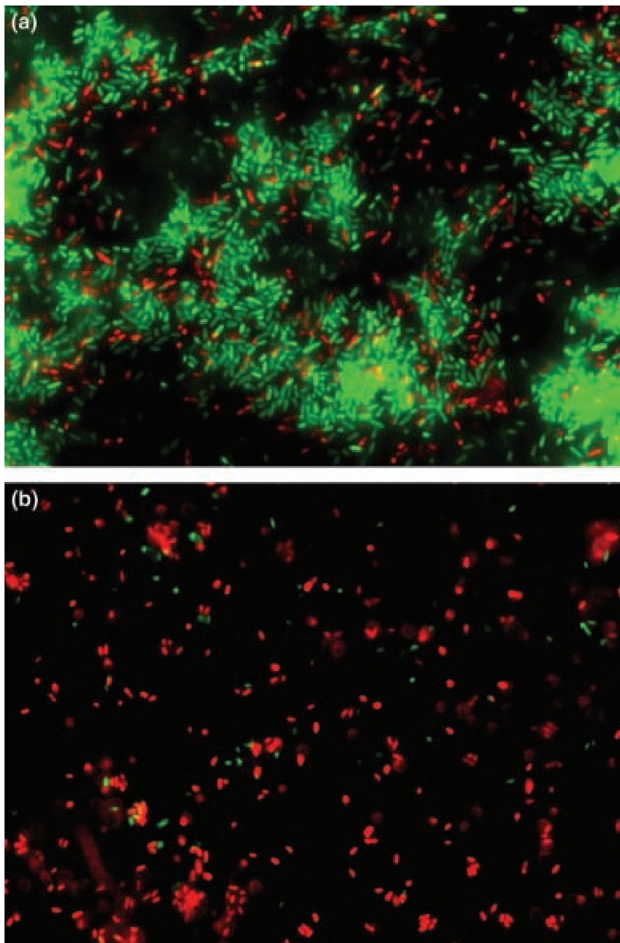

Fig. 2 Fluorescent microscope micrographs of *Salmonella typhimurium* after 3 h of incubation. Control population (a) and L-arginine, N^{α}-lauroyl ethylester-treated population; (b) cells were stained with Syto-13 and propidium iodide. Green cells stained with Syto-13 and in red cells stained with propidium iodide

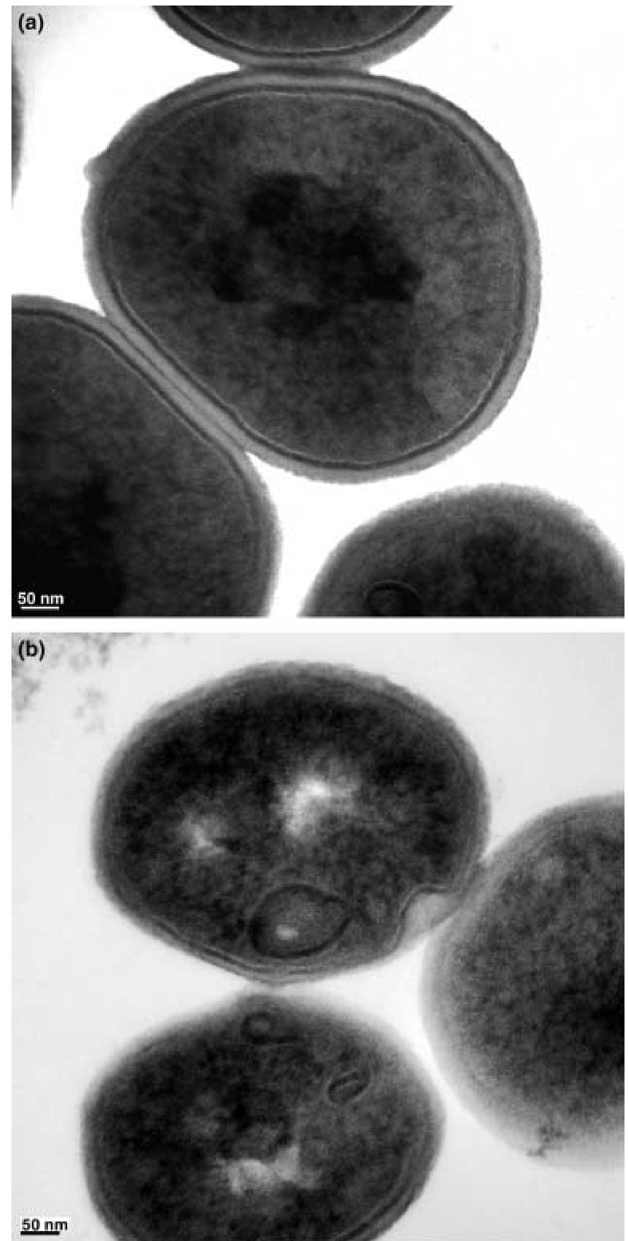

Fig. 3 Electron transmission microscopy of *Staphylococcus aureus* ATCC 6538. (a) control cells, bar: 50 nm; (b) cells treated with L-arginine, N^{α}-lauroyl ethylester (8 μg ml^{-1}) 3 h, bar: 50 nm

population of *Staph. aureus* is shown in Fig. 3. In this case, LAE causes multiple alterations in the population (Fig. 3b) compared with the control population (Fig. 3a). Different effects were observed (Fig. 3b): mesosome-like formations (structures derived from the cell membrane), intracytoplasmic white spots, clear zones and multiseptated cells were observed, although it should be stressed that the integrity of the membrane and the cytoplasm content are maintained. Finally, although some damage occurred in the membrane, cell integrity was confirmed by FM observations, in which almost all the treated population was stained with PI (Fig. 4).

In both cases, *S. typhimurium* and *Staph. aureus,* cells remained intact: cell lysis was not observed in the micrographs and the integrity of the cells was clear in the fluorescent micrographs.

Flow cytometry

Two fluorochromes, which bind nucleic acids were used, SYTO-13, which penetrates all types of cellular membranes and PI, which only penetrates the bacterial membrane when it is damaged. In double-cell staining (Syto/PI), PI removes SYTO-13 and consequently the damaged cell retain progressively PI fluorochrome (Lopez-Amorós *et al.* 1995; Vives-Rego *et al.* 2000).

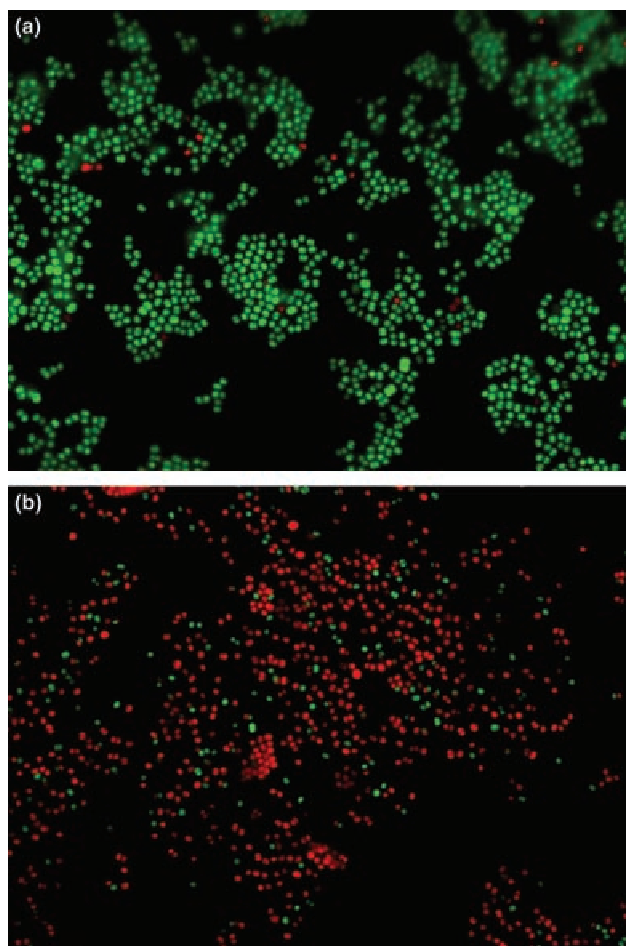

Fig. 4 Fluorescent microscope micrographs of *Staphylococcus aureus* ATCC6538 after 3 h of incubation. (a) Control population; (b) cells treated with L-arginine, N^{α}-lauroyl ethylester (8 μg ml^{-1}). Cells were stained with Syto-13 and propidium iodide. Green cells stained with Syto-13 and in red cells stained with propidium iodide

The results with *S. typhimurium* are shown in Fig. 5. The population was successfully stained. Most of the control population (94%) was stained with SYTO-13, whereas only a small proportion was stained with PI (3·48%), or double stained (2·52%). This indicates that these cells had damaged membranes. However, the control population recovered (98·4%) at 24 h of incubation (Fig. 5a). After being treated with 32 μg ml^{-1} of LAE, the *S. typhimurium* suspension showed a different pattern (Fig. 5b): after 30 min of contact most of the population (90·7%) was stained with PI and only 6·3% retained SYTO-13. The proportion of membrane-damaged cells increased with contact time to about 97% at 24 h of incubation. At that time, only a small fraction of the population (2·1%) retained both fluorochromes, thus indicating that the membranes of these cells were partially damaged. Cells were counted to examine the relationship between cell viability and cell damage. As seen in Fig. 6, 99·8% reduction of viability was found after 30 min of contact. Equivalent results were observed by FC (Fig. 6), strongly indicating that losses in viability may be associate with membrane damage rather than with cell lysis.

Dual parameter fluorescence histograms of *Staph. aureus* indicated that 97·8% of the control population was stained with SYTO-13 (Fig. 7); only 1·6% was stained with PI. The most striking effect was observed when *Staph. aureus* was treated with 8 μg ml^{-1} of LAE. Three sub-populations were easily detected (Fig. 7b) unlike the control population (Fig. 7a). After 30 min of contact, the largest fraction of the population (42·9%) was stained with PI, and a fairly important sub-population (21·4%) was double-stained, indicating that this sub-population was partially damaged. During the 24 h of contact, the fraction stained with PI increased to 56·3%. Parallel cell counts (Fig. 8) indicated that the correlation between PI-stained cells (severely damaged cells) and viability was very weak. Nevertheless, close correlation was found between viability and PI-stained cells plus a double-stained subpopulation (SYTO-13/PI), suggesting that in the case of *Staph. aureus* even partially damaged cell membranes had reduced viability.

LAE-induced transmembrane ion fluxes

Intracellular potassium leakage resulting from exposure to LAE of *S. typhimurium* and *Staph. aureus* at the corresponding MIC (32 μg ml^{-1} and 8 μg ml^{-1}, respectively) is shown in Fig. 9. A major effect of LAE was found for *Staph. aureus*. The increase of potassium-ion concentration in the supernatant after 30 min of contact was 7·7 ppm, whereas in *S. typhimurium* the increase was 3·34 ppm. Moreover, this value remained fairly constant throughout the experiment.

Specific ion flux was studied by measuring the proton flux across the membrane, induced in the presence of LAE by the deliberate imposition of ion gradients and subsequent monitoring of their product-induced dissipation. The alteration of the proton flux in the case of *S. typhimurium* is presented in Fig. 10. As observed, a decrease of the proton flux was observed in *S. typhimurium* after 1 min of contact, and this effect remained constant throughout the experiment (5 min of contact). A similar effect was observed in *Staph. aureus* after 1 min of contact, although the decrease was smaller and it remained fairly constant during the experiment, compared with the control population.

DISCUSSION

Cationic surfactants have a general antimicrobial action. This leads to the disruption of the cell wall and

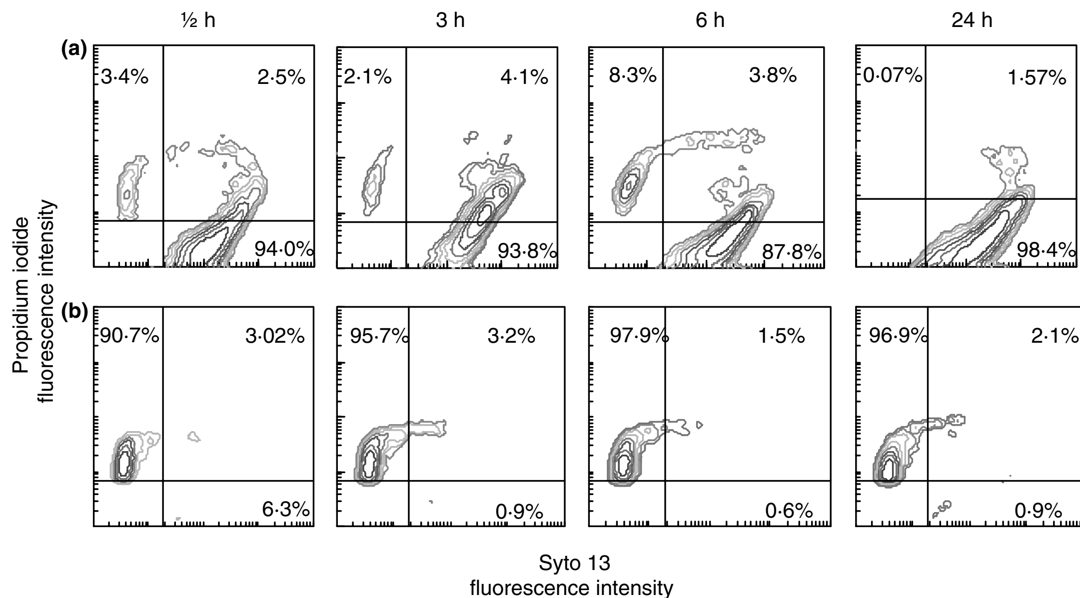

Fig. 5 Dual-parameter SYTO-13/propidium iodide fluorescence histograms obtained by staining *Salmonella typhimurium* ATCC 14028. (a) Control cells; (b) cells after treatment with L-arginine, N$^{\alpha}$-lauroyl ethylester (32 μg ml^{-1}) (logarithmic scale)

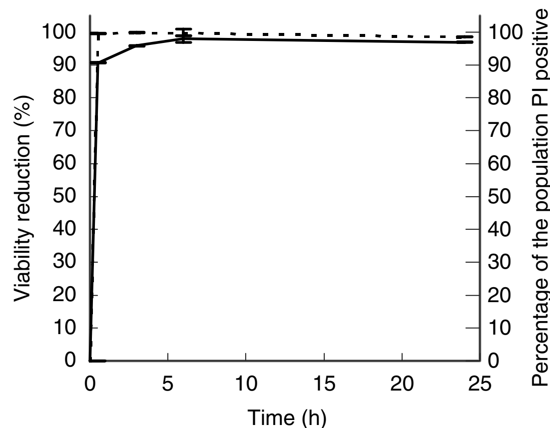

Fig. 6 Effect of the contact time on viable counts (······) and total counts by flow cytometry after propidium iodide staining (——) in L-arginine, N$^{\alpha}$-lauroyl ethylester-treated *Salmonella typhimurium* cells

membranes and, consequently, to the depolarization of the cytoplasmic membrane and the leakage of the cytosol components (Kanazawa *et al.* 1995; Shimoda *et al.* 1995; Langsrud and Sundheim 1996; Paulsen *et al.* 1996 and Tattawasart *et al.* 2000). Ultrastructural changes may be induced by the action of an antimicrobial compound, producing dramatic effects on the bacterial envelopes and causing lysis or massive leakage of cell components (Tattawasart *et al.* 2000). Both epifluorescent microscopy and TEM provide cytological evidence that helps to explain the action of LAE. Ultrastructural differences were observed on comparison with the untreated population of *S. typhimurium* and *Staph. aureus*.

The effect of LAE on *S. typhimurium* at 3 h of contact is clearly seen (Fig. 1b), compared with the dense and compact membrane structure in the control cells (Fig. 1a). However, the cytoplasmic membrane was not disrupted and membrane-enclosed vacuoles were also observed. No sphaeroplasts were observed, suggesting that no plasmolysis occurred (Beveridge *et al.* 1991). Tobramycin-treated cells induced similar damage in *Pseudomonas aeruginosa*. However, in this case, massive cell lysis was reported (Gilleland *et al.* 1998), whereas in other cases filamentous growth was induced in chlorhexidine-treated *Pseudomonas stutzeri* (Tattawasart *et al.* 2000).

Different effects of LAE were observed in the Gram-positive *Staph. aureus*: the mesosome-like structures observed in treated cells were induced by LAE, as nontreated cells did not contain them. Similar alterations were described when *Staph. aureus* was treated with cationic peptides, defensines or trimethoprin (Shimoda *et al.* 1995; Friederich *et al.* 2000). However, in these cases thinning and disintegration of cell wall were also observed. In contrast with the action of cationic peptides described, cellular lysis in LAE-treated cells did not occur as no sphaeroplasts or 'ghosts' were observed. The most relevant effect on the cell wall that we observed was the abnormal septation and irregular cross-wall formation, similar to those induced by trimethoprin (Friederich *et al.* 2000). Intracytoplasmic

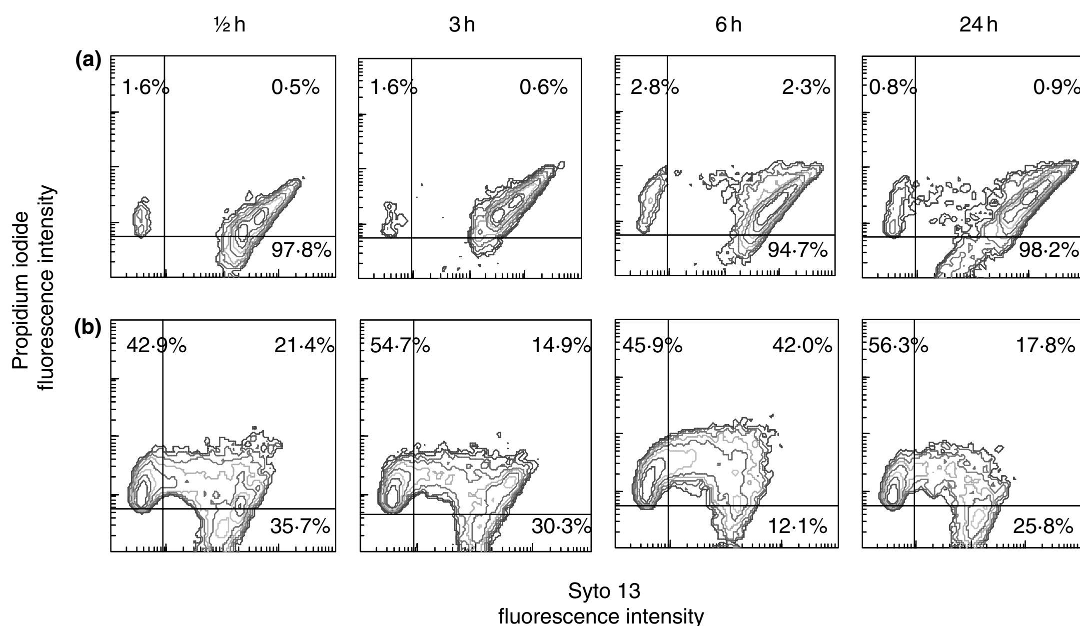

Fig. 7 Dual-parameter SYTO-13/propidium iodide fluorescence histograms obtained by staining *Staphylococcus aureus* ATCC 6538. (a) Control cells; (b) cells after treatment with L-arginine, N^{α}-lauroyl ethylester (8 μg ml^{-1}) (logarithmic scale)

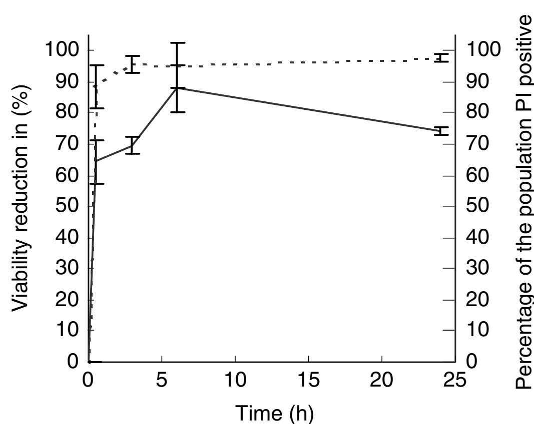

Fig. 8 Effect of the contact time on viable counts (·······) and total counts by flow cytometry after propidium iodide staining (——) in L-arginine, N^{α}-lauroyl ethylester-treated *Staphylococcus aureus* cells. Cells labelled with propidium iodide, include double-labelled cells, in the assay conditions used for flow cytometry (see text)

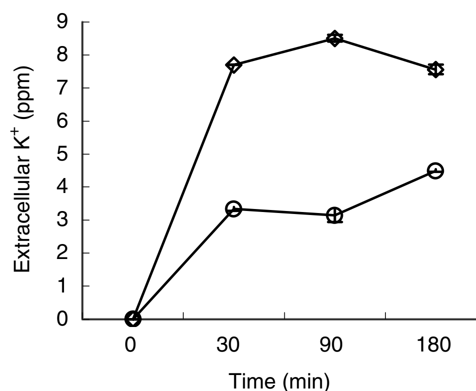

Fig. 9 Potassium leakage of the cell suspension of *Salmonella typhimurium*(○) and *Staphylococcus aureus* (◇) treated with L-arginine, N^{α}-lauroyl ethylester

white spots appeared in the treated population (Fig. 3b), suggesting solubilization of part of the cytoplasm material by the surfactant properties of LAE and hence the loss of viability of the treated cells.

We can conclude that LAE acts at a different level and the cellular effect depends on the structure of the bacterial cells. In Gram-negative cells, the alterations involve both the cytoplasm membrane and the external membrane. In contrast, in Gram-positive cells, alterations were observed in the cell membrane and in the cytoplasm. In both cases, cells remained intact: cell lysis was not observed in the treated population.

During the last few years, a large number of fluorescent-based assays for evaluating bacterial viability, membrane depolarization, enzyme activity or the physiological state of bacteria have appeared (Lebaron *et al.* 1998; Shapiro 2000). However, very little information has been published on the

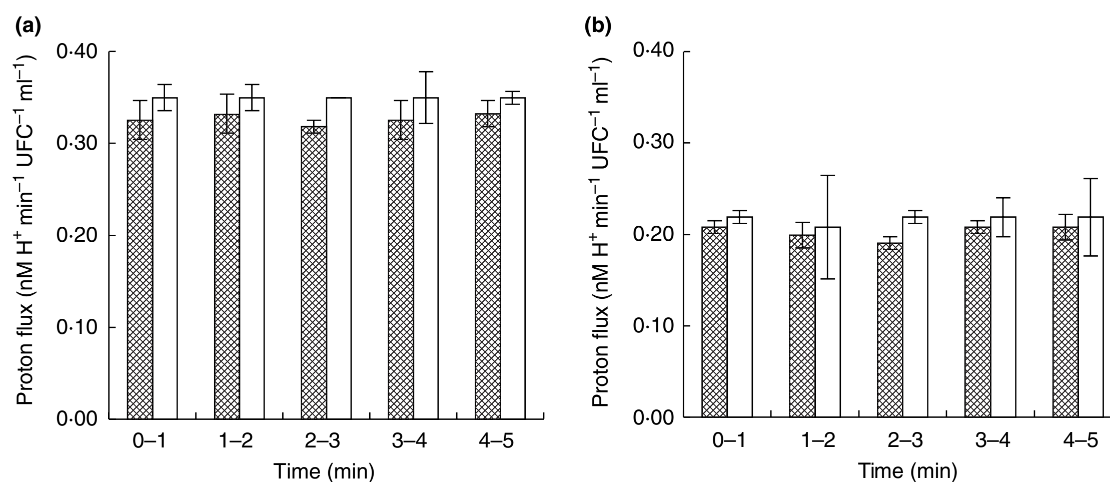

Fig. 10 Rate of proton flow associate with cell suspensions of *Salmonella typhimurium* (a) and *Staphylococcus aureus* (b) untreated and treated with L-arginine, N^{α}-lauroyl ethylester, in the conditions stated in the text. Control population open bar, treated population shaded bar

effect of surface-active compounds on bacterial populations (Comas and Vives-Rego 1997). The advantage of FC is that it analyses the individual characteristics of cells. Coupled to adequate fluorochromes, FC may distinguish between viable and nonviable cells to assess the bactericidal activities of biocides and antibiotics. This assessment is based on the fact that not all cells are in the same metabolic state, and so it is of the utmost importance to select the right fluorescent probes. Two fluorochromes were used in this study, SYTO-13, which penetrates all types of cellular membranes and binds nucleic acids of the cell, and PI, which only penetrates the bacterial membrane when the membrane is damaged, i.e. when the membrane is depolarized.

The consequence is that three types of stained cells can be observed: intact cells, severely damaged and partially damaged cell membranes. In these conditions the heterogeneity of the bacterial cultures for either *S. typhimurium* or *Staph. aureus* was clear in the treated populations. LAE induced severe damage in the bacterial membranes of *S. typhimurium* at 30 min of contact: up to 94% of the population was stained with PI, indicating that the membrane was severely damaged. Moreover, after 24 h a slight increase of the damage occurred. The detection of a double-stained population suggests progressive damage in the cell envelopes; however, the effect of LAE seemed to take place in the first minutes of contact and then remain fairly stable. As stated earlier, cytometry and fluorescent probes allow the experimenter to differentiate culturable and nonviable cells by observing different metabolic stages (Vives-Rego *et al.* 2000). In this regard, cell-counts indicated that cells stained with PI had lost their viability. However, cytosol material remained inside the cell. A different pattern was found in *Staph. aureus*, and after 30 min only 43% of the population was stained with PI. In this case, a fairly important subpopulation (21·4%) was double stained after 30 min of exposure. The double-stained subpopulation, according to the results presented, consisted of nonculturable material. However, cells remained intact as the fluorochromes were retained inside the cytoplasm.

As expected, the loss of K^+ was rapid and monophasic in both strains, although different patterns were observed for the Gram-negative and the Gram-positive bacteria. The amount of potassium ions released was 2·5 times higher in *Staph. aureus* suspensions than in *S. typhimurium*. No correlation was found between potassium leakage and viability. Similarly, no correlation between drug concentration and loss of viability was found in *Staph. aureus* treated with triclosan (Suller and Russel 2000).

The chemical gradient of hydrogen ions is another means of observing changes in membrane permeability, given that any such alteration is reflected in a perturbation of the delicate balance in ion gradient across the membrane. Given that the assessment is of net exchange, it is impossible to determine the direction of proton flow. The flow of protons in the population treated with LAE is slightly less than in the untreated population, though no substantial alteration was detected in the populations at the concentrations studied (Denyer and Hugo 1991).

The effect of LAE on the *S. typhimurium* and *Staph. aureus* has been documented by electron microscopy, FC, ion leakage and disruption of membrane potential. The results indicate that LAE caused disturbance in membrane potential and structural changes and loss of cell viability, although no disruption of cells was detected.

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