Effects of the antimicrobial peptide temporin L on cell morphology, membrane permeability and viability of *Escherichia coli*

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Antimicrobial peptides are produced by all organisms in response to microbial invasion and are considered as promising candidates for future antibiotics. There is a wealth of evidence that many of them interact and increase the permeability of bacterial membranes as part of their killing mechanism. However, it is not clear whether this is the lethal step. To address this issue, we studied the interaction of the antimicrobial peptide temporin L with *Escherichia coli* by using fluorescence, confocal and electron microscopy. The peptide previously isolated from skin secretions of the frog *Rana temporaria* has the sequence FVQWFSKFLG-RIL-NH_2. With regard to fluorescence microscopy, we applied, for the first time, a triple-staining method based on the fluorochromes 5-cyano-2,3-ditolyl tetrazolium chloride, 4′,6-diamidino-2-phenylindole and FITC. This technique enabled us to identify, in the same sample, both living and total cells, as well as bacteria with altered membrane permeability. These results reveal that temporin L increases the permeability of the bacterial inner membrane in a dose-dependent manner without destroying the cell's integrity. At low peptide concentrations, the inner membrane becomes permeable to small molecules but does not allow the killing of bacteria. However, at high peptide concentrations, larger molecules, but not DNA, leak out, which results in cell death. Very interestingly, in contrast with many antimicrobial peptides, temporin L does not lyse *E. coli* cells but rather forms ghost-like bacteria, as observed by scanning and transmission electron microscopy. Besides shedding light on the mode of action of temporin L and possibly that of other antimicrobial peptides, the present study demonstrates the advantage of using the triple-fluorescence approach combined with microscopical techniques to explore the mechanism of membrane-active peptides in general.

Key words: amphibian skin, antimicrobial peptide, *Escherichia coli*, fluorescence microscopy, membrane permeability, temporin L.

INTRODUCTION

Antimicrobial peptides are gene-encoded polypeptides of various lengths and structures found in all organisms including humans and plants [1–5]. These molecules are essential components of the innate immune system of all these organisms, providing them a weapon against microbial infections before the adaptive immunity is activated [6–8]. In animals, antimicrobial peptides are mostly found in circulating leucocytes and in those parts that are most likely to encounter pathogens. These parts include the skin and epithelial surfaces, where they are stored in granules destined for extracellular secretion [9,10]. Mobilized shortly after microbial invasion, antimicrobial peptides target and rapidly neutralize a broad range of micro-organisms, being relatively non-specific [11]. The widespread use of conventional drugs has led to the development of many antibiotic-resistant strains, which constitutes a major health concern worldwide. This prompted many studies towards the development of alternative antibiotics with new modes of action that could overcome resistance. Antimicrobial peptides are among the most promising candidates [4,5,12], and hundreds of them have been isolated from different biological sources (see an updated list at http://www.bbcm.univ.trieste.it/~tossi/pag1.htm), and many of them have been subjected to mode of action studies.

Although antimicrobial peptides differ significantly in their sequences, most of them share similar features, such as a net positive charge and a potential to adopt an amphipathic α-helix and/or β-sheet structures on their interaction with membranes. Many studies on their mode of action have provided compelling evidence that a common step in the killing mechanism is their interaction with the bacterial cytoplasmic membrane [13–15]. However, it is not yet clear whether the actual killing of bacteria is the result of one of the following processes: (i) the peptide induces membrane permeation either via pore formation or via membrane disintegration [16]; (ii) the peptide binds to cellular nucleic acids [17–19] and interferes with DNA and protein synthesis machinery [20]; or (iii) the peptide binds to other intracellular targets and thus inhibits various vital functions [21,22].

We addressed these questions using temporin L. Temporins constitute a large and growing family of short (10–14 residues) and linear antimicrobial peptides synthesized in the skin of frogs belonging to *Rana* species, but they are also found in the venom of wasps. They were isolated initially from the European red frog *Rana temporaria* [23], and, to date, more than 40 temporin-like peptides are currently known [24,25]. The consensus sequence of the frog-derived temporins is FLPLIASLLSKLL-NH_2. One of them, temporin L, has been found to be considerably active against both Gram-positive and Gram-negative bacteria [26] and,

Abbreviations used: CFU, colony-forming units; CLSM, confocal laser scanning microscopy; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; DAPI, 4′,6-diamidino-2-phenylindole; Gal-ONp, 2-nitrophenyl β-D-galactoside; LB, Luria–Bertani broth; PTA, phosphotungstic acid; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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therefore, was subjected to an in-depth investigation to understand its mode(s) of action. So far, it has been shown that temporin L binds and permeates artificial membranes with different lipid compositions, and is also capable of increasing the permeability of the bacterial inner membrane [26,27]. However, the actual killing mechanism is not clear.

In the present study, we report on a study aimed at understanding the mode of action of temporin L on *Escherichia coli* D21 cells by determining whether (i) the membrane permeation process is a lethal step and (ii) the killing of bacteria is accompanied by DNA release and cell lysis. For this purpose, we used fluorescence/phase-contrast, confocal and electron microscopy, which enabled us to visualize the effects of temporin L on the viability and membrane integrity of individual *E. coli* cells. In these studies, we applied, for the first time, a triple-staining method based on the following fluorochromes: CTC (5-cyano-2,3-ditolyl tetrazolium chloride), DAPI (4′,6-diamidino-2-phenylindole) and FITC. This technique enabled the microscopic identification, in the same sample, of both total and living cells, as well as of bacteria with altered membrane permeability. The results, supported by *in vitro* data on the bactericidal capacity and membrane permeation of the peptide, are discussed with respect to the proposed mode(s) of action of temporin L and possibly that of related antimicrobial peptides. Furthermore, the present study illustrates the advantage of using the three fluorescent-dye approach combined with microscopic techniques to explore the mechanism of membrane-active peptides in general.

MATERIALS AND METHODS

Materials

Synthetic temporin L was purchased from Synt:em (Nîmes, France). The purity of the peptide and its sequence and concentration were determined as described previously [28]. Gal-ONp (2-nitrophenyl β-D-galactoside), CTC, DAPI and FITC were all from Sigma (St. Louis, MO, U.S.A.). Poly(L-lysine), glutaraldehyde and PTA (phosphotungstic acid) were from Fluka (Buchs, Switzerland). All other chemicals used were of reagent grade.

Antibacterial activity: time-killing curves

E. coli D21 was grown at 37 °C in LB (Luria–Bertani broth) medium until an absorbance A_{590} 1 was reached. The cells were centrifuged, washed and resuspended in 10 mM sodium phosphate buffer (pH 7.4). Approx. 2×10^7 cells were incubated with temporin L at different concentrations at 30 °C. Aliquots of 10 μl were withdrawn at different intervals and spread on LB-agar plates. After overnight incubation at 30 °C, the surviving bacteria, expressed as CFU (colony-forming units), were counted. Controls were run without peptide and in the presence of peptide solvent [20 % (v/v) ethanol].

Permeation of the cytoplasmic membrane

The ability of temporin L to permeate the cytoplasmic membrane was determined by measuring the β-galactosidase activity using Gal-ONp as a substrate, as described previously [29]. Briefly, *E. coli* D21 was grown at 37 °C in LB medium supplemented with 1 mM isopropyl β-D-thiogalactopyranoside to an $A_{590} \sim 1$, washed and resuspended in sodium phosphate buffer. Approx. 2×10^7 cells (20 μl of $\sim 10^9$ CFU/ml) were incubated with different concentrations of temporin L for 60 min at 30 °C. The same amount of bacteria without peptide was used as a control. At the end of the incubation time, 2 μl of aliquots were withdrawn, diluted (1:100) in PBS and spread on LB plates containing streptomycin (100 mg/l) for counting. The bacterial culture was then passed through a 0.2 μm filter and the hydrolysis of Gal-ONp was recorded on the filtrate at 420 nm using a spectrophotometer (Hitachi U-1100).

Staining protocols: fluorescent probes for bacteria counting, viability and assessment of membrane integrity

A triple-staining method was developed to visualize, in a single preparation, the total (i.e. viable plus non-viable) and live *E. coli* cells, and to detect, at the same time, the membrane permeabilization induced by temporin L on bacteria. The following fluorochromes were used: (i) the double-stranded DNA-binding dye DAPI, to stain all bacterial cells irrespective of viability; (ii) the vital dye CTC, used to stain viable micro-organisms; and (iii) the green fluorescent probe FITC, which is unable to traverse the cytoplasmic membrane of cells unless permeabilized by a peptide. Both CTC and DAPI were made up as stock solutions in water at a concentration of 6 mg/ml (20 mM) and 10 mg/ml (28 mM) respectively. After exposure to temporin L at 2 and 50 μM for 60 min, the bacterial cells were incubated with 900 μl of CTC (5 mM in PBS) for 90 min at 37 °C with agitation (150 rev./min). The cell suspension was poured on to poly(L-lysine)-coated glass slides previously placed in the wells of a polystyrene Costar plate (Corning, Acton, MA, U.S.A.). Subsequently, the plates were kept at 30 °C for 45 min to allow the adhesion of peptide-treated *E. coli* cells to the glass slides. The plates were then washed with sodium phosphate buffer, and 1 ml of DAPI solution (10 μg/ml in PBS) was added to the wells. After 30 min at 30 °C, the DAPI solution was removed and the slides were rinsed again with sodium phosphate buffer. An FITC stock solution (10 mg/ml in acetone) was diluted to 6 μg/ml in sodium phosphate buffer and added to the wells. The plates were incubated at 30 °C for 30 min and washed as described above. In all cases, controls were run in the absence of peptide and in the presence of peptide solvent. The slides were then examined by phase-contrast, fluorescence and CLSM (confocal laser scanning microscopy). Phase-contrast images as well as images under fluorescence light were recorded using a Zeiss Axioplan 2 optical microscope equipped with an oil-immersion objective ($\times$ 100). CLSM observations were made using a Sarastro 2000 (Molecular Dynamics; Sunnyvale, CA, U.S.A.) apparatus adapted to a Nikon Optiphot microscope (objective PLAN-APO 60/1.4 oil) and equipped with an argon-ion laser (25 mW). FITC was excited at 488 nm and laser power set at 1 mW. Serial optical sections were assembled in depth-coding mode. Acquisition and processing were performed using Image Space software (Molecular Dynamics).

Scanning electron microscopy (SEM)

E. coli D21 cells were grown in LB broth to an exponential phase, harvested by centrifugation, washed twice with 10 mM sodium phosphate buffer and resuspended in the same buffer. Approx. 2×10^7 cells were incubated at 30 °C for up to 30 min with 2 and 50 μM temporin L. Controls were run in the presence of the peptide solvent. The volume was adjusted to 500 μl and each sample was spread on a poly(L-lysine)-coated glass slide (18 mm $\times$ 18 mm) to immobilize bacterial cells. Glass slides were incubated at 30 °C for 90 min. Slide-immobilized cells were fixed with 2.5 % (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer, extensively washed with the same buffer and dehydrated with a graded ethanol series. After critical-point drying and gold coating, the samples were observed with a Philips XL 30 CP instrument.

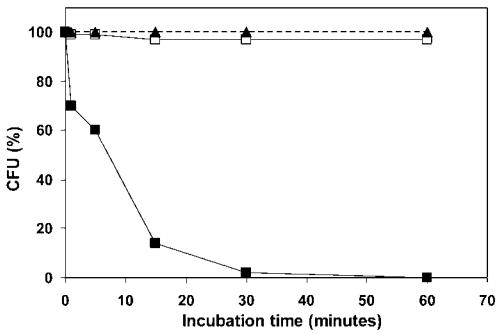

Figure 1 Dose-dependent killing of *E. coli* D21 by temporin L

E. coli D21 cells (2×10^7) were grown in LB at 37 °C, diluted in 10 mM sodium phosphate buffer (pH 7.4) and incubated with 2 μM (□) or 50 μM (■) temporin L at 30 °C. The number of surviving cells, at different incubation times, is expressed as the percentage of total cells. The control (▲) is bacteria without peptide. The values are means for three independent measurements.

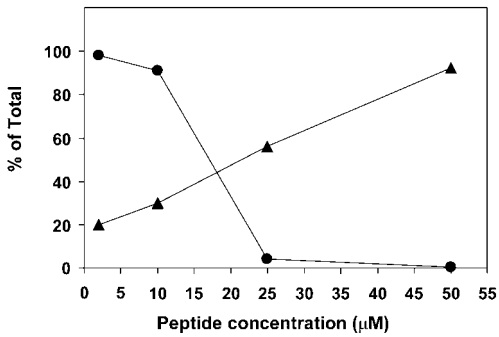

Figure 2 Bacterial viability and β-galactosidase activity of *E. coli* D21 culture after treatment with temporin L

Bacterial cells (2×10^7) were grown in LB at 37 °C, diluted in 10 mM sodium phosphate buffer (pH 7.4) and incubated with temporin L at different concentrations for 60 min at 30 °C. The number of surviving cells (●) is given as the percentage of the total. β-Galactosidase activity was measured in the culture filtrate following the hydrolysis of 2 mM Gal-ONp at 420 nm. Enzymic activity detected in the control (bacteria without peptide) was subtracted from all values, which are expressed as percentage of the total (▲). Complete enzyme activity was determined by treating bacteria with 0.1 % SDS/chloroform. The values are the means for three independent measurements.

Transmission electron microscopy (TEM)

Samples containing *E. coli* D21 (2×10^7 cells) in LB medium were incubated with 2 and 50 μM temporin L for 30 min, and then centrifuged at 300 ***g*** for 10 min. Controls were made in the presence of peptide solvent. The pellets were resuspended; a drop containing the bacteria was deposited on to a carbon-coated grid and negatively stained with 2 % (w/v) PTA (pH 7.0). The grids were examined using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

RESULTS

Rate of bactericidal activity and permeation of the cytoplasmic membrane

The antimicrobial activity of temporin L on *E. coli* D21 was measured in experiments assessing the rate of killing by using low (2 μM) and high (50 μM) peptide concentrations (Figure 1). At 2 μM, temporin L causes only a 2 % decrease in the number of CFU within 60 min, according to the epifluorescent images, which reveal the absence of dead cells under these conditions. However, at 50 μM, the bactericidal effect is rapid and an 86 % decrease in CFU was observed within 15 min. No viable colonies were detected after 60 min. These results are in agreement with the finding that the conventional minimal inhibitory concentration value (the concentration at which 100 % inhibition of bacterial growth is observed after 18 h incubation) for the tested strain determined in LB medium is 8 μM. Thus 2 and 50 μM temporin L correspond to sublethal and killing concentrations respectively.

We also examined the effect of temporin L on the integrity of the *E. coli* D21 inner membrane by monitoring the leakage of the cytoplasmic β-galactosidase, as described previously in the study of the membrane-perturbing activity of antimicrobial peptides [21,30–33] and lysozyme [34]. These results denote that temporin L increases the permeability of the *E. coli* inner membrane under conditions in which it shows significant bactericidal activity (Figure 2). Thus the enzymic activity measured in the supernatant was approx. 90 % of the total at 50 μM, the highest peptide concentration tested. This finding is in agreement with the notion that the bacterial inner membrane is indeed a site of action for temporin L [26], as was also found for other temporin-like peptides [28]. The decrease in bacterial viability is concomitant with the enhancement of membrane permeability and leakage of intracellular compounds.

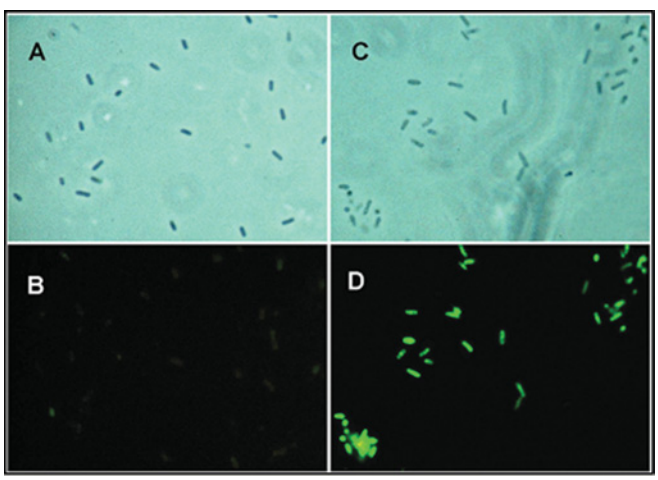

Figure 3 Bacterial inner-membrane permeation induced by temporin L and visualized by FITC fluorescence

E. coli D21 cells were grown as indicated in Figures 1 and 2, incubated with 50 μM temporin L at 30 °C for 60 min, and immobilized on poly(L-lysine)-coated glass slides. FITC (500 μl of a diluted solution in 10 mM sodium phosphate buffer) was spread on the slides which were incubated at 30 °C for 30 min. Controls were run in the presence of peptide solvent. After washing, the slides were examined by phase-contrast and fluorescence microscopy to assess the effect of the peptide on FITC influx inside the bacterial cells. Phase-contrast images of control (**A**); fluorescent images of control (**B**); phase-contrast images of bacteria treated with temporin L (**C**); fluorescent images of bacteria treated with temporin L (**D**).

Triple-staining assay for the detection of total, viable and membrane-perturbed bacteria

To visualize further the membrane-perturbing activity of temporin L, we used FITC, a low-molecular-mass (389.4 Da) green fluorescent probe. FITC was unable to traverse the cytoplasmic membrane of control intact cells. Indeed, when *E. coli* D21 cells were incubated with the probe without pretreatment with the peptide, no appreciable fluorescent signal was discerned, as visualized by comparing the phase-contrast and fluorescence microscopy images (Figures 3A and 3B). In contrast, FITC was readily accumulated in bacteria after their exposure to 50 μM temporin L

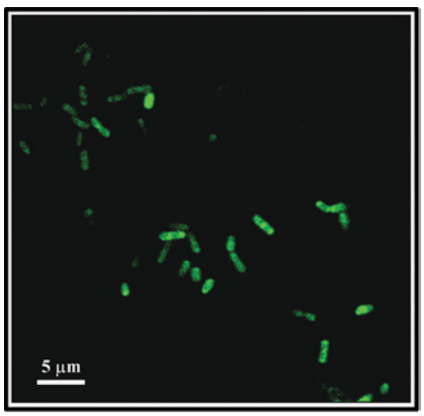

Figure 4 CLSM image of temporin L-treated *E. coli* D21

Cells were grown, treated with temporin L and stained with FITC, as indicated in the legend to Figure 3. To enable CLSM observations, FITC was excited at 488 nm.

(Figures 3C and 3D), suggesting that the peptide increases the permeability of the bacterial membrane. CLSM analysis confirmed that the intense green fluorescence is indeed the result of FITC uptake, i.e. the probe was internalized in peptide-treated cells (Figure 4), rather than localized on the surface of the bacterial membrane. CLSM also showed that the probe is not distributed evenly inside the bacteria but instead tends to cluster in discrete patches, often situated at the cell poles and at the sites of bacterial division (Figure 4).

Simultaneous microscopic numbering of total, viable and membrane-perturbed *E. coli* cells was achieved by their exposition to triple staining with the fluorescent probes CTC, DAPI and FITC. We tested both control bacteria and bacteria treated with sublethal and lethal concentrations of temporin L. CTC is a monotetrazolium redox dye, which is practically colourless and non-fluorescent unless it is exposed to an electron-transport chain. This readily reduces the compound to an insoluble red fluorescent CTC-formazan salt that accumulates intracellularly [35]. Therefore the presence of red CTC-formazan crystals, which can be easily detected by long-wave UV illumination (> 350 nm) [36], indicates the presence of metabolically active (respiring) bacteria. The other probe, DAPI, binds to a double-stranded DNA, forming a stable complex, which gives a blue fluorescence when excited by UV light. Counterstaining CTC with DAPI, as described previously by Cappelier et al. [37], allows identification of either the total number of cells (blue fluorescence) or the number of metabolically active bacteria (red fluorescence) within the same preparation. In the present study, we show that combining the double CTC–DAPI staining with the green fluorescent probe FITC, makes it possible to monitor simultaneously three types of *E. coli* cells in the same glass slide: (i) bacteria with an altered membrane permeability (cells with a bright green fluorescence due to FITC uptake); (ii) bacteria in which membrane perturbation does not affect their viability (assessed by the red CTC-formazan crystals); and (iii) bacteria in which the killing process and/or membrane permeation causes DNA leakage (presence or absence of blue fluorescence due to DAPI binding to DNA).

The results obtained with the triple-staining approach are illustrated in Figure 5. When *E. coli* cells were incubated with 2 μM temporin L, which causes only approx. 2% reduction in CFU (Figure 1) and a limited β-galactosidase efflux (Figure 2), all bacteria, as detected by blue DAPI staining (Figure 5A), also emit a bright green fluorescence (Figure 5B), indicating the intracellular influx of the small probe FITC. These findings suggest

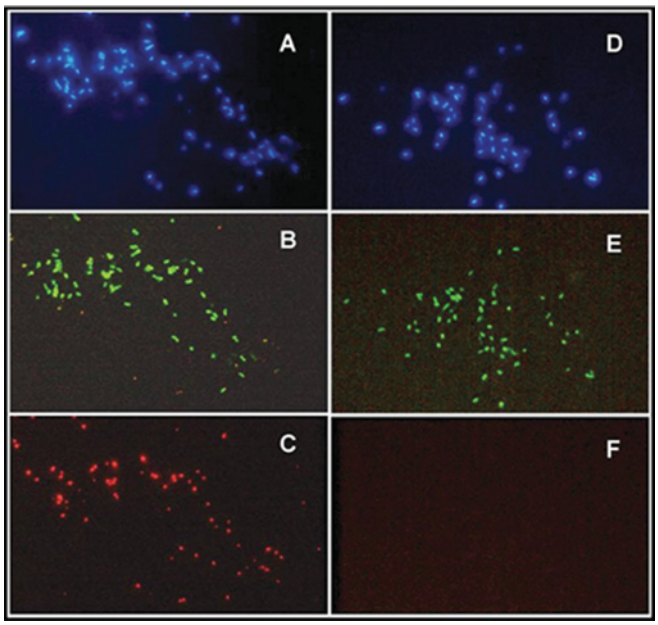

Figure 5 Detection of total, viable and membrane-perturbed bacteria using triple staining with fluorescent probes

E. coli cells were grown and treated with temporin L at 2 or 50 μM for 60 min, immobilized on poly(L-lysine)-coated glass slides, and incubated with CTC, DAPI and FITC, as described in the Materials and methods section. In all cases, controls were run in the absence of peptide and in the presence of the peptide solvent. The slides were then examined by fluorescence microscopy. Left panels: bacteria treated with 2 μM temporin L; right panels: bacteria treated with 50 μM temporin L; staining with DAPI (**A**, **D**), FITC (**B**, **E**) and CTC (**C**, **F**). See the Results section for a detailed description of the images.

that even a sublethal peptide concentration is capable of leading to a significant alteration of membrane integrity, which becomes permeable, at least to low-molecular mass compounds. Interestingly, this membrane alteration is not accompanied by the killing of bacteria, as demonstrated by the red CTC fluorescence within the same cells (Figure 5C), and also by the antimicrobial assays (Figures 1 and 2). Surprisingly, at a high peptide concentration (50 μM), sufficient to kill approx. 2×10^7 CFU and to induce the leakage of approx. 90% of the β-galactosidase (Figures 1 and 2), all the treated bacteria maintain the typical stick shape without losing the nucleic acids content, as manifested by their clear, intense blue fluorescence (Figure 5D). As expected, microbial cells have an increased membrane permeability to FITC (Figure 5E) and no red fluorescence could be detected (Figure 5F), thus proving that all bacteria are dead. This is in agreement with the peptide bactericidal activity reported in Figures 1 and 2.

Electron microscopy experiments

To gain insight into the direct effect of temporin L on the morphology of *E. coli* D21, we used SEM and TEM at sublethal and killing peptide concentrations. The exposure of bacteria to 2 μM peptide for up to 30 min causes a remarkable modification of their cell shape, as shown by SEM. Untreated bacteria display a rough bright surface, typical of this *E. coli* strain [38], with no apparent cellular debris (Figure 6A). In contrast, peptide-exposed cells exhibit a wide range of significant abnormalities. These include deep roughening of the cell surface (Figure 6B), the collapse of the cell structure (Figure 6C) and a ghost-like appearance in which the cells seem transparent, looking empty and flat, with cell debris arising from them (Figures 6D and 6E). Although occurring

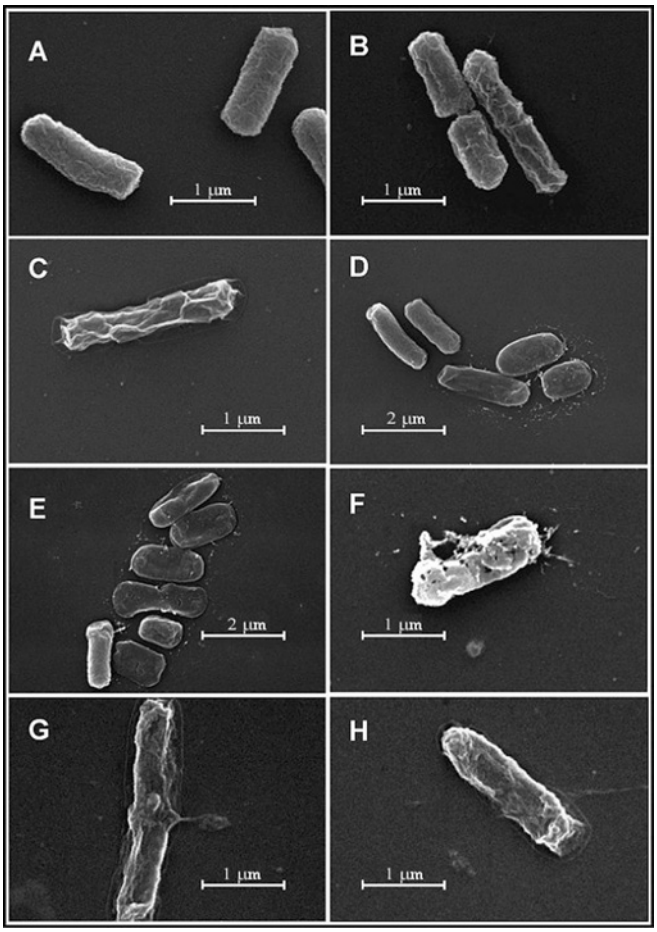

Figure 6 SEM of temporin L-treated *E. coli* D21

Control bacteria (**A**); bacteria after treatment with temporin L at a sublethal concentration (2 μM) for up to 30 min (**B**–**F**); bacteria after treatment with temporin L at a high concentration (50 μM) for 15 min (**G**, **H**). See the Results section for other experimental details and descriptions of the images. Each Figure has been magnified $\times$ 15 000 or $\times$ 30 000.

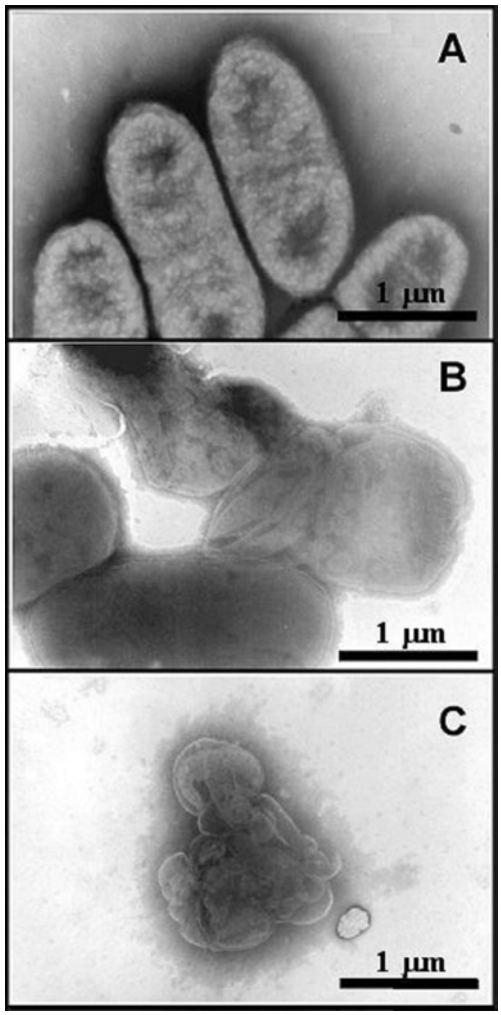

Figure 7 Electron micrographs of negatively stained *E. coli* D21 cells treated with temporin L

Cells were grown in LB medium and incubated with 2 and 50 μM temporin L for 30 min. Controls were run in the presence of the peptide solvent (20 % ethanol) alone. A drop containing the bacteria was deposited on to a carbon-coated grid and negatively stained with 2 % PTA. The grids were examined using TEM, as described in the Materials and methods section. Untreated *E. coli* cells (**A**); cells treated with 50 μM temporin L (**B**, **C**).

rarely, lysed bacteria were also observed (Figure 6F). In contrast with temporin L, most antimicrobial peptides studied so far do not cause these kinds of shapes but rather blebs on the microbial surface. Note that under these conditions (i.e. at 2 μM temporin L), most of the bacteria were found to be metabolically active and with an altered membrane permeability to low-molecular-mass compounds, as shown by the triple-colour-staining assay (Figure 5C). Moreover, the bacterial viability, examined by CFU counting, decreases by approx. 2 % in 60 min (see above). At a lethal temporin L concentration (50 μM), a more frequent and prominent collapse of the cell structure was noticeable already within 15 min and the cells seemed to have lost their content, although the overall cell shape was still recognizable (Figures 6G and 6H). This is also in agreement with the fluorescence microscopy data (Figures 3C, 3D, 4, 5D and 5E).

The effects of temporin L at 2 and 50 μM on *E. coli* were also visualized using TEM. At both concentrations, the morphology of the treated bacteria is altered (shown only for 50 μM) (Figures 7B and 7C) compared with the control (Figure 7A). More specifically, the whole cell shape is modified significantly, and ghost-like structures similar to those observed in the SEM images (Figures 6D and 6E) are also visible (Figures 7B and 7C). Furthermore, neither serious damage to the bacterial walls nor discrete holes on their surface is apparent. Instead, cell collapsing and membrane folding occur (Figures 7B and 7C). In all cases tested, no blebs were formed after peptide treatment.

DISCUSSION

Although hundreds of antimicrobial peptides have been isolated so far, only a limited number of them have been investigated extensively for their mode of action, and most of these studies included a wide range of biophysical techniques mainly with model phospholipid membranes. However, the details of the mode(s) of action and the molecular mechanism that causes the death of the target cell are not fully understood, thus delaying the emergence of antimicrobial peptides as a new class of antibiotics. This is particularly crucial in the case of Gram-negative bacteria, which give rise to the highest mortality and morbidity, for which our arsenal of effective therapeutic agents is rapidly waning.

The current and generally accepted view of the mode of action of cationic antimicrobial peptides on Gram-negative bacteria envisages that, in the first step, the peptide interacts with the highly

negatively charged surface of the outer membrane consisting of the anionic lipopolysaccharide [12]. Then, the peptide inserts and translocates across the outer bilayer via a 'self-promoted uptake' pathway to reach and bind the anionic inner membrane through electrostatic and hydrophobic interactions (reviewed in [39]). This is followed by the permeation/disruption of these membranes. However, it is still not clear whether this is actually the lethal step or if peptide-mediated bacterial killing is a more complex event [40].

The present study addresses the question of whether and to what extent temporin L, a small cationic and amphiphilic peptide, affects the membrane permeation of *E. coli*, and how this relates to cell viability and a change in morphology. Furthermore, this study also deals with the controversial issue of whether bacterial killing always results in cell lysis or has a different outcome. To address this, we developed a new approach based on the use of three different fluorescent probes (CTC, DAPI and FITC), which allowed for the first time a direct microscopic visualization, within the same sample, of total and living micro-organisms, as well as bacteria with an altered membrane permeability. We applied this method together with electron microscopy to monitor concomitantly the action of temporin L against bacteria. Interestingly, we found that FITC does not enter non-treated control living bacteria but is easily accumulated in all microbial cells, both alive and dead, after their exposure to temporin L. This indicates that membrane permeation caused by temporin L does not necessarily lead to bacterial death, which takes place at higher peptide concentrations, and is accompanied by the leakage of cytoplasmic components, such as β-galactosidase, but not DNA. These results prove that the extent of membrane damage increases with increasing peptide concentration, until a significant disruption of the membrane structure occurs, followed by death of the bacteria. These results are in accordance with the previous experiments on model membranes, demonstrating that temporin L induces the release of vesicle-entrapped markers in a manner that is dependent on both the size of the probe and the peptide concentration used [26]. Studies with lipid vesicles also indicated that the peptide does not have a detergent-like effect on the membrane, but rather perturbs the bilayer organization on a local scale [26], which agrees with our present fluorescence microscopy results, revealing that even dead cells retain their original shape and DNA content. These studies support the notion of a 'pore-forming' mechanism for bacteria killing by temporin L, as suggested by fluorescence studies with model membranes [27,41], revealing that the peptide inserts deeply into the membrane core, increases the membrane acyl chain order and has a perpendicular orientation with respect to the plane of the membrane surface. The unique effect of temporin L on *E. coli*, compared with many other antimicrobial peptides, was further demonstrated by using SEM and TEM, which showed no bleb formation at any stage of the treatment. Instead, only ghost-like bacteria were noted.

Previous studies on the alteration of the surface morphology of *E. coli* cells after treatment with antimicrobial peptides, such as sarcotoxin I [42], hecate-1 [43], melittin [43], PGYa [44], SMAP-29 [45], CEMA [12] and magainin [46], mostly reported blebbing and blistering of the outer membrane. Furthermore, under conditions similar to those used in the present study, melittin [47], the human antimicrobial peptide LL-37 [48] and D,L-amino acid-containing peptides [49,50] also lead to the formation of blebs in *E. coli* and other bacterial strains, followed by cell lysis. All of these examples further demonstrate the different modes of action of temporin L compared with most currently investigated antimicrobial peptides.

In conclusion, this study emphasizes the advantage of using the triple-staining approach combined with microscopic visualization of cell morphology for investigating the effect of antimicrobial peptides on living bacteria. Our results support the hypothesis that the main target of temporin L is the bacterial inner membrane, since the peptide clearly causes appreciable membrane permeabilization, but not total disruption of cells. Most interestingly, at a sublethal peptide concentration, the inner membrane becomes permeable to small compounds, but at this stage the bacteria are still alive. Instead, at a high peptide concentration, the membrane also becomes leaky to large cytoplasmic components, which is concomitant with death of bacteria. Therefore it is reasonable to speculate that the actual mechanism of bacterial killing differs at high and low peptide concentrations. At high temporin L concentration, a rapid killing occurs owing to a serious loss of membrane integrity. Nevertheless, at low peptide concentrations, membrane perturbation would not be the lethal step. Under these conditions, a small percentage of *E. coli* might be killed because of other additional events. Among them, peptides could bind to intracellular components as proposed for other natural antimicrobial peptides, such as bactenecin [18], Bac-7 [51], abaecin [52], apidaecin [53], PR-39 [54], indolicidin [55], buforin II [17] and several synthetic peptides [22]. At this stage, however, it cannot be ruled out that temporin L hits multiple potential targets in the bacteria (besides the inner membrane) even at high peptide concentrations.

Besides shedding light on the mode of action of temporin L and proposing a new approach to study the modes of action of antimicrobial peptides on living bacteria, we have observed that treatment of bacteria with a low temporin L concentration can alter the membrane permeability without killing the bacteria, which suggests that this peptide (and probably others) also functions as a 'helper agent'. The peptide might be suitable for facilitating the ingress of impermeant drugs, such as most of the conventional antibiotics, inside microbial targets. Furthermore, the new triple-staining approach presented in this study could be useful, from a pharmaceutical point of view, to screen peptides or other molecules that have the capacity to modify the bacterial membrane permeability without affecting cell viability.

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