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KKKKPLFGLFFGLF: A cationic peptide designed to exert antibacterial activity

Emilie Duval [a], Céline Zatylny [a], Mathieu Laurencin [b], Michèle Baudy-Floc'h [b], Joël Henry [a,*]

[a] UMR 100 IFREMER Physiologie et Ecophysiologie des Mollusques Marins, Université de Caen, 14032 Caen cedex, France
[b] Ciblage et Auto-Assemblages Fonctionnels, ICMV, UMR CNRS 6226, Université de Rennes I, 263 Av. du Général Leclerc, F-35042 Rennes Cedex, France

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ABSTRACT

With 14 residues organized as two domains linked by a single proline, the *de novo* peptide called K4 was designed, using Antimicrobial Peptide Database, to exert antibacterial activity. The N-terminal domain is composed of four lysines enhancing membrane interactions, and the C-terminal domain is putatively folded into a hydrophobic α-helix. Following the synthesis, the purification and the structural checking, antibacterial assays revealed a strong activity against gram-positive and gram-negative bacteria including human pathogenic bacteria such as *Staphylococcus aureus* and some marine bacteria of the genus *Vibrio*. Scanning electron microscopy of *Escherichia coli* confirmed that K4 lyses bacterial cells. The cytotoxicity was tested against rabbit erythrocytes and chinese hamster ovary cells (CHO-K1). These tests revealed that K4 is non-toxic to mammalian cells for bacteriolytic concentrations. The peptide K4 could be a valuable candidate for future therapeutic applications.

1. Introduction

Pathogens resistances to antibiotics are becoming one of major public health problem. Almost all antibiotics were baffled by the great ability to adaptation of pathogens and the emergence of new agents, discovered with up-dated technologies. The development of new antibiotic compounds like antimicrobial peptides (AMPs) is necessary.

Antimicrobial peptides are currently used clinically in two topical and two systemically applied formulations for the treatment of several diseases, as well as prophylactically to prevent infections in neutropenic or cystic fibrosis patients [11]. While thousands of AMPs are identified, only few of them are developed in view of clinical applications. The high cost of manufacturing peptides is arguably the principal problem preventing the widespread clinical use of this class of antibacterial therapeutic. The size of peptides is an important issue for reducing manufacturing costs [6].

Strategies have been devised very recently to develop biologically active molecules with a potential role as anti-infective agents. These new synthetic compounds are either based on the amino acid sequences of naturally occurring AMPs or *de novo* peptides and pseudopeptides. Numerous synthetic peptides have been developed based upon the structure of antimicrobial agents to enhance the activity [12].

Tossi et al. (1997) [13] developed a method for the design of synthetic α-helical AMPs based on the comparison of the sequences of known, naturally occurring peptides which contain α-helical domains, and extraction of sequence patterns which favor formation of this type of peptide. The patterns were then used to construct sequence templates for potential α-helical antibacterial peptides. Among the strategies, the development of synthetic combinatorial libraries (SCLs) represented a major advance in the discovery of new compounds for drug development [3,2,8]. SCL approaches allow a better understanding of the mechanisms underlying the lytic specificity of peptides, i.e., discrimination between eukaryotic and prokaryotic cell membranes [1].

In this study, we have developed a *de novo* antibacterial peptide using a strategy different from those outlined above. Its structure is based on characteristics of naturally occurring AMPs: short, preferably cationic, with α-L-amino acids, and with more than 30% of hydrophobic residues. This peptide was obtained using the Antimicrobial Peptide Database (APD: http://aps.unmc.edu/AP/main.html [14]). This web tool offers a research interface to select peptides with search criteria such as length, structure, hydrophobic

* Corresponding author. Tel.: +33 2 31 56 55 96; fax: +33 2 31 56 53 46.
E-mail address: joel.henry@unicaen.fr (J. Henry).

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percentage, net charge and the target organism and provides statistical information on peptide sequence, structure and function, average peptide length, average net charge per peptide and frequency of each amino acid.

In addition, APD has a prediction interface, which allows input of peptide sequences, and prediction of whether the new peptide has the potential to be antimicrobial based on known factors. The program carries out a residue analysis on the peptide and in terms of structure, simple predictions can be made. When hydrophobic residues appear every two or three residues in the peptide sequence, an amphipathic helix will be predicted. K4 is a 14 residue linear peptide including 3 Leu and 4 Phe (50% hydrophobicity), and 4 Lys (positive net charge +4). K4 appeared to be a potent antibacterial peptide against gram-positive and gram-negative bacteria without any toxicity for mammalian cells at its bacteriolytic concentration.

2. Material and methods

2.1. Peptide

K4 was synthesized using classical Fmoc (*N*-[9-fluorenyl]-methoxycarbonyl) solid-phase chemistry using a commercial automatic peptide synthesizer (Advanced Chem. Tech. 440 Mos synthesizer) by coupling Fmoc-α-amino acids on preloaded Wang resin. Protected amino acids were coupled by *in situ* activation with diisopropylethylamine (DIEA) and *N*-hydroxybenzotriazole (HOBt). *N*α-Fmoc deprotection was performed with 20% piperidine in dimethylformamide (DMF). Side chain deprotection and cleavage of the peptide from the solid support were performed by treatment with reagent (95% trifluoroacetic acid (TFA)/2.5% triisopropylsilane (TIS)/2.5% water) for 2 h at 20 °C. The peptide was purified by reversed-phase HPLC (RP-HPLC) using a Waters semi-preparative HPLC system with an X Terra 10 μm column (300 mm × 19 mm). The elution was achieved with a linear gradient of aqueous 0.08% TFA (A) and 0.08% TFA in acetonitrile (B) at a flow rate of 8 ml/min (5–60%B in 30 min) with photodiode array detection at 210–440 nm. The purity of the peptide was controlled by analytical RP-HPLC on the same instrument with an X Terra 5 μm column (250 mm × 4.6 mm) using a linear gradient of 0.08% TFA in water and acetonitrile containing 0.08% TFA at a flow rate of 1 ml/min (5–60%B in 20 min). Finally, K4 was assessed by LC–ESI-MS/MS analysis.

2.2. Micro LC–ESI-MS/MS analysis

Analyses were performed with a HPLC Surveyor chain connected on-line to an orthogonal electrospray source (Deca XP MS-n Thermofinnigan) operated in the positive electrospray ionization mode (ESI+). The ions were focused into an ion trap, capable of MS and MS/MS analyses. The mass spectra were acquired during 35 ms from 300 to 2000 *m*/*z*. The capillary exit of the electrospray ion source was set at 70 V, the octapole at 3 V and the capillary temperature at 200 °C. A counter flow of nitrogen was used as nebulizing gas. Xcalibur data system was used to acquire the data, which were further processed with a Turbo Sequest data system. The synthetic peptide was resuspended in 0.1% acetic acid in water and injected onto a C18 Thermo electron corporation C18 column (0.5 mm × 50 mm, 5 μm) with an acetonitrile linear gradient of 3% per minute in 0.1% acetic acid, from 2% to 60%. A split ratio of 30:1 was used to perfuse the column at a flow rate of 10 μl/min. The HPLC column was rinsed with 90% acetonitrile in 0.1% acetic acid between each injection. The MS data was acquired in scan mode considering the positive ion signal.

2.3. Antimicrobial assays

Antimicrobial activity of K4 was assayed against several bacteria summarized in the Table 1. The minimal growth inhibition concentration (MIC) was determined in triplicate by liquid growth inhibition assays [7]. Briefly, 10 μl of peptide solution were incubated in microtiter plates with 100 μl of a suspension of bacteria at a starting optical density of D_{600} = 0.001 in Poor-broth nutrient medium (PB: 1% peptone, 0.5% NaCl, w/v, pH 7.5). Pathogenic bacteria were grown in PB supplemented with 0.3% of beef extract (BD) except *Enterococcus faecalis* and *Listeria monocytogenes* being grown in brain heart infusion (BD), and marine bacteria being grown in Saline PB (SPB: 1% peptone, 1.5% NaCl, w/v, pH 7.2). Bacterial growth was assayed by measurement of optical density at *A*595 after a 20-h incubation at 30 °C or 18 °C for marine bacteria. MICs are expressed in μg/ml as a concentration interval [a] – [b], where [a] is the highest concentration tested at which microbial growth was observed and [b] is the lowest concentration tested that caused 100% growth inhibition.

2.4. Scanning electron microscopy

A mid-logarithmic phase culture of *Escherichia coli* was exposed in duplicate to 100 μg/ml K4 or water as a control, in PB for 2 h at 37 °C. Bacteria were pelleted by centrifugation at 3000 g for 5 min and washed 2 times in phosphate-buffered saline (PBS, pH 7.4). The supernatants were removed and the pellets were fixed in 800 μl of 2.5% glutaraldehyde in 0.1 M phosphate Sorensen buffer (pH 7.4) for 18 h. During the fixation, the samples were mounted onto poly-

Table 1
Strains of bacteria and medium used for determining MICs.

	Bacteria	Origin	Culture medium	Test medium
Gram-positive	*Bacillus megateriurm*	CIP 51.17	LB	PB
	Enterococcus faecalis	CIP 76.117	BHI	BHI
	Listeria monocytogenes	LME, Caen	BHI	BHI
	Staphylococcus aureus	CIP 53.1 56	BHI	PB + Beef extract 0,3%
Gram-negative	*Escherichia coli*	CIP 54 .8	LB	PB
	Klebsiella pneumoniae	CIP 52.1 45	LB	PB + Beef extract 0,3%
	Pseudomonas aeruginosa	CIPA22	LB	PB + Beef extract 0,3%
	Salmonella typhimurium	LME, Caen	LB	PB + Beef extract 0,3%
	Vibrio aestuarianus	IFREMER	Zobell	SPB
	Vibrio alginolyticus	IFREMER	Zobell	SPB
	Vibrio harveyi	IFREMER	Zobell	SPB
	Vibrio splendidus	IFREMER	Zobell	SPB

LME: Laboratoire de Microbiologie de l'Environnement, Caen, France IFREMER: French Research Institute for Exploitation of the Sea CIP: Collection of ''Institut Pasteur'' BHI: Brain Heart Infusion (BD): 37 g/l, pH 7,4 LB: (Luria-Bertani): peptone 10 g/l. yeast extract 5 g/l, NaCl 10 g/l. pH 7,5 PB (Poor Broth): peptone 10 g/l, NaCl 5 g/l, pH 7,4 SPB (Saline Poor Broth): peptone 10 g/l, NaCl 15 g/l. pH 7,2 Zobell: peptone 4 g/l, yeast extract 1 g/l, sea salts (Instant Ocean) 30 g/l Beef extract (BD)

L-lysine coated coverslips. The bacteria on the coverslips were washed 3 times in 0.1 M phosphate buffer and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4 °C in the dark. The bacteria were then washed 3 times in 0.1 M phosphate buffer and dehydrated in a graded alcohol series. The samples were vacuum dried (Balzers CPD 020) and sputter coated with platinum (Jeol JFC1300) and viewed with a Jeol (JSM 6400 F) scanning electron microscope at the Electron Microscopy Centre of the University of Caen (CMABio).

2.5. Cytotoxicity assay

The colorimetric MTT (3-[4,5 dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide) dye reduction assay was used to determine the cytotoxicity of K4 on CHO-K1 cells. Briefly, 1.34×10^4 cells/well in Ham F12 supplemented with L-glutamine (Gibco) and 10% foetal calf serum (Eurobio) were placed into 96-well plates. After incubation for 24 h under a fully humidified atmosphere of 95% room air and 5% CO_2 at 37 °C, K4 was added to cell cultures at a final concentration of 10 μg/ml, 40 μg/ml, 160 μg/ml and 640 μg/ml. Toxicity was evaluated after 24 h of incubation by measuring the optical density of the culture at 570 nm using the cell growth determination kit (Sigma) based on conversion of the yellow tetrazolium salt MTT into purple formazan crystals by metabolically active cells.

2.6. Hemolytic activity

The hemolytic activity of the peptide K4 was determined based on hemolysis of rabbit red blood cells (rRBCs). The rRBCs were isolated from rabbit blood: 1 ml of blood was deposited into a tube containing 2 ml of phosphate buffer saline (PBS).

The rRBCs were then isolated by centrifugation at 1000 g for 5 min and washed 3 times with PBS. The washed rRBCs were resuspended in 10 ml of PBS to attain a dilution of about 1% of the erythrocyte volume initially collected. 90 μl of rRBCs solution were incubated with 90 μl of different peptide concentrations dissolved in PBS buffer for 1 h at 37 °C. The samples were centrifuged for 5 min and hemolysis was determined by measuring the optical density of the supernatant at 415 nm. Zero hemolysis (blank) and 100% hemolysis were determined in PBS buffer and 1% Triton X-100, respectively.

2.7. Statistical analysis

The results are expressed as mean ± standard deviations. Student's *t* test for (paired or unpaired) data was used to determine statistical significance. *P*-value < 0.05 was considered significant.

3. Results

3.1. Peptide design and synthesis

The C-terminal segment is constituted of 3 Leu and 4 Phe residues which may form a α-helix and which may have at least 7 residues on the same hydrophobic. Pro has long been recognized as a helix-breaking amino acid and the Pro[5] may therefore form a flexible hinge connecting the N-terminal 4 Lys and the C-terminal α-helical segment. The positive net charge (+4) of the peptide due to the 4 Lys should facilitate interaction with the negatively charged bacterial cell membrane. The peptide was purified by rp-HPLC and its sequence as well as its purity (>90%) were checked by microLC–ESI-MS/MS. The calculated molecular weight (MW 1668.9) was recovered (*m*/*z* 1670.12) (Fig. 1A) and MS/MS spectrum confirmed the expected primary sequence (Fig. 1B).

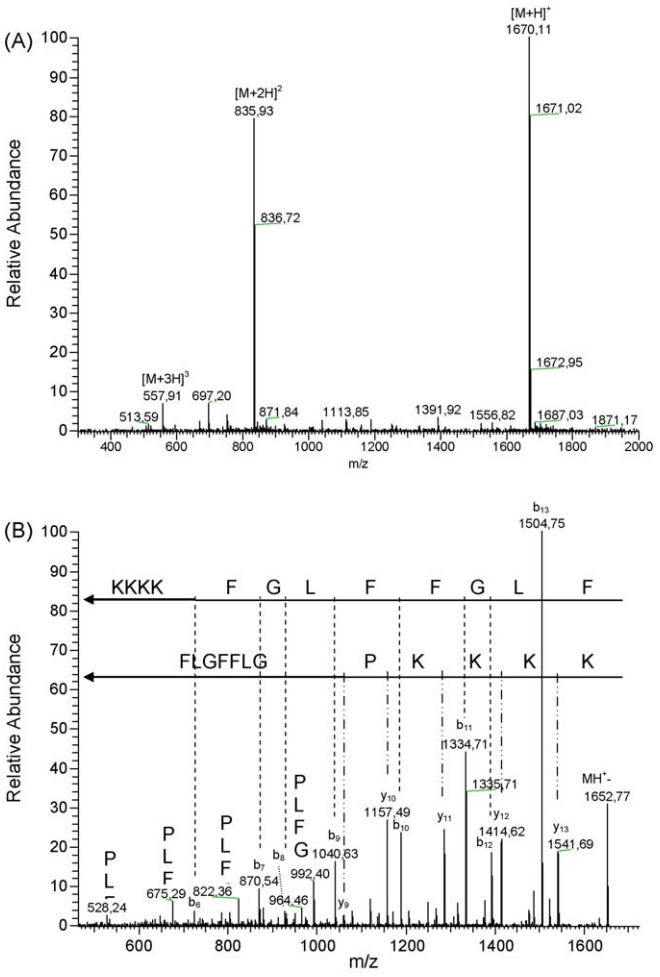

Fig. 1. analytical data of purified peptide K4. (A) MS spectrum and (B) structural checking by MS/MS analysis.

3.2. Antibacterial studies

The MICs of K4 obtained for *Bacillus megaterium*, *Enterococcus faecalis*, *L. monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella thyphimurium*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio aestuarianus* and *Vibrio splendidus* can be compared with those of ampicillin and gentamicin, two common antibiotics. K4 had a broad-spectrum activity which applied to all strains tested. K4 inhibited the growth of gram-positive bacteria such as *B. megaterium* (5–10 μg/ml (3–6 μM)), *S. aureus* (10–20 μg/ml (6–12 μM)), *L. monocytogenes* (20–40 μg/ml (12–24 μM)) and *E. faecalis* (80–160 μg/ml (48–96 μM)). K4 was also effective against gram-negative bacteria: for *E. coli* and the marine bacteria *V. harveyi*, *V. aestuarianus* and *V. splendidus* the MIC was of 5–10 μg/ml (3–6 μM). *V. splendidus* was less sensitive than other marine bacteria, the MIC of K4 being 10–20 μg/ml (6–12 μM) for this species. K4 was less effective against the human pathogenic gram-negative bacteria. For *S. thyphimurium*, *P. aeruginosa* and *K. pneumoniae*, its MIC ranged from 40 μg/ml to 80 μg/ml (24–48 μM). K4 showed a significantly higher potency against *B. megaterium*, *P. aeruginosa*, *V. harveyi* and *V. alginolyticus* in comparison to ampicillin.

3.3. Scanning electron microscopy

SEM was used to examine if K4 significantly altered *E. coli* membranes. SEM of *E. coli* treated with K4 (100 μg/ml) showed a

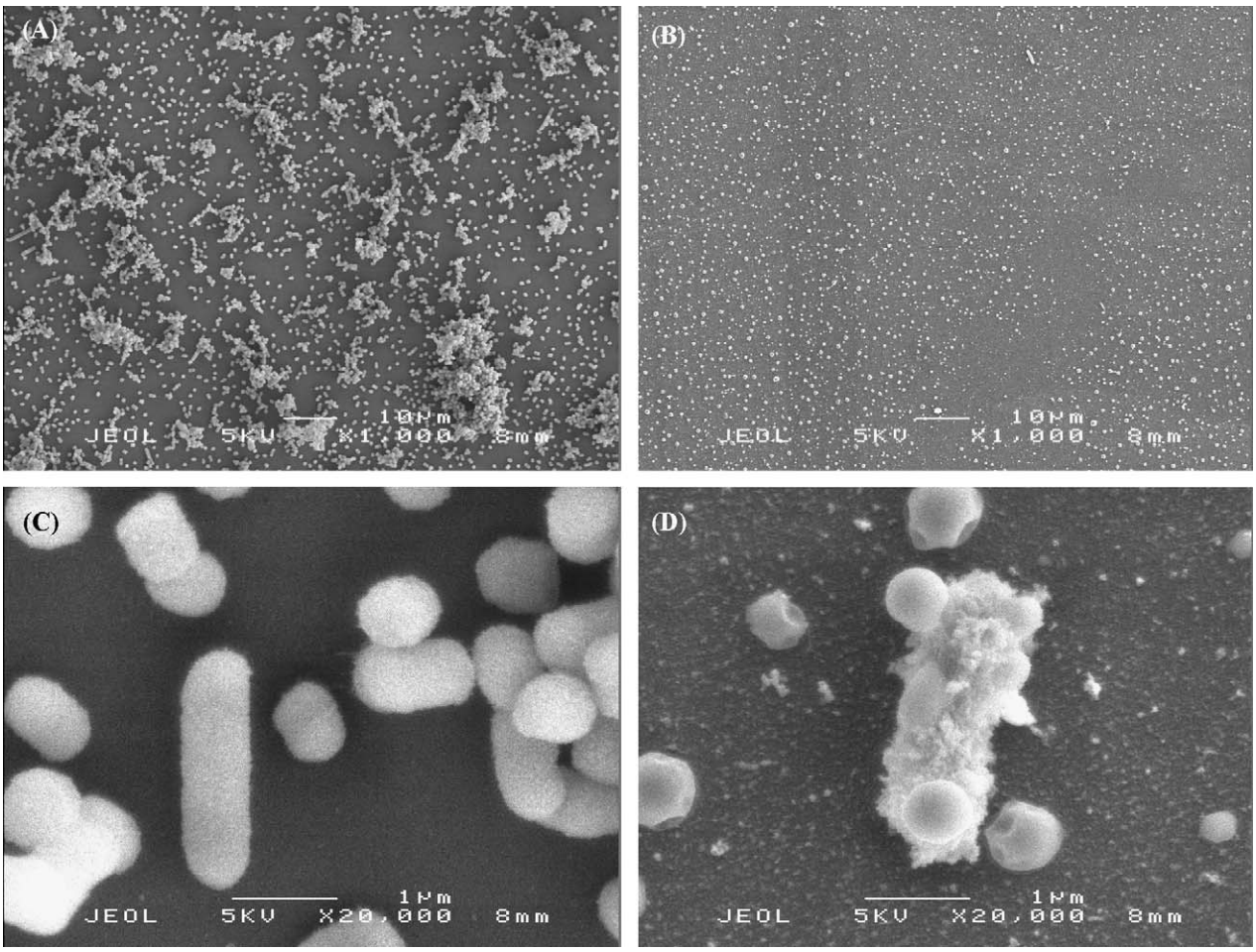

Fig. 2. Scanning electron micrographs of *E. coli* treated with K4. *E. coli* in mid-logarithmic growth was incubated with antibacterial peptide K4 for 2 h. (A) and (C) Water only; (B) and (D) K4, 100 μg/ml.

considerable lytic effect of the *de novo* peptide (Fig. 2A and B) with almost all bacteria denatured. Numerous cellular fragments were observed in samples of treated bacteria (Fig. 2D), whereas there were no fragments for control untreated bacteria (Fig. 2C). *E. coli* treated with K4 were covered by a thick coating (Fig. 2D). Furthermore, numerous spherical elements whose diameter ranged from 0.2 μm to 0.7 μm were observed around bacteria (Fig. 2B).

3.4. Hemolytic assay

K4 was tested for hemolytic activity against rabbit erythrocytes at 10 μg/ml, 40 μg/ml and 160 μg/ml. In rabbit erythrocytes, no significant hemolytic activity (<3%) was observed at 10 and 40 μg/ml. K4 showed slight hemolysis (6.65%) at 160 μg/ml. However, this dose is higher than the MICs measured for the 12 bacteria strains tested.

3.5. Cytotoxicity assay

The cytotoxic activity of K4 on CHO-K1 cells at 10 μg/ml, 40 μg/ml, 160 μg/ml and 640 μg/ml is illustrated in Fig. 3. The smallest doses of K4 tested (10 μg/ml and 40 μg/ml) were not cytotoxic to CHO-K1 cells. The peptide was slightly cytotoxic at 160 μg/ml, cell viability being 76%. However, cytotoxic activity was high at 640 μg/ml (384 μM), which is 8–128 times the MIC of this peptide.

4. Discussion

In view of the emergence of multidrug-resistant bacteria such as methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus* sp., there is currently considerable interest in developing new anti-infective agents. In this context, AMPs, which are an essential component of innate immunity in all living organisms, are interesting candidates.

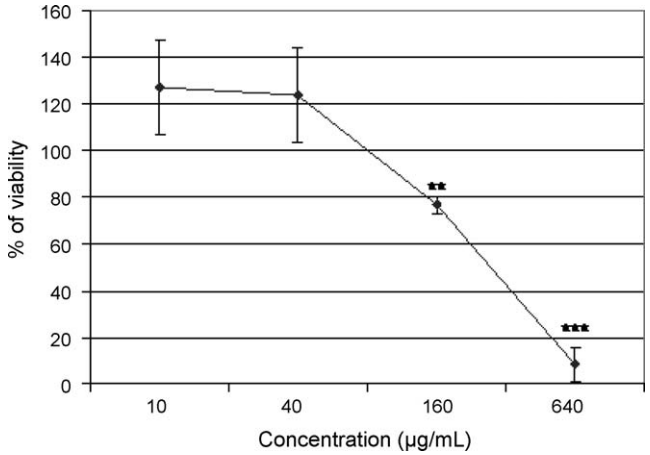

Fig. 3. Viability of Chinese hamster ovary cells (CHO-K1) incubated with increasing concentrations of peptide K4. $^{**}P$-value < 0.01 and $^{***}P$-value < 0.001.

In this paper, we report the design of a linear *de novo* peptide that may form a α-helix. K4 possesses a broad spectrum of activity, affecting the growth of all gram-positive and gram-negative bacteria tested at concentrations lower than 160 μg/ml (96 μM). For gram-positive bacteria, MICs of K4 depended on the strain tested. It was generally less potent than ampicillin, except against *B. megaterium*, growth of which was inhibited at 5–10 μg/ml (3–6 μM). K4 inhibited growth of the gram-negative bacteria *E. coli* at 5–10 μg/ml (3–6 μM), while a MIC of 40–80 μg/ml (24–48 μM) was effective against other gram-negative pathogens tested. Overall, classical antibiotics were generally more potent than K4, with some exceptions such as the MIC measured for gentamicin against *E. faecalis*, but the MICs of K4 were, on the whole, very close to those of antibiotics.

For *E. coli*, the MIC of K4 being so low, a SEM study was conducted to observe the structural effects of the peptide on these bacteria. Marked changes were observed on the cell surface of the bacteria treated with K4: a thick coating was present, in contrast to the smooth surface of untreated bacteria (Fig. 2). The numerous fragments as well as spherical element, probable microsomes, observed in *E. coli* cultures treated with K4 indicate cell lysis. K4 could form transmembrane pores resulting in membrane permeabilization, as described for many α-helical antibacterial peptides [9,15]. Membrane disruption could be associated with leakage of ions and metabolites, depolarization and ultimately cell death.

K4 was active against marine bacteria belonging to the genus *Vibrio*. All of the MICs measured were lower than 20 μg/ml (12 μM). K4 was often more potent than AMPs isolated from marine invertebrates. For example, for *V. alginolyticus*, the MIC of K4 ranged from 10 μg/ml to 20 μg/ml (6–12 μM), that is between 3 and 6 times lower than those of respectively, defensine Cg-def isolated from the oyster *Crassostrea gigas* [5] and penaeidine Pen-2 isolated from the shrimp *Penaeus vannamei* [4]. Furthermore, K4 was more potent than ampicillin against marine bacteria. Ampicillin was inactive at concentrations corresponding to MICs of K4 for *V. harveyi* (5–10 μg/ml (3–6 μM)) and *V. alginolyticus* (10–20 μg/ml (6–12 μM)). Since K4 appears to particularly affect the growth of marine bacteria, it could be used in aquaculture to protect hatcheries and rearing systems against microbially induced infectious diseases. Antibacterial peptides such as CEME and amidated pleurocidin have already been demonstrated to be efficient in this context. These two peptides have the ability to protect coho salmon *Oncorhyncus kisutch* against *Vibrio anguillarium* infection *in vivo* [10] Before the peptide K4 can be used in the field of aquaculture, additional experiments are required, including tests on survival of larvae and cytotoxicity tests on invertebrate and/or fish cells.

This *de novo* peptide contains only 14 amino acids and shows a broad spectrum of activity, affecting gram-positive as well as gram-negative bacteria. In addition, at 40 μg/ml (24 μM) K4 has no lytic effect on rabbit erythrocytes and CHO-K1 cells *in vitro*. Due to these characteristics, potential applications in human and animal health can be envisaged, such as use as an antiseptic or a disinfectant.

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