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The interaction of arginine- and tryptophan-rich cyclic hexapeptides with *Escherichia coli* membranes‡

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Abstract: Cyclization of R- and W-rich hexapeptides has been found to enhance specifically the antimicrobial activity against Gram-negative *Escherichia coli*. To gain insight into the role of the bacterial outer membrane in mediating selectivity, we assayed the activity of cyclic hexapeptides derived from the parent sequence c-(RRWWRF) against several *E. coli* strains and *Bacillus subtilis*, L-form bacteria, and *E. coli* lipopolysaccharide (LPS) mutant strains, and we also investigated the peptide-induced permeabilization of the outer and inner membrane of *E. coli*. Wall-deficient L-form bacteria were distinctly less susceptible than the wild type strain. The patterns of peptide-induced permeabilization of the outer and inner *E. coli* membranes correlated well with the antimicrobial activity, confirming that membrane permeabilization is a detrimental effect of the peptides upon bacteria. Truncation of LPS had no influence on the activity of the cyclic parent peptide, but the highly active c-(RRWFWR), with three adjacent aromatic residues, required the complete LPS for maximal activity. Furthermore, differences in the activity of the parent peptide and its all-D sequence indicated stereospecific interactions with the LPS mutant strains. We suggest that, depending on the primary sequence of the peptides, either hydrophobic interactions with the fatty acid chains of lipid A, or electrostatic interactions disturbing the polar core region and interference with saccharide-saccharide interactions prevail in the barrier-disturbing effect upon the outer membrane and thereby provide peptide accessibility to the inner membrane. The results underline the importance of tryptophan and arginine residues and their relative location for a high antimicrobial effect, and the activity-modulating function of the outer membrane of *E. coli*. In addition to membrane permeabilization, the data provided evidence for the involvement of other mechanisms in growth inhibition and killing of bacteria. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial hexapeptides; cyclic peptides; lipopolysaccharides (LPS); L-form bacteria; membrane permeabilization; outer membrane; inner membrane

INTRODUCTION

Because of a broad spectrum of activity against Gram-negative and Gram-positive bacteria, some fungi, viruses and parasites, antimicrobial peptides have been considered as a potential source of a new class of antibiotics [1]. The diversity in amino acid composition and conformation of such peptides, as well as their membrane active properties predicted by their biophysical characteristics, are compatible with a mechanism of action that involves common cellular properties rather than specific molecular targets. Different mechanisms of membrane perturbation and permeabilization have been discussed as the principal mode of action. More recently, an increasing number of peptides are being described that act also on intracellular targets [2], but all mechanisms still involve an initial interaction with the outer and/or inner membrane of bacteria.

An important class of antimicrobial peptides comprises short sequences that are rich in particular amino acid residues such as arginine (R) and tryptophan (W) [3]. Different strategies to improve their activity and bacterial selectivity have been described. However, neither the key factors that provide bacterial specificity nor the mode of action of short peptides are fully understood. Recent studies of an antimicrobial R- and W-rich hexapeptide, identified from combinatorial libraries [4], demonstrated that single amino acid substitutions and induction of conformational constraints can enhance or abolish activity and alter Gram-positive and Gram-negative selectivity [5,6]. While conserving the low haemolytic activity, head-to-tail cyclization and clustering of aromatic residues resulted in a 62-fold increase in activity against *Escherichia coli*, whereas such an increase against *Bacillus subtilis* did not exceed 8-fold. The activity was related to a pronounced amphipathicity conferred upon the conformationally constrained peptide by the cationic arginine side chains and the clustered tryptophan residues [7]. In contrast to the

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activity on biological cells, the permeabilizing effect towards electrically neutral lipid bilayers mimicking the erythrocyte's membrane was high and decreased distinctly towards bilayers composed of negatively charged lipids, which are characteristic of bacterial membranes [5,6]. The selective *E. coli* activity increase after peptide cyclization and the inverse relationship between activity against cells and cell membrane-modeling lipid bilayers indicated an important role for the outer membrane (OM) of Gram-negative bacteria. Its major components, the lipopolysaccharides (LPS), have two potential barriers, the hydrophilic, highly negatively charged and densely packed oligosaccharide core [8], and the hydrophobic region of hydrocarbon chains [9]. A mechanism of 'self promoted uptake' across the complex OM has been proposed to make the inner membrane (IM) accessible to the antimicrobial peptide [10].

Most antimicrobial peptides show affinity to acidic phospholipids as well as to LPS. However, it is not clear whether interactions between the peptides and LPS are specific or merely electrostatic as in the case of peptides and acidic phospholipids. The cyclic β-sheet antimicrobial peptide tachyplesin I, which is rich in R and aromatic residues, was found to show a 280-fold higher affinity for LPS than for acidic phospholipids, whereas a linear analog and a helical magainin peptide could not discriminate between LPS and negatively charged phospholipids [11]. The recognition site was the lipid A moiety and the cyclic structure was crucial to this specific binding. The cyclic structure also endowed the peptide with very rapid OM permeabilization. Studies with lactoferricin-derived peptides performed with LPS mutant strains underlined the importance of W and R, and their appropriate location for activity against *E. coli*. It has been suggested that the peptide first interacts with the negative charges present in the inner LPS core leading to the disorganization of the OM and thereby facilitates the approach of W residues to the lipid A in order to promote hydrophobic interactions [12]. Reduction of activity of lactoferricin-derived peptides against LPS mutant strains carrying modifications in the O-antigen and core regions points towards an activity-modulating role for these LPS regions [13].

The present studies address the question of how the *E. coli* OM contributes to the pronounced cyclization-induced activity increase of hexapeptides. We analyzed the effect of cyclic peptides derived from the parent sequence cyclo-(RRWWRF) against several strains. Their activity was determined against *E. coli* and *B. subtilis* strains and cell-wall deficient L-form bacteria. Their permeabilizing effect upon the OM and IM and their activity against *E. coli* LPS mutant strains was also evaluated. The results underline the important role of W and R residues and their position within the peptides for a high antimicrobial effect and demonstrate the activity-modulating function of the OM of *E. coli*. In addition to permeabilization, our results suggest that other mechanisms are involved in the antimicrobial effect.

MATERIALS AND METHODS

Materials

Linear peptides were synthesized automatically using standard Fmoc chemistry and cyclization was performed manually as described elsewhere [5,6]. Nitrocefin (NCF) was purchased from Oxoid (Basingstoke, UK). Luria Broth (LB), ampicilllin and HEPES buffer, *O*-nitrophenyl-β-galactoside (ONPG), Polymyxin B sulphate (PMX), cell culture additives (saccharose, yeast extract) and agar were from Sigma-Aldrich/Fluka (Steinheim, Germany). Mueller Hinton Broth medium was from Difco (BD, Heidelberg, Germany) and BBL brain heart infusion medium (BHI) was from BD (Heidelberg, Germany).

The *E. coli* strain ML-35p was provided by Prof. Robert I. Lehrer (Center for Health Sciences, Los Angeles, CA). The lipid A mutant strains MLK53, 986 and 1067 were kindly donated by Prof. C. R. Raetz (Duke University Medical Center, Durham, NC, USA).

Antimicrobial Activity Against Wild Type and L-Form Bacteria

The minimal inhibitory concentration (MIC) of bacterial growth was determined using a microtiter plate assay as previously described [14]. The peptide activity was tested against Gram-positive *B. subtilis* (PY22) and Gram-negative *E. coli* (DH5α) and (ML-35p) cultivated in LB. The inoculum was prepared from mid log phase cultures ($OD_{600} = 0.4 \pm 0.1$). The final number of cells per well was 2.5×10^5. The final concentrations of peptides, tested in duplicate, ranged from 1.5 to 200 μM. L-form *E. coli* W*1655 F+* cells (LWF+) and *B. subtilis* (L170) were cultivated in BHI containing 0.6 mg/ml penicillin, 30 mg/ml saccharose and 10 mg/ml yeast extract to an OD_{600} of 0.8 and diluted to OD_{600} 0.4. After adding 180 μl samples of the cell suspension to the wells of a microtiter plate containing 20 μl of serial dilutions of the peptide, the cell concentration in the wells was 3.5×10^8 LWF + cells/ml and 1.4×10^8 L170 cells/ml and the final peptide concentration ranged between 0.05 and 100 μM. After incubating the plates overnight at 37 °C with shaking at 180 rpm the absorbance was read at 600 nm (Safire Microplate Reader, Tecan, Crailsheim, Germany). The MIC was defined as the lowest concentration of peptide for which no change in optical density could be observed.

Outer- and Inner-Membrane Permeabilization Assays

The peptide-induced permeabililzation of the IM and OM of *E. coli* ML-35p was determined essentially as recently described [15]. The cells were grown in LB containing 100 μg/ml ampicillin to an OD_{600} of 0.3, rinsed twice using centrifugation at 4700 rpm for 1 min and suspended in HEPES buffer to an OD_{600} of 0.3. The peptides were dissolved in HEPES buffer. An NCF stock solution was prepared by dissolving 1 mg NCF in DMSO and diluting with HEPES buffer at a concentration of 60 μg/ml. ONPG was dissolved in HEPES buffer to 300 μg/ml.

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Membrane permeabilization was assayed in 96-well microtiter plates. The OM permeabilization assay was carried out with wells filled with 50 µl peptide solution and 50 µl of the NCF stock solution. NCF, a substrate of β-lactamase localized within the periplasmic space, is normally excluded from *E. coli* by the outer LPS layer. To assay IM permeabilization, the wells contained 50 µl peptide solution and 50 µl ONPG solution. ONPG can be cleaved by β-galactosidase localized within the cytoplasm, but it is blocked from cell entry by the IM since the strain lacks *lac* permease. The plates were prepared shortly before the experiment. Finally, 50 µl of cell suspension (OD 0.3) was added to the wells to give a final concentration of 20 µg/ml NCF or 100 µg/ml ONPG. Depending on the MIC of the peptide, the concentrations ranged from 1 to 200 µM.

After warming to 37 °C the plates were positioned in the plate reader at 37 °C. NCF entry and cleavage by β-lactamase was followed by optical density measurement at 500 nm over 10 min, and ONPG uptake and cleavage by β-galactosidase within the cytoplasm was characterized by monitoring absorption over a period of 60 min at 420 nm. Complete permeabilization was induced in the presence of 5 µM PMX as a positive control and wells lacking peptides served as negative control. The concentration of half-maximal membrane permeabilization (EC_{50}) was derived from dose–response curves giving the difference in the absorption values measured for peptide-exposed cells and peptide-free wells at 500 nm after 5 min (OM permeabilization) and at 420 nm after 40 min (IM permeabilization).

Bacterial Killing Assay

Overnight cultures of ML35p *E. coli* cells were diluted in LB containing 100 µg/ml ampicillin and grown to an OD_{600} of about 0.3 as described above. The cells were washed twice and resuspended in HEPES to an OD_{600} of 0.3. The assay was performed as recently described [16]. The peptide was dissolved in HEPES and added to the cell suspension to give an OD_{600} of 0.1 corresponding to 10^7 cfu/ml and final peptide concentrations corresponding to the MIC or the EC_{50} of OM permeabilization. The suspension was incubated at 37 °C. At time intervals of 1, 5, 10, 15, 20 and 30 min, samples were removed, diluted 50-fold (peptide-treated) or 5000-fold (untreated control), and 50 µl samples of the dilution were plated onto agar plates. After overnight incubation at 37 °C the colonies were counted.

Activity Towards LPS Carbohydrate and Lipid A Mutant Strains

The peptides were assayed against wild type and rough mutant strains of *E. coli* with structurally different LPS as described elsewhere [13]. The strains can be grouped according to the affected region of their LPS. The relevant genotype of the K12 strains is as follows: TG1 (wild type), D21 (rfa+), D21e19 (rfa-11), D21e7 (rfa-1), D21f1 (rfa-1, rfa-21), D21f2 (rfa-1, rfa-31). D21e19, D21e7, D21f1 and D21f2 carry different mutations in the rfa operon which encodes for transferases and other biosynthetic enzymes involved in biosynthesis of the hexose region of the LPS core. The composition of their LPS has been biochemically defined by gas chromatography analysis of the carbohydrates [17]. In strains D21e7 and D21e19, the lack of rhamnose and galactose with the reduced content of glucose has been suggested to lead to mutations affecting a step in the incorporation of galactose (from UDPgal) and galactose (from UDPG) [17]. Their different LPS composition implied that different genes were affected within the rfa operon, with a higher content in galactose for D21e19 indicating that the region affected was higher within the LPS, before the outer core and next to the O-antigen region. D21f1 is a glucose- and heptose-bound phosphate-less LPS strain and D21f2 is a heptose-less LPS strain (Figure 1). The lipid A mutant strains MLK53, 986 and 1067 are defective in late acetylations of lipid A and are knockout mutants. MLK1067 lacks the $(KDO)_2$-(lauroyl)-lipidIV_A myristoyltransferase and produces penta-acetylated lipid A that is devoid of the myristic acid residue. MLK 53 lacks the $(KDO)_2$-lipidIV_A lauroyltransferase and produces a penta-acetylated lipid A that is devoid of the lauric acid residue. The double mutant MLK986 produces lipid A that lacks the lauric and myristic acid residues (Figure 1).

Cultures were grown overnight in Mueller Hinton Broth medium at 37 °C. Strains MLK53, MLK986 and MLK1067 are thermo-sensitive and were therefore cultured at 32 °C. MICs were obtained using the recently modified method used in the Hancock laboratory from University of British Columbia, Vancover, (http://cmdr.ubc.ca/bobh/showmethod.php?methodid=79) based on the classical microtiter broth dilution recommended by the National Committee of Laboratory Safety and Standards (NCLSS). All assays were performed in duplicate using a range of ten different concentrations from 1 to 512 µM.

RESULTS

Antimicrobial Activity of Wild Type Strains and L-form Bacteria

MICs of several recently described hexapeptides [3,4] are summarized in Table 1. The data show that the ratio of MIC_c/MIC_l (subscripts c and l refer to cyclic and linear peptides, respectively) as a measure of cyclization-improved activity reached a value >62-fold for the c-RW3 and *E. coli* but was not higher than 8-fold for *B. subtilis*. To check whether this *E. coli*-specific activity-enhancing effect was related to the OM of bacteria, we determined the peptide activity against L-form bacteria. The negatively charged LPS of the *E. coli* OM are a physical barrier. Their removal was expected to enhance the peptide accessibility to the inner target membrane. In contrast, the susceptibility of both the LWF+ and the L170 strain decreased. Despite differences in the composition of the cytoplasmic membrane between Gram-negative and Gram-positive bacteria, no distinct differences in the MIC towards their L-form strains were found. The activity pattern of the investigated peptides was conserved, with c-RW2 and c-RW3 being the most active and c-RY the least active compound (Table 1). The results underline the important role of the outer envelope of Gram-negative and Gram-positive bacteria for the antimicrobial effect. The negative charge provided by anionic lipids, LPS and the peptidoglycan layer of the *E. coli* outer wall

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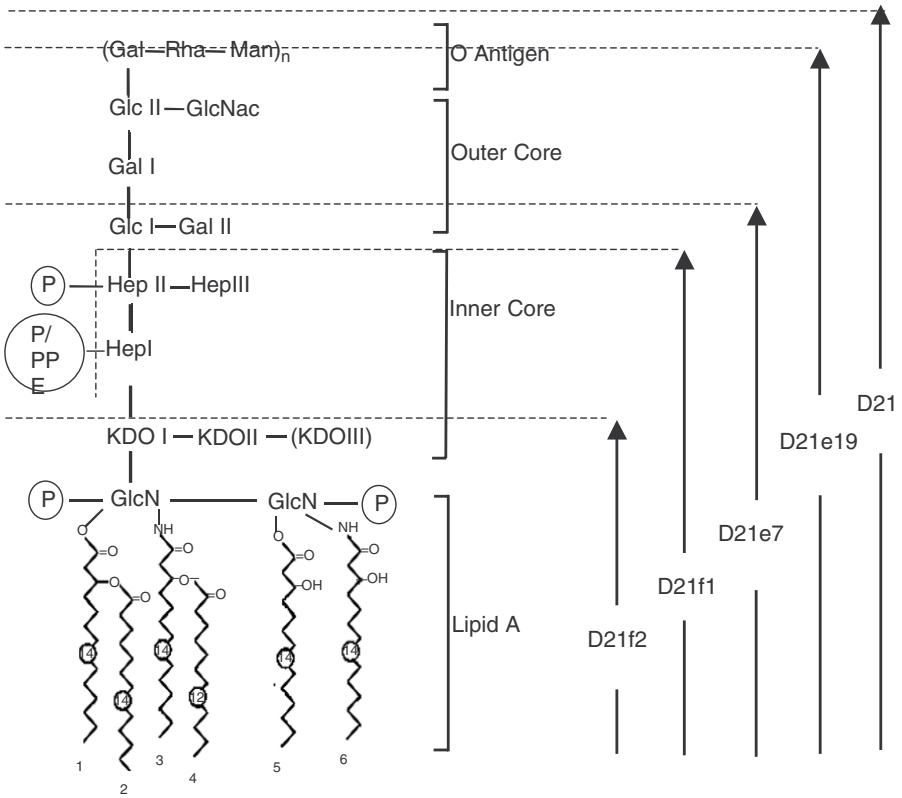

Figure 1 LPS-mutant phenotypes. The mutants derived from the *E. coli* parent strain D21 are as follows: D21e19 is deficient in carbohydrates close to the O-antigen region, D21e7 is galactose-less, D21f1 is a glucose- and heptose-bound phosphate-less LPS strain and D21f2 is a heptose-less LPS strain. The lipid A mutant strain MLK53 lacks the lauric acid residue (2), MLK 1067 is devoid of the myristic acid residue (4) and the double mutant MLK986 lacks both residues.

Table 1 Amino acid sequence of peptides, abbreviations and antimicrobial activity against wild type and L-form bacteria

Sequence	Name		MIC (μM) *E. coli*				MIC (μM) *B. subtilis*			
			DH5α		MIC_l/MIC_c	*LWF+*	*PY22*		MIC_l/MIC_c	*L170*
	linear	cyclic	lin	cyc		cyc	lin	cyc		cyc
RRNalNalRF	RNal	c-RNal	16	8	**2**	12.5	4	2	**2**	10
RRYYRF	RY	c-RY	>100	>100		>100	>100	>100		>100
KKWWKF	KW	c-KW	>100	25	**>4**	50	>100	25	**>4**	50
RRWWRF	RW	c-RW	>100	6	**>16**	25	25	3	**8**	40
rrwwrf	rw	c-rw	>100	12	**>8**	25	25	6	**4**	50
RRWWFR	RW2	c-RW2	>125	4	**>31**	12.5	31	4	**8**	12.5
RRWFWR	RW3	c-RW3	>125	2	**>62**	10	31	4	**8**	12.5

The linear peptides were *N*-terminally acetylated and *C*-terminally amidated. Small letters stand for D-amino acid residues and Nal is ß-(naphth-1-yl)alanine.
MIC values give the minimal inhibitory concentration of the growth of *E. coli* DH5α, *E. coli*-derived L-form LWF+ cells and *B. subtilis* PY22 and outer wall-deficient L170 cells. MIC_l/MIC_c is the ratio of the MIC of the linear and corresponding cyclic peptide and is a measure for the cyclization-induced improvement of antibacterial activity.

or the teichoic acids attached to the peptidoglycan layer of *B. subtilis* likely favor peptide accessibility to the cytoplasmic membrane. The composition of the cytoplasmic membrane seems to be less important for the peptide structure-dependent activity.

Outer and Inner Membrane Permeabilization

To further elucidate the role of the *E. coli* envelope, we determined the antimicrobial activity against strain ML-35p and the peptide-induced permeabilization of the

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OM and IM (Figure 2). Comparable to the peptide effects against strain DH5α (Table 1), the remarkable activity of the highly hydrophobic Nal-containing sequence was only slightly enhanced with cyclization and the less hydrophobic Y-containing peptide was inactive up to a concentration of 200 μM in the linear as well as cyclic form (Figure 2(A)). The all-L and all-D RW peptides showed equal potency. The linear peptides showed low permeabilizing efficiency, and the cyclization-induced activity increase was most pronounced for c-RW3.

To test the peptide effect upon the outer LPS-rich membrane, NCF was used as a probe. It is normally excluded by the OM, but following the barrier permeability enhancement of peptides, it can then be cleaved by ß-lactamase localized in the periplasmic space of ML-35p cells. Similarly, permeabilization of the IM was monitored using ONPG as a probe which is normally blocked from cell entry, but can be cleaved by ß-galactosidase after entry into the cytoplasm. PMX is a highly efficient membrane-permeabilizing cyclic peptide that mediates maximal NCF cleavage at 5 μM after about 3 min (Figure 3(A)). An OD value of 0.58 was equivalent to maximal OM permeabilization. All the investigated hexapeptides were much less efficient. Even at 40 μM concentration, the most active c-RW3 was not able to completely permeabilize the OM within 10 min. High c-RW3 concentrations were required to induce partial IM permeabilization, whereas PMX at 5 μM was also highly efficient (Figure 3(B)). The peptide effect on the OM (Figure 2(B)) and IM (Figure 2(C)) correlated well with the antimicrobial activity profile (Figure 2(A)), suggesting that comparable structural motifs determine the interaction with the two *E. coli* membranes.

Bacterial Killing Assay

The fact that significant OM and IM permeabilization was achieved only at 3–8 times the MIC of peptides (Figure 2) motivated studies to correlate membrane permeabilization and bacteriocidal activity under permeabilization assay conditions. At the MIC of c-RW3 (6 μM), the number of surviving cells was reduced to 10^5 cfu/ml compared to 2×10^7 cfu/ml of the control. No time dependence of cell survival was observed over an incubation period of 1 to 30 min, suggesting that the peptide acted very rapidly. At 25 μM concentration, bacterial survival after 30 min was rare. The results imply that membrane permeabilization is not the only event responsible for the cytotoxic mechanism.

Peptide Activity Against LPS-Mutant Strains

It has been found that the O-antigen, the core oligosaccharides, and the highly conserved lipid A of LPS are important features in *E. coli* resistance/susceptibility to peptides. To investigate the role of LPS in mediating the pronounced cyclization-induced activity increase of R- and W-containing peptides against *E. coli*, we

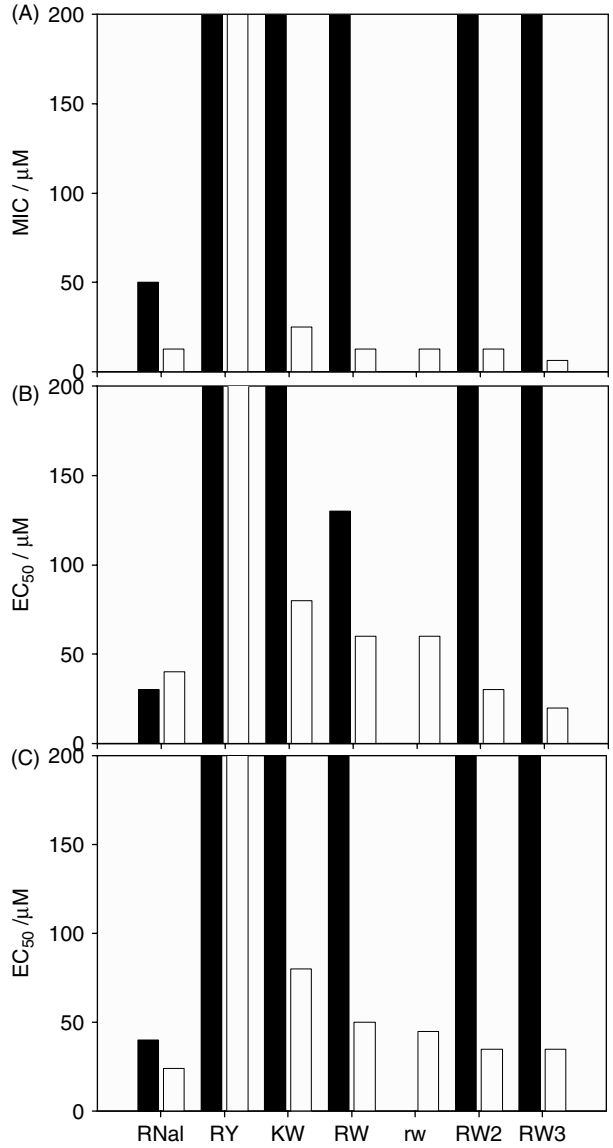

Figure 2 MIC of linear (black) and cyclic (white) peptides towards *E. coli* ML-35p (A) and concentrations of half maximal permeabilization, EC_{50} of the outer (B) and inner (C) membrane. The EC_{50} for the permeabilzation of the outer membrane was derived from changes in the optical density at 500 nm caused by NCF (20 μg/ml) cleavage after 10 min of cell incubation with peptides at 37 °C. The EC_{50} of inner membrane permeabilization was obtained from absorption measurements at 420 nm after 60 min monitoring ONPG (100 μg/ml) cleavage.

determined the peptide activity towards different LPS-carbohydrate and lipid A mutant strains (Table 2). No activity was detected for the linear peptides up to the concentration of 512 μM (data not shown). The activity of the cyclic peptides against the wild type strain TG1 followed the patterns observed for the DH5α and ML-35p strains (Table 1 and Figure 2), with the exception of the enantiomer c-rw, which was distinctly more active than c-RW. These results suggest different membrane properties for the three wild type strains and

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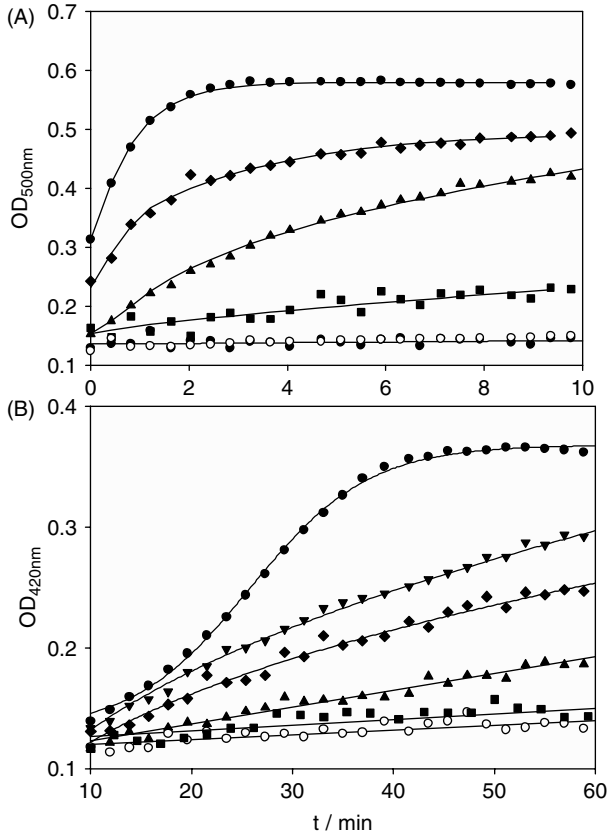

Figure 3 Time dependence of c-RW3-induced permeabiliziation of the outer and inner membrane of *E. coli* ML-35p. The kinetics of peptide-mediated NCF passage across the outer membrane (A) and ONPG passage across the inner membrane (B). The concentrations of the substrates were 20 and 100 μg/ml, respectively. The symbols indicate trials in the presence of 5 μM PMX (closed circles) to induce maximal response, untreated cells (open circles) as control and c-RW3 at 5 (hexagons), 10 (squares), 20 (up triangles), 40 (diamonds) and 80 (down triangles) μM concentrations.

specific peptide interactions with the LPS of TG1 bacteria. Truncation of the LPS-carbohydrate chains did not influence the activity of c-RW and c-KW but resulted in an increase in activity for the most hydrophobic peptide c-RNal. In contrast, the activity of c-rw was the lowest against the D21e7, D21f1 and D21f2 mutants. For c-RW2 and c-RW3 the activity was the highest against the wild type strain and decreased with the truncation of the LPS carbohydrate chain to be the lowest against the galactose-deficient D21e7 and the glucose- and heptose-bound phosphate-deficient D21f1 mutants, suggesting the involvement of the O-antigen and outer core in mediating peptide activity.

No differences were observed for the high activity of c-RW3 against lipid A mutant strains (Table 2). A decrease in MIC for c-RNal, c-KW, and c-RW against the tetra-acetylated lipid A mutant strain MLK986 leads to the suggestion that alteration of the tight fatty acid packing of the hexa-acetylation of lipid A results in an increase in fluidity which could facilitate hydrophobic interactions [12].

DISCUSSION

In this paper we demonstrate that the pronounced sequence-dependent and cyclization-induced activity increase of R- and W-rich hexapeptides towards Gram-negative *E. coli* is related to the permeabilization of both OM and IM. Secondly, their decreased activity towards cell-wall-deficient L-form bacteria, and the reduction of activity of the peptides containing three adjacent aromatic and charged residues with progressive decrease in the LPS-polysaccharide chain, both suggest the OM as a main modulator of activity. Thirdly, the observation that peptide-induced membrane damage seems to lag behind the time required to cause cell death points to a contribution of permeabilization-independent mechanisms in cell killing.

The OM of Gram-negative bacteria consists of the hydrophilic and highly negatively charged oligosaccharide core and a hydrophobic barrier provided by the six fatty acid chains per LPS molecule [10]. The role of Mg^{2+} ions in tightening LPS packing and the capacity of

Table 2 MIC of cyclic peptides towards LPS-carbohydrate and lipid A mutants

Peptides	MIC (μM) LPS carbohydrate mutants						MIC (μM) lipid A mutants			
	TG1	D21	D21e19	D21e7	D21f1	D21f2	W3110	MLD 53	MLK 1067	MLK 986
c-RNal	256	256	256	128	128	128	128	128	128	64
c-KW	512	512	512	512	512	512	256	256	256	32
c-RW	256	256	256	256	256	256	256	256	256	64
c-rw	64	64	64	128	128	128			n.d.	
c-RW2	64	128	128	128	128	64			n.d.	
c-RW3	16	64	64	128	128	64	32	32	32	32

n.d. not determined.

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peptides to interfere with ion binding to LPS have been shown [18]. The peptides appear to induce disorders in the LPS organization by interfering with the divalent ions and by insertion into the hydrocarbon chain region. Although this is not the lethal step in the mechanism of action of peptides, it is their means to gain access to the IM [10] and to disturb its organization which leads to the breakdown of cellular processes and final cell death.

Our studies show that permeabilization of *E. coli* OM and IM are based on the same structural motifs in this series of peptides. R and W confer significant affinity and perturbing activity upon the cyclic peptide, which correlated well with the recently reported retention times in reverse-phase HPLC and the permeabilizing activity against lipid bilayers [5,7]. However, unlike peptides such as melittin, in which OM permeabilizition is immediately followed by IM permeabilization [15], our peptides permeabilized the OM rapidly but the IM rather slowly. Thus, peptide passage across the OM is not rate-limiting for the weaker permeabilization of the IM. The activities of the cyclic peptides resembled that of PMX. This cationic, cyclic, acetylated decapeptide is highly active against the OM [19] but shows weaker IM activity [15,20]. Its high OM perturbation capacity is related to rapid competitive displacement of divalent cations from the negatively charged binding sites followed by insertion into the hydrophobic fatty acid chain region of LPS [21]. Hydrophobic interactions with OM LPS are also important for the activity of the cyclic hexapeptides. The effect of c-RNal and the less hydrophobic c-RW and c-KW on the different LPS mutant strain could be related to hydrophobic interactions with the acyl chain region of lipid A. The high activity of all peptides against the MLK986 mutant with only four fatty acid chains supports the suggestion that disturbance of the hydrophobic barrier is important for efficient peptide passage across the OM.

Several findings lead to the suggestion that specific interactions with the oligosaccharide core might also be involved in the lesion of the OM barrier. Firstly, changes in the stereochemistry (c-rw) and clustering of the aromatic and charged residues (c-RW2, c-RW3) improved peptide activity against the TG1 wild type strain whereas the activity towards the truncated LPS mutant strains D21e7 and D21f1 was little modified. Secondly, there was a distinct difference in the activity of c-RW and its all D-enantiomer c-rw against TG1 bacteria which was not observed against *E. coli* DH5α and ML-35p strains. Thirdly, a high activity of c-RW3 against the wild type strains and an independence of MIC from fatty acid packing of lipid A mutants were observed.

Recently, a decrease in activity with truncation of LPS carbohydrate chains was reported for lactoferricin-derived peptides [13]. Unmasking of the negative charges carried by the phosphate groups of the inner core was suggested to improve electrostatic interactions between the lactoferricin peptides and LPS. As a consequence, peptide transport to the target membrane should be inhibited. Such observations with R-, W-containing peptides are distinctly different from the findings with melittin and protegrin, which show improved activity towards core LPS mutants, [22] or the peptide NK2 whose antimicrobial activity is also strongly dependent on the length of the sugar chains, with LPS rough mutants being the most sensitive [9]. Positioning of the side chains seems be a determinant of the activity of the R- and W-containing peptides. Recent studies with compounds based on trimesic acid as a scaffold, and attached indole rings and guanidino or amino groups, support the idea that the accessibility of the negatively charged moieties of LPS for the cationic peptide residues is important for selectivity. A large linker between cationic guanidino or amino moieties and the scaffold was highly favorable for *E. coli* selectivity, whereas compounds with a short linker were inactive [23].

Besides ionic and hydrophobic interactions, saccharide–saccharide interactions have been proposed to promote tight association of LPS molecules [8,24]. Disturbance of interactions between the sugar moieties might contribute to the distinctly improved activity of c-rw, c-RW2 and c-RW3. Thus, tryptophan and tyrosine (Y) residues in carbohydrate-binding modules of enzymes participate in hydrophobic stacking interactions with polysaccharide substrates [25,26] and selective sugar–aromatic ring interactions have been reported for β-cyclodextrin and the Y- and W-bearing third domain of turkey ovomucoid [27].

In studies with L-form bacteria, no distinct differences were found in peptide activity towards *E. coli*- and *B. subtilis*-derived strains. This was quite different to observations with highly membrane active amphipathic helical model peptides, which were more active towards L170 compared to LWF+ [14]. The comparable activities of the cyclic hexapeptides against the two strains suggest that the composition of the cytoplasmic membrane might be of reduced importance for the biological effect. Implications for the involvement of other mechanisms in addition to peptide-induced membrane permeabilization were provided in studies of killing kinetics. At the MIC, the most active c-RW3 peptide induced rapid killing but was unable to permeabilize the OM and IM of *E. coli* within 10 and 60 min, respectively. Comparable findings have been reported for *E. coli* incubated with a defensin peptide, which killed the cells in 15 min but continued to damage the membrane 30–120 min after exposure [28]. Also, membrane permeabiliazition does not seem to be the ultimate mode of action of gramicidin S, which induced maximal depolarization of the *E. coli* IM by channel formation below the MIC [29]. For several antimicrobial peptides, including W-rich indolicidin and lactoferricin

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B, membrane translocation and interaction with cytoplasmic components with the consequence of inhibition of intracellular synthesis of nucleic acids and proteins have also been reported [30,31]. Recent studies of a linear R- and W-rich hexapeptide with fungi showing morphological alterations at sub-MIC [32] imply that the inhibition of bacterial growth induced by our cyclic RW-peptides might not also be solely a consequence of membrane perturbation.

In conclusion, these studies show that the cyclization-induced activity increase of R- and W-rich hexapeptides is related to an enhanced permeability increase of the OM and IM of bacteria. The efficient, structure-dependent disturbance of the OM is based on hydrophobic interaction with lipid A fatty acid chains, ionic interaction with phosphate groups located in lipid A and the inner core and probably peptide–saccharide interaction in the outer core and O-antigen region. The OM modulates the peptide accessibility to the cytoplasmic membrane. In addition to membrane damage, other mechanisms might be involved in the antimicrobial effect. Further studies are under way to analyze the individual contributions in detail.

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