Flo antibacterial peptide from the tropical tree *Moringa oleifera*:

A template for novel antibacterial agents

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RÉSUMÉ

Flo, un peptide antibactérien isolé de l'arbre tropical *Moringa oleifera*:
Une base pour de nouvelles substances antibactériennes

Les peptides antimicrobiens sont essentiels à la survie d'organismes multicellulaires. Ils servent à lutter contre des microbes pathogènes et à contrôler les communautés commensales inoffensives. Jusqu'à aujourd'hui ces peptides n'ont pas permis aux microbes de développer de véritables résistances. Pour ces raisons, les peptides antimicrobiens seraient des substances intéressantes comme alternative aux antibiotiques classiques.

La cible des peptides antimicrobiens est probablement la membrane cellulaire. Une structure amphipathique du peptide (charges positives séparées de parties hydrophobiques) est considéré essentielle pour l'activité. Son interaction avec les lipides membranaires (eux-mêmes amphipathiques) déstabilise la membrane, ce qui peut aller d'un simple changement de la perméabilité jusqu'à la rupture complète de la membrane.

Le polypeptide "Flo" a été isolé des graines de l'arbre *Moringa oleifera* Lam. L'extrait de ces graines est utilisé dans des pays africains pour purifier l'eau. Flo a d'abord été identifié comme substance coagulante, avant qu'une activité antibactérienne ait été démontrée.

Les buts du présent projet sont de comprendre le mécanisme antibactérien de Flo et de trouver un sous-fragment très court ayant la même activité que Flo. Ceci permettra de produire le peptide d'une manière biotechnologique à des coûts raisonnables.

Nous nous sommes servi d'un modèle tridimensionel produit par un outil bioinformatique. Nous avons synthétisé des sous-fragments du peptide Flo pour ensuite effectuer deux tests. D'abord l'effet antibactérien a été testé sur une culture d'*Escherichia coli*. Le taux de survie a été déterminé en comparant le nombre de cellules survivantes dans l'expérience à un contrôle. Ensuite, l'interaction avec des membranes a été testée par des vésicules artificielles qui libèrent un fluorochrome lors de la rupture de leur membrane induite par des peptides antibactériens.

Nos résultats suggèrent que des boucles hydrophobes et des hélices-α sont des éléments structuraux importants pour l'activité antibactérienne. En plus, nous avons découvert un peptide court, dérivé de Flo, qui a une activité antibactérienne 1000 fois plus haute que ce dernier. Nous proposons donc un modèle simple d'éléments structuraux minimaux pour le fonctionnement des peptides antibactériens. Cela permettra de développer des stratégies pour améliorer l'activité antibactérienne des peptides existantes et la création de nouvelles substances hautement antimicrobiennes.

SUMMARY

Antimicrobial peptides represent an important mechanism of innate immunity and are necessary for the survival of multicellular organisms. Biotechnically produced, antimicrobial peptides provide a powerful tool for treating infections with bacterial strains resistant against multiple common antibiotics. Antimicrobial peptides are reported to act on membranes with hydrophobic and positively charged patches. "Flo" is an antibacterial peptide isolated from seeds of a tropical tree (*Moringa oleifera* Lam.). The goals of this study were to elucidate the mechanism of action of Flo and to find short fully active Flo derived peptides that may be produced at a large scale. Based on a bioinformatical 3-dimensional model, subfragments of Flo were synthesised. Their antibacterial activity was tested by incubating *Escherichia coli* with the peptides and counting surviving cells. The effect on membranes was tested using artificial vesicles releasing a self-quenching fluorescencent dye when disrupted. Results show that a hydrophobic loop and stabilising α-helices are key factors to the antibacterial activity. In this study, we propose a simple model for the structural requirements of antibacterial peptides and we report the finding of a very short, highly active sub fragment that improves the antibacterial activity of Flo by a factor of 1000.

INTRODUCTION

It is crucial for the survival of multicellular organisms that they are able to defend themselves against microorganisms. This includes the elimination of pathogenic microbes and the control of non-pathogenic commensal communities. Antimicrobial peptides are an important part of the innate immune system of many different multicellular species from plants to mammals. Unlike common antibiotics, over a long period of time antimicrobial peptides did not permit microbes to develop resistance mechanisms against them (at large scale). This makes them interesting for the development of novel agents for the treatment of emerging multiresistant bacterial strains.

Antimicrobial peptides have been reported to act directly, and non-specifically, on membranes, which seems to be the reason for the difficulties microbes face in becoming resistant to them. Target microorganisms include: gram positive bacteria, gram negative bacteria, fungi and enveloped viruses. The membranes of multicellular species are rarely affected by antimicrobial peptides. Antimicrobial peptides probably interact with the membranes in two stages. First, cationic amino acids are attracted by negative charges (e.g. phospholipid headgroups) on the surface. Second, hydrophobic and positively charged patches of the peptide interact with the aliphatic fatty acids and the anionic components respectively. This induces membrane destabilisation, and bacteria are thought to be killed by the leakage of cytoplasmic contents, the loss of membrane potential, the change of membrane permeability, the change of lipid distribution, the entry of the peptide and blocking of anionic cell components or the triggering of autolytic enzymes [1, 2]. The membrane is the location of many important biochemical processes, which may be disturbed in this way.

In the literature there is a consensus about the requirement of a specific structural property for an antimicrobial peptide to be fully active, but it is rarely specified. There are three models proposed for the membrane destabilisation mechanism [2]: 1) In the barrel-stave model the peptides aggregate and form ordered channels which render the membrane leaky [3]. 2) The aggregate channel model predicts that unstructured peptide aggregates penetrate the membrane where they form transient pores and enter into the cell plasma [4]. 3) The carpet model hypothesises, that the membrane is covered by the peptide which induces a bending of the membrane and thus induces a breaking and disaggregation [5, 6].

The polypeptide "Flo" was isolated by cation exchange chromatography from the seed extract of the tropical tree *Moringa oleifera* Lam. The extract is traditionally used for water clarification in African countries such as Sudan and Malawi [7]. The peptide was found to act as a coagulant and its primary sequence was determined [8, 9]. Both recombinantly produced and synthetic versions of the peptide have been found to retain the coagulant activity [10, 11]. In addition it has been found to exert an antibacterial effect on many pathogenic bacterial strains, including antibiotic-resistant isolates of *Staphylococcus*, *Streptococcus* and *Legionella* species [11].
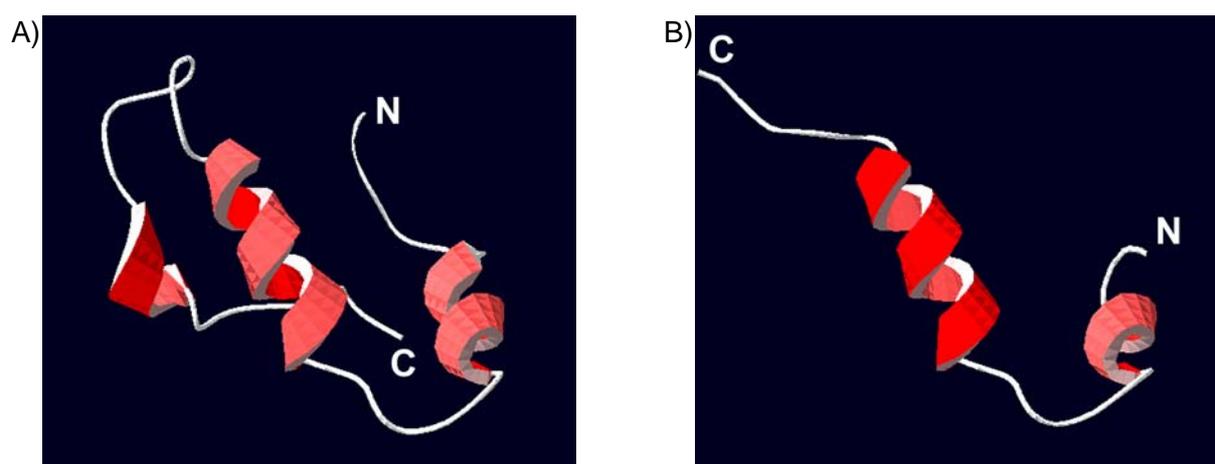

Fig. 1. 3-dimensional bioinformatical model based on the NMR structure of napin, a homologous 2S-albumin. (in red: α-helices)

A) Polypetide Flo, B) Sub fragment p4

Antibacterial peptides are structurally highly divergent with a typical size of 12-45 amino acids. They can be classified as amphipathic α-helices, disulfide bond stabilised β-sheet structures, peptides with predominant amino acids or peptides with loop structures [2]. In plants, antimicrobial peptides have been known for a long time and are classified into different families [12, 13]. Flo cannot be clearly associated with any one of the proposed classes.

Flo has sequence similarities to the larger B chain of 2S albumin seed proteins such as napins and mabilins [14]. We used the 3-dimensional structure of a napin, as determined by NMR [15], to design a homology based, bioinformatical model of Flo. The putative structure represents three α-helices linked by two loop regions (Fig. 1). The existence of three α-helical regions has been confirmed by secondary structure prediction [14]. These predictions could not be supported by circular dichroism analysis of Flo in aqueous solution, where the peptide seemed to adopt a random coil structure. However the presence of artificial membranes induced a shift of the spectrum to a more α-helical conformation [14]. Induced structuring is also reported for other α-helical antimicrobial peptides [16].

We had two major goals in our study. A) To elucidate the mechanism of the antibacterial activity of the Flo polypeptide, and B) to find short, but fully active, derivatives of Flo, that may be synthesised at a reasonable cost [17].

Herein we report the discovery of two short and very active fragments of Flo and propose a model for the antimicrobial activity of the Flo polypeptide.

RESULTS AND DISCUSSION

Antibacterial activity

We used an antibacterial test for our synthetic peptides derived from Flo. We incubated *E. coli* culture with each peptide during two hours. To estimate the number of surviving bacterial cells we made dilution series and plated cultures on petri-dishes. After an overnight growth we counted colonies on the dishes and compared their number to a control experiment.

Table 1. Amino acid sequences of Flo and derived sub fragments.

2° structure		pI
peptide	amino acid sequence	pI
Flo	QGPGRQPDFQRCGQQLRNISPPQRCPSLRQAVQLTHQQQGQVGPQQVRQMYRVASNIPST	11.61
p2	PQRCPSLRQAVQLTHQQQGQV	10.39
p4	RCGQQLRNISPPQRCPSLRQAVQLTHQQQGQ	11.53
p4G40R	RCGQQLRNISPPQRCPSLRQAVQLTHQQQ**R**Q	11.83
p4a	RCGQQLRNISPPQRCPSLRQAVQLTHQ	11.53
p4b	LRNISPPQRCPSLRQAVQLTHQQQGQ	11.70
p4bPP21NN	LRNIS**NN**QRCPSLRQAVQLTHQQQGQ	11.70
p4bR17Q	L**Q**NISPPQRCPSLRQAVQLTHQQQGQ	10.35
p4c	RCGQQLRNISPPQRCPSLRQ	11.53
p4d	LRNISPPQRCPSLRQAVQLTHQ	11.70

2° structure is showed according to the 3-dimensional model in Fig. 1. (+: positive charges) Charged arginine residues are marked with a grey background. Substitutions are highlighted in bold.

Flo is predicted to be composed of 3 α-helices. The Flo sub fragment p2 (Table 1) containing the second α-helix was shown to be the most active of three sub fragments which were made previously [14]. As p2 does not exert the full effect of Flo and the N-terminal fragment has a slight activity, it is likely that the helix 1, containing two positive charges, is necessary to complete full activity. To test this hypothesis, p4, which contains all of helix 2 plus the C-terminal portion of helix 1, was synthesised (Table 1, Fig. 1). As expected p4 was more active than p2 [14]. We could confirm these results (Fig. 2).
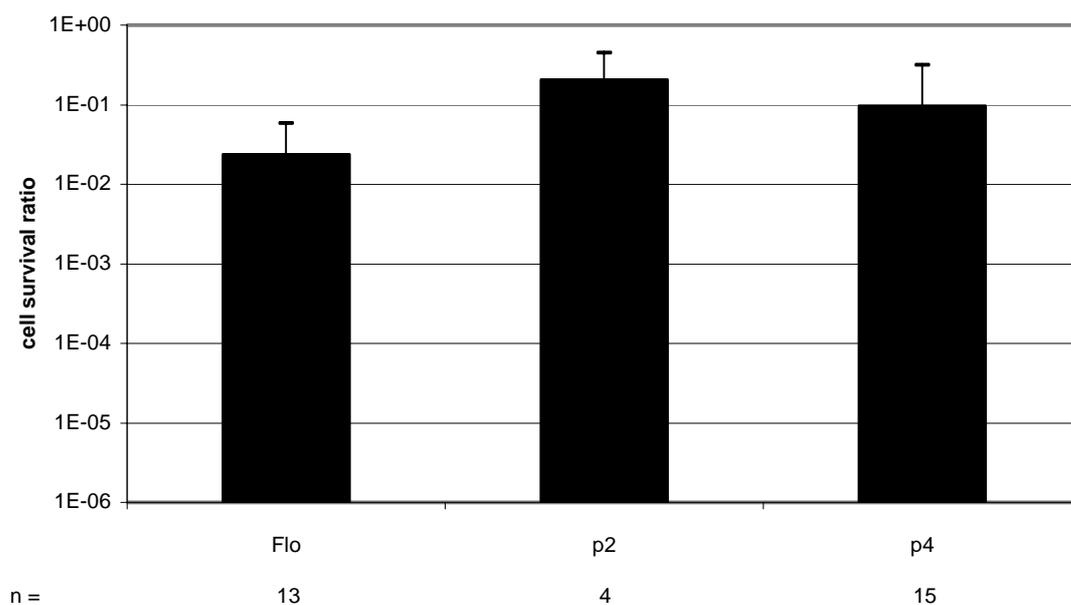
Fig. 2. Effect of Flo and sub fragments on *E. coli* survival after a 2h incubation time at 300μM peptide concentration. (Error bars: standard deviation.)
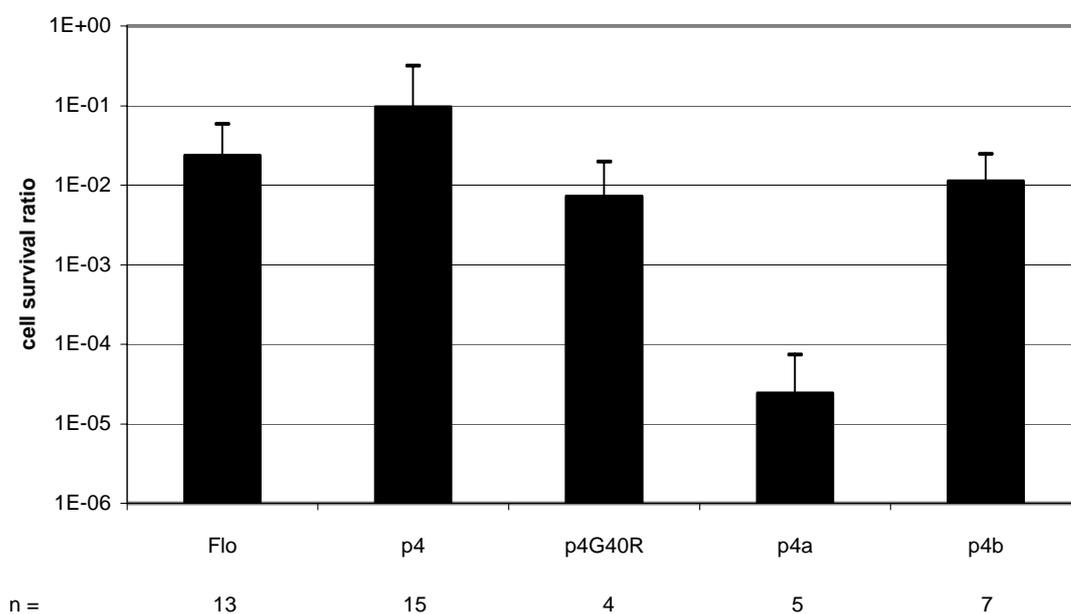

Fig. 3. Effect of p4 derived peptides on *E. coli* survival after a 2h incubation time at 300μM peptide concentration. (Error bars: standard deviation.)

Sequence analysis showed that the residues between helix 2 and helix 1 (which is present in p4 but not in p2) may be capable of forming a loop structure. In the literature it has been reported that loops may be important for activity of antibacterial peptides [18, 19, 20, 21, 22]. In order to test if the putative loop structure between helix 1 and 2 is important for the antibacterial activity of p4, we decided to synthesise fragment p4b (Table 1). This peptide contains helix 2 and the loop, but is missing all of helix 1. One of two positively charged arginines of helix 1 is left in the fragment. p4b does not lose activity compared to p4 and may even have an increased activity (Fig. 3).

Based on these observations it appeared that the putative loop is important part for the antibacterial activity of Flo. We therefore decided to examine its predicted 3-dimensional structure (Fig. 4). A special feature within the loop are the two proline residues, Pro21 and Pro22. Prolines give structural constraints to a polypeptide because of their particular cyclic side chain which is hydrophobic. This double-proline introduces a hydrophobic kink into the peptide which is exposed to the solvent according to the 3-dimensional structure prediction (Fig. 1). Again, according to this prediction, arginines Arg17 and Arg24 enclose the hydrophobic kink with their positively charged groups. In addition, this loop could potentially be stabilised by hydrophobic interactions and hydrogen bonds between the residues. This orientation could anchor Arg17 and Arg24 to the negatively charged bacterial membrane and exert a force that favours the interaction of the hydrophobic prolines with the hydrophobic chains of the membrane lipids or proteins (Fig. 4). This would be in agreement with the carpet model [6], where interactions of hydrophobic and positively charged patches on antibacterial peptides interact with the respective parts of the membrane compounds (e.g. phospholipid headgroups) to destabilise the membrane and induce a leakage of cytoplasm.

If the our model is correct we should be able to eliminate the observed antibacterial activity by disrupting the loop structure. To do so, we designed two peptides derived from p4b. In one we replaced the hydrophobic and rigid proline residues by hydrophilic and flexible asparagine residues of approximately the same size: p4bPP21NN (Table 1). In the second we removed the previously added positively charged arginine-anchor and replaced it by an uncharged glutamine which retains a hydrophilic character and is of approximately the same size: p4bR17Q (Table 1). Initial results indicate that both of these peptides show reduced antibacterial activity compared to p4b (Fig. 5). This supports the hypothesis that the loop is an important part of the antibacterial activity of Flo. The prolines seem to be a necessary loop component, and may act either via their hydrophobicity or by their rigidity (or both). The arginine might be necessary to anchor the loop on both sides. Without this anchoring the whole loop may be too flexible to be able to displace membrane components.
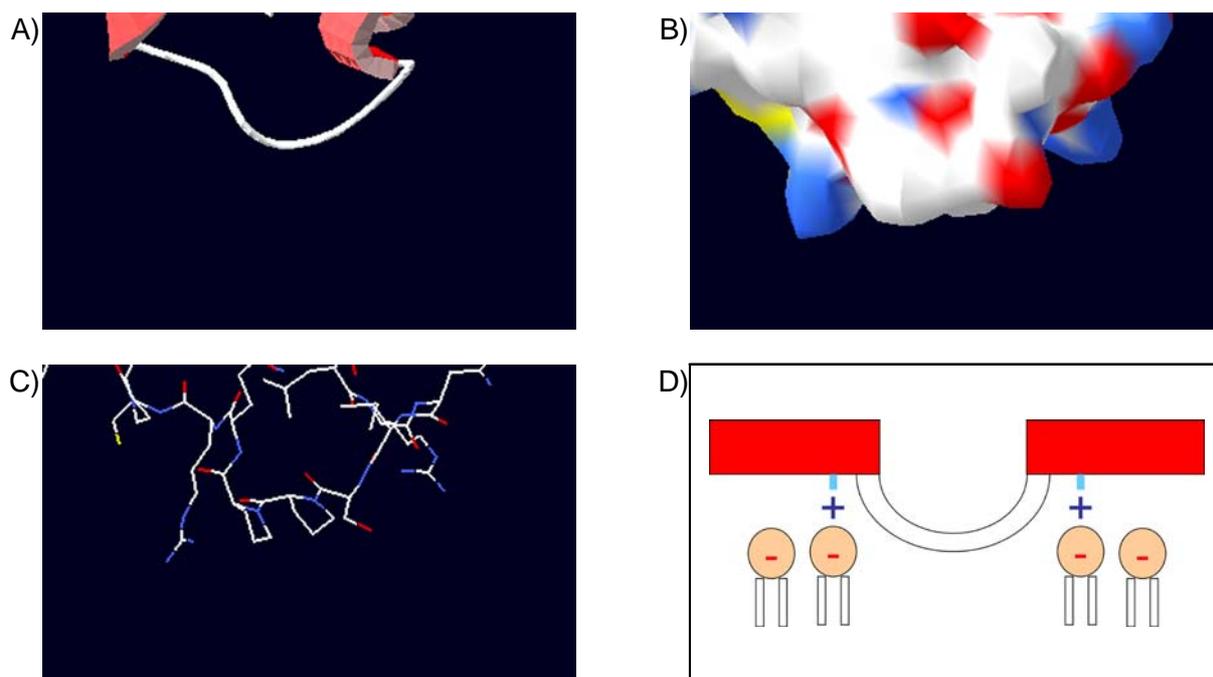
Fig. 4. 3-dimensional bioinformatical model of the loop region.

A) Backbone model (in red: α-helix ends)

B) Space fill model (in white: C-atoms, in blue: N-atoms, in red: O-atoms, in yellow: S-atoms)

C) Stick model (in white: C-atoms, in blue: N-atoms, in red: O-atoms, in yellow: S-atoms)

D) Model of hypothetical mechanism of action of the loop region. The charged arginines (blue +) on the adjascent α-helices (red bars) of the loop sticks to the negatively charged phospholipids (red -) of the bacterial membrane. Thereby, the rigid and hydrophobic kink formed by the two prolines in the loop (white arc) can be lead to interact with the hydrophobic interior of the membrane and therefore destabilise it.

Peptide p4 which is 31 amino acids long has a similar antibacterial activity to the full length Flo peptide which is 60 amino acids. The goal of finding short, active fragments in order to produce them at reduced cost is already being approached by the fragment p4. As other potentially commercial antibacterial peptides are much shorter than 30 residues the active portion of Flo needs to be further defined. This is why we eliminated some amino acids at the C-terminal end of p4. According to the 3-dimensional model, this region has no particular secondary structure. The resulting peptide is p4a (Table 1) which is not expected to lose activity by this reduction. Experimental results showed that p4a was highly active (Fig. 3). Compared to Flo it is significantly more active and represents on average a 1000 fold increase. This indicates that the C-terminal end of p4 may be inhibitory to the antibacterial activity. It is probably too short to cover an active portion of the peptide. We therefore think that unstructured parts destabilise the structured α-helices what could lower their activity.
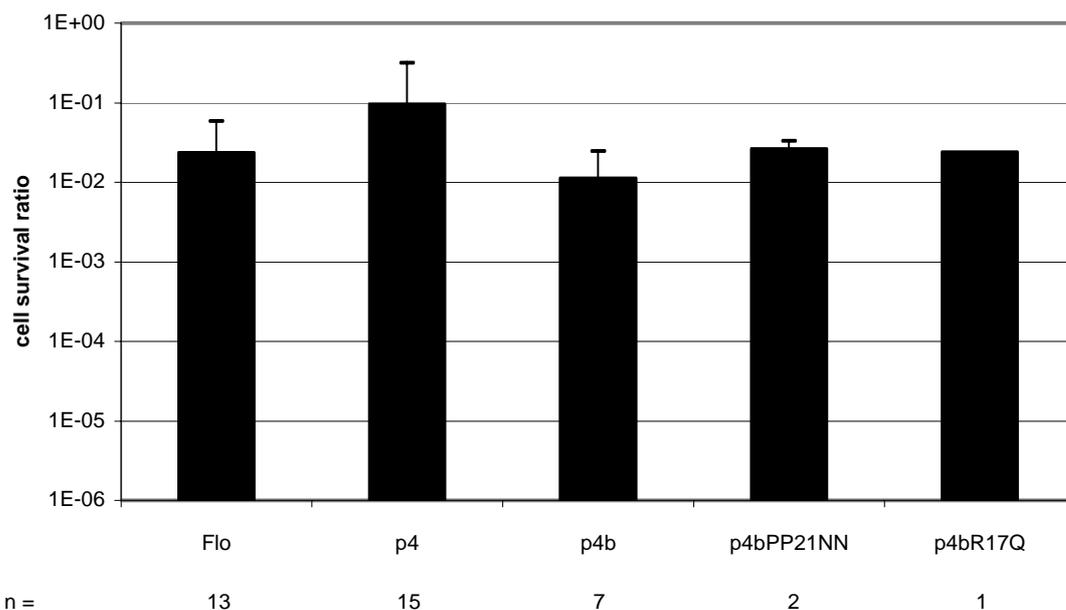
Fig. 5. Effect of peptides with altered loop region on *E. coli* survival after a 2h incubation time at 300μM peptide concentration. (Error bars: standard deviation.)
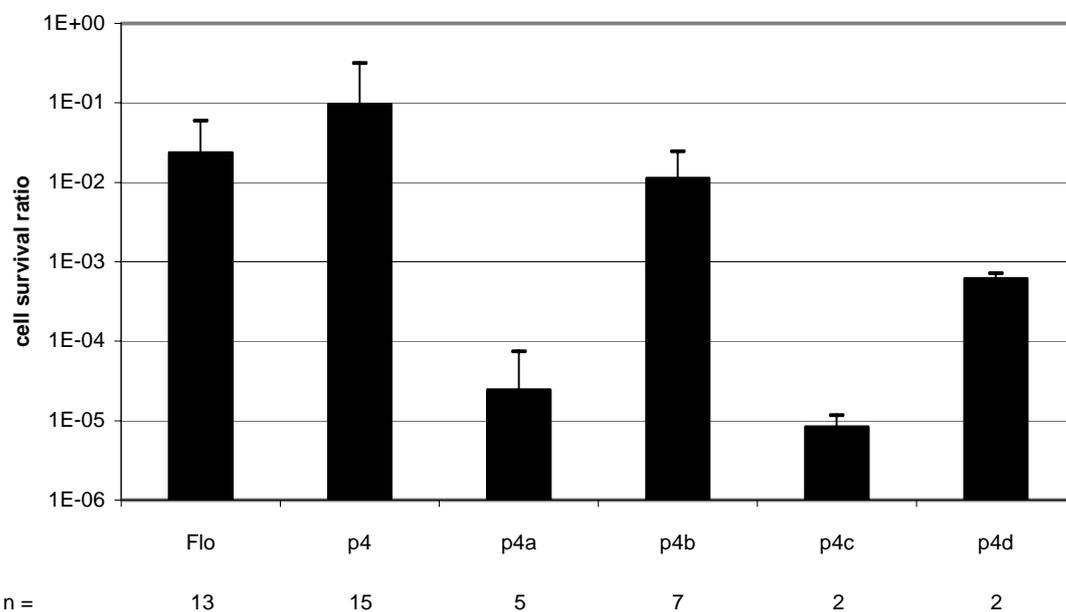

Fig. 6. Effect of shortest fragments of Flo on *E. coli* survival after a 2h incubation time at 300μM peptide concentration. (Error bars: standard deviation.)

As the C-terminal shortening of p4 had the effect of increasing its activity, we decided to apply the same approach to p4a and synthesise peptide p4c (Table 1). In this peptide we removed residues until close to the first positive charge (Arg29) in the core of helix 2. The remaining peptide has a total length of only 20 residues. It corresponds just to the loop, flanked by two short, doubly charged helices. According to our model of the loop and the hypothesis of required α-helix stability we expected p4c to be fully active. Results indicate that this peptide is as active or even more active than p4a (Fig. 6). This suggests that the inactive C-terminal part of p2 is relatively long. The full length of helix 2 is not needed for the full activity of p4a. This result supports our hypothesis that the loop is playing an important part in the antibacterial activity of the Flo-derived peptides. The deletion of C-terminal amino acids in p4 resulted in an increase of activity suggesting that this portion of the molecule may exert an inhibitory effect. We wished to determine if removing this region in the context of p4b would also act to increase this peptide's antibacterial activity. We therefore synthesised p4d (Table 1). This fragment corresponds to p4b with the putative inhibitory region removed. It also corresponds to p4a with helix 1 removed. If our hypothesis is correct, p4d should lose no activity compared to p4a and gain activity compared to p4b. Our results (Fig. 6) indicate that p4d does gain activity compared to p4b but that it has lower activity compared to p4a. This confirms that the C-terminal region is inhibitory in p4b. This also suggests that helix 1 was dispensable for antibacterial activity in the context of p4 but not in the context of p4a. Unstructured regions seem to reduce the activity of the antibacterial peptides. There even seems to be a minimal amount α-helical content needed. As structured α-helices confer stability to a peptide structure, stability is probably an important parameter of antibacterial effect.

The stability of the helices may be important. It was shown that the introduction of a charge in p2 (Gly40 replaced by Arg40) may stabilise the helix 2 and introduce more amphipathicity, thereby increases antibacterial activity [23]. We observed that the same substitution in p4 also enhances the antibacterial activity (see p4G40R, Table 1, Fig. 3). Here we provide another argument to underline the importance of the stability to antibacterial peptides.

It should be noted that the antibacterial test showed some problems of reproducibility. In addition some results obtained for the most recently produced peptides are preliminary as the number of repetitions carried out has not allowed us yet to generate statistically significant results (Supplemental data).

We used two different syntheses of the peptide p4 in our experiments. If the antibacterial activity of p4 is separated into two different data sets corresponding to these two syntheses a considerable difference is noted (Fig. 7). The most recently synthesised p4 sample is much more active than the previously synthesised sample. Analysis by HPLC and mass spectrometry of the two syntheses would lead us to conclude that the most recent synthesis more reliably reflects the true antibacterial properties of peptide p4. This would influence the interpretation of the results obtained from the other peptides. In the comparison of p4 with p4b which is depleted of the helix 1, the loop would considerably lose importance compared to the helix 1 (Fig. 5). As p4b has still more antibacterial activity compared to p2 (helix 2 only) the loop would still remain an active element of the activity. In the comparison of p4 with p4a which is missing the unstructured C-terminal end, the region could not be inhibitory to the activity (Fig. 5). Nevertheless, this region would be dispensable for the activity of p4. The peptide p4d has indicated that in the context of p4b this region is inhibitory. Therefore the inhibitory effect of the C-terminal region of p4 and p4b would depend on the context. This can be interpreted as a question of the stability of the peptide. In a peptide with enough α-helix content (as in p4) the unstructured end is dispensable whereas in a less α-helical peptide another unstructured region is inhibitory. As the peptide p4G40R (with more charges) shows similar variation as p4, it is cautious not to continue speculating about other interpretations.
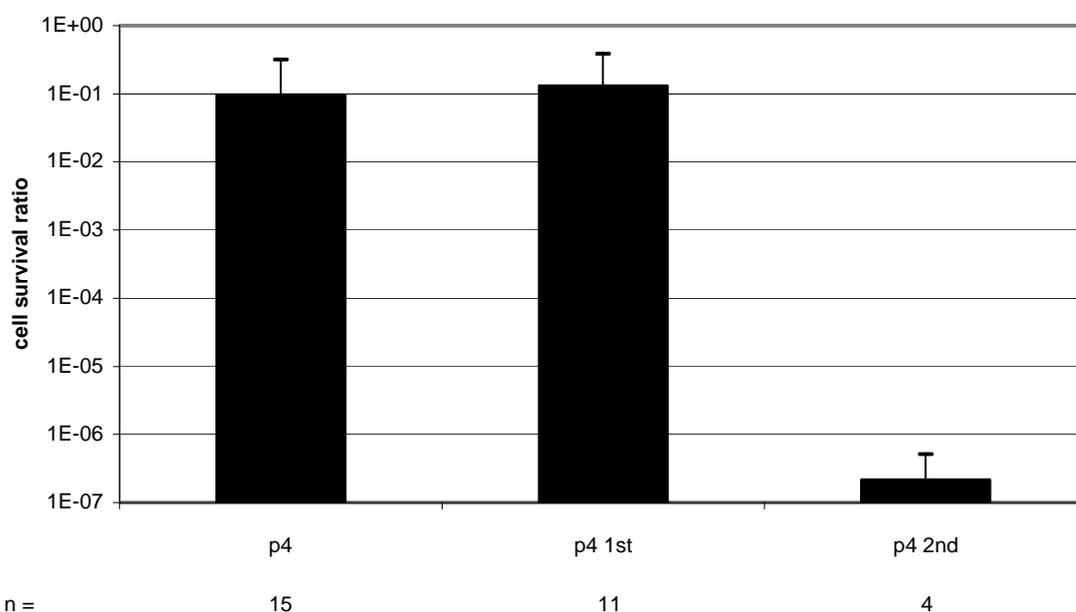

Fig. 7. Effect of two different syntheses of p4 on *E. coli* survival after a 2h incubation time at 300μM peptide concentration. (Error bars: standard deviation. Note the scale going downto 1E-07.)

All the results presented herein were obtained using peptide at a concentration of 300μM. the experiments were also performed at a concentration of 30μM. At the lower concentration, no clear cut antibacterial activity was observed (Supplemental data). Therefore the concentration threshold for the antibacterial activity might be located above 30μM.

In some experiments of p2 and p4bR17Q there was no dilution effect visible on the platings (Supplemental data). For p2 we know that it has coagulation effect at 100 fold lower concentrations which could stick together bacteria particles. This could result in an agglomeration of several cells to form 1 CFU which results in an underestimation of cells. With subsequent dilution series p2 could be washed away and particles can separate and form individual CFUs. It was shown [11] that there is not just a coagulation but also a real killing process going on in when bacteria are incubated with Flo.

Liposome disruption activity

In the literature it is thought that antibacterial peptides act to destabilise the bacterial membrane [1, 4, 5, 6, 24, 25, 26]. In the carpet model, which fits well with our proposed mode of action of Flo, a covering, unstructured interaction is predicted. This would lead to the formation of holes or rupture in membrane. As a consequence cytoplasmic contents would be released and membrane potential would be lost. To confirm the model, we need to prove the action of the peptides on the membrane. One way to do this is to test the leakage of vesicles. We produced unilamellar vesicles with phosphatidylcholine of about 60nm in diameter containing carboxyfluorescein (CF), a fluorescent dye that self-quenches at high concentrations. If the peptide induces membrane leakage, CF is released and an increase of fluorescence can be measured. We performed 1000 second time course experiments (where Triton X-100 was used to completely disrupt membranes attain maximum fluorescence at the end of the time course).

In a preliminary experiment we tested Flo, p4a (the most antibacterially active peptide) and p4G40R (representing an intermediate number of positive charges) (Table 1). Antibacterial activity is a rapid mechanism. We expect therefore the fluorescence to increase rapidly and to obtain the highest level using p4a. We observed a clear, prolonged and constant increase of fluorescence induced by Flo (Fig. 8). Fluorescence increase merely observed for Flo, but p4 and p4G40R remain largely inactive. This indicates that Flo is not completely disrupting membranes but inducing a high leakage of vesicles. The results show that CF release of phosphatidylcholine vesicles by the Flo derived peptides is not correlated to their antibacterial activity. Therefore leakage of cytoplasmic contents with a size of CF (~500Da) or bigger may not be the lethal event for bacteria. We can speculate that smaller particles as ions would be released by peptides like p4a what induces a lethal event. It could be lethal for bacteria to lose membrane potential which important membrane processes depend on.

In an other preliminary experiment we tested Flo at different concentrations, which we thought to be correlated to the activity. The results indicate the highest activity at $600\mu M$. Starting from $300\mu M$ on we observed an effect, which increases enormously (about 16 fold), when peptide concentration is doubled. As the end level of fluorescence is lower than the initial level, we cannot see whether at $900\mu M$ the effect is higher than for $600\mu M$. These results suggest, that Flo acts highly cooperatively on membranes. We expect this effect to be even stronger at $900\mu M$. The absence of an effect below $300\mu M$ confirms the observation that at $30\mu M$ the antibacterial test did not show a significant effect. We therefore suspect a threshold concentration for our antibacterial peptides between.

As the end level of fluorescence of the respective peptides are different, there must be an interaction between the CF and the peptide. The fluorescent activity of CF is highly sensitive to the pH, we therefore suspect the effect of direct interaction to be due to an acidification of the buffer. Even though the peptide is strongly basic, the powder dissolved in water is markedly acid. As the peptide is purified in acid solutions, the arginines are all protonated. These protons are probably responsible for the acidification of the buffer. As in Flo there are seven arginines they reach to the same range of number of protons as there are buffer molecules, and the latter will be dominated. This explains the differences of end fluorescence of the Flo titration and as well those of the respective peptides (Flo, p4a, p4G40R) in agreement with their different content in arginines.
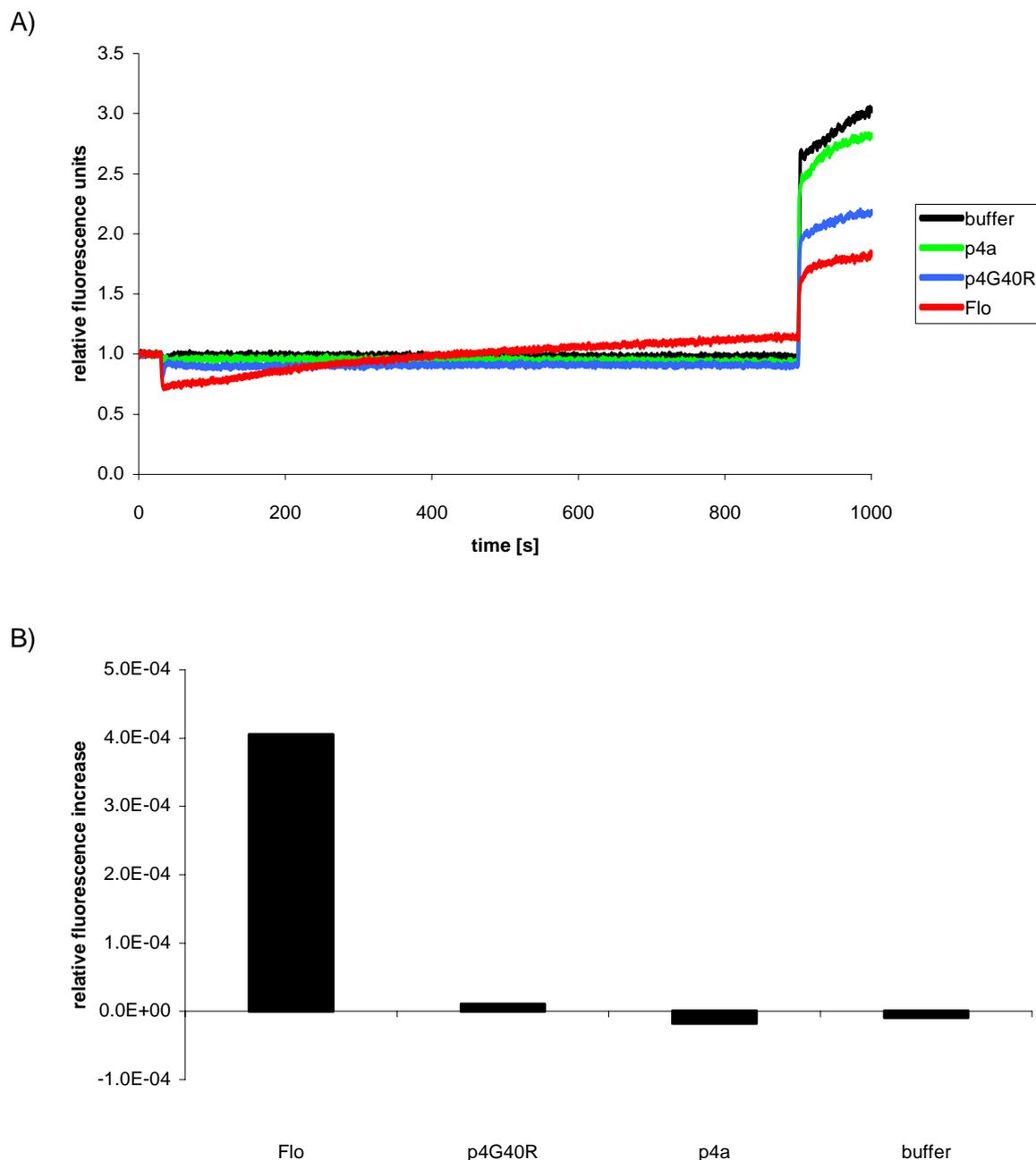
A)

B)

Fig. 8. Liposome disrupting activities of different peptides.

Preliminar results of 1 experiment. Carboxyfluorescein inside unilamellar phosphatidylcholine liposomes is quenched and when vesicles are leaking or destroyed, fluorescence in the solution increases. Fluorescence was measured during 1000s. After 30s the peptide solutions were added to the final concentration of $600\mu M$. After 900s a detergent is added to completely destroy all vesicles and reach maximal fluorescence.

A) Evolution of the fluorescence signal relative to the initial fluorescence values.

B) Slope of linear regression of curve between time point 30s and 900s normalised by the difference of maximal signal (at 1000s) and the minimal signal (after 30s). Negative values result from bleaching of carboxyfluorescein after multiple measurements.

A)

B)
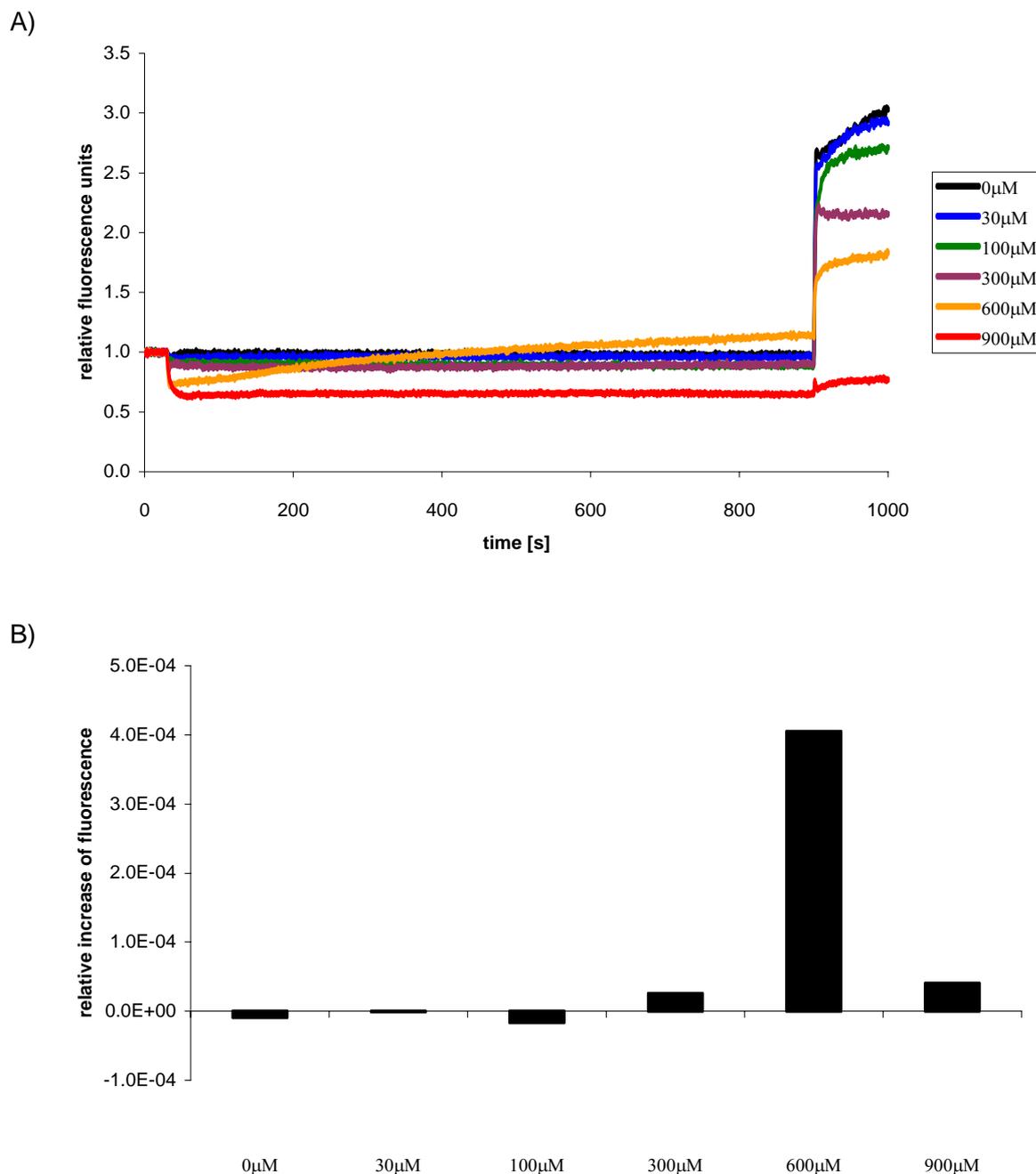
Fig. 9. Liposome disrupting activities of Flo at increasing concentrations.

Preliminar results of 1 experiment. Carboxyfluorescein inside unilamellar phosphatidylcholine liposomes is quenched and when vesicles are leaking or destroyed, fluorescence in the solution increases. Fluorescence was measured during 1000s. After 30s the peptide solution is added to indicated final concentration. After 900s a detergent is added to completely destroy all vesicles and reach maximal fluorescence.

A) Evolution of the fluorescence signal relative to the initial fluorescence values.

B) Slope of linear regression of curve between time point 30s and 900s normalised by the difference of maximal signal (at 1000s) and the minimal signal (after 30s). Negative values result from bleaching of carboxyfluorescein after multiple measurements.

We also produced 10% phosphatidylglycerol containing vesicles. These lipids contain a negatively charged phosphate group. According to the model, these vesicles should be more sensitive to disruption by our peptides. Preliminary results indicate that this is not the case. This suggests that the presence of phosphatidylglycerol is not critical for the membrane disrupting activity of Flo derived peptides. However natural bacterial membranes that contain negative charges in lipopolysaccharides (Gram negatives) or teichoic acids (Gram positives) may be necessary.

CONCLUSION

With the present study we confirmed previous results on the antibacterial activity of Flo peptide sub fragments. We showed an increase of activity when adding helix 1 to p2 resulting in p4. Thereby it was possible to regain nearly full activity of Flo with half the peptide length.

The generally accepted assumption that membranes are the target of antimicrobial peptides could be confirmed by the liposome disruption assay. We showed that Flo has an effect of releasing the content of phosphatidylcholine vesicles which mimic the bacterial membrane. This excludes the "aggregate channel" model as explanation of antibacterial mechanism of Flo. We showed that the threshold of this releasing activity is at the same peptide concentration as our antibacterial assay and that Flo acts in a highly cooperative manner. However, the bacteria killing mechanism must be different, because a the highly bactericidal p4a, did not show a vesicle leakage inducing activity. Electronmicrographs could be used to observe the effect of the peptides on natural membranes.

We found that the loop between helix 1 and helix 2 is an important element of the antibacterial activity of Flo. We showed that the hydrophobic proline-kink within the loop and that the charges on each side of the loop may be important for the activity. We propose that the interaction of positive charges on the peptide with the negatively charged bacterial membrane may occur in a stabilised context (provided by the α-helices) thereby leading to the interaction of a hydrophobic kink with the hydrophobic interior of the membrane. This would not be in agreement with a model of highly organised pores (as in "barrel-stave" model) but more with generally destabilising peptide-phospholipid interaction as proposed in the "carpet" model. Our "loop" model can be tested with artificially produced helix-loop-helix peptides. The peptide p4c may serve as a template.

In view of a biotechnological application, the discovery of short and active peptides is crucial to the development of antibiotic drugs or water disinfectants. In p4a we developed a peptide that was more than 1000 fold more active than Flo and that kills more than 99.99% of *E. coli* when used at a concentration of $300\mu M$. In addition we synthesised the shorter peptide p4c, composed of only 20 amino acids which may be even more active. As the length of the α-helices must be maintained for stability, one possible future direction would be to continue to shorten peptide p4c by reducing the length of the inter helical loop to a minimum

In summary we report the synthesis of a novel highly active antibacterial peptide, derived from a naturally occurring seed protein, and we present a simple model of the minimal structural requirements necessary for a peptide to have an antibacterial activity. This may allow for the development of strategies aimed at the improving the activity of existing antibacterial peptides, or designing *de novo* highly active antimicrobial compounds.

METHODS

Peptide synthesis and purification

The amino acid sequence of Flo and its subfragments were based on *Moringa oleifera* Lam. seed component M.O. 2.1 [9] (Swiss-Prot primary accession number: P24303). The peptides were synthesised using Fmoc-chemistry solid phase peptide synthesis (Novabiochem, Germany), on an Abimed (Germany) AMS 422 multiple peptide synthesiser, generously provided by the Laboratoire de Biotechnologie Chimique (EPFL, Switzerland). Cleavage of the resin and deprotection of the side chains were performed during 2h employing 94% TFA, 2.5% EDT, 1% TES (protocol for cysteine residues, Novabiochem, Germany).

Individual synthesis products were checked by mass spectrometry analysis (ESI-TOF) on a LCT instrument (Micromass), generously provided by the Laboratoire de Biotechnologie Chimique (EPFL, Switzerland). The lyophilised peptides were diluted in a mixture of water/acetonitrile 1:1 (v/v) and sprayed at a flow rate of 10μL/min. The mass spectrometer was tuned according to the size of the peptide of interest but with a broad range in order to allow the identification of contaminants.

Individual synthesis products of the same peptide species were pooled, further analysed and purified by HPLC employing a monolithic Chromolite™ Performance RP-18e column 4.6x100mm (Merk, Germany). An isocratic interval of 100% solution A (0.1% TFA in water) was followed by a gradient from 100% solution A to 100% solution B (0.085% TFA in Chromasolv® acetonitrile (Riedel-deHaen, Seelze, Germany).

Eluted fractions were lyophilised. Peptides were dissolved in water to create 10mM stock solutions and purity was estimated by mass spectrometry. A minimum purity of 70% was required. The exact concentration was checked by analytical HPLC at a wavelength of 215nm corresponding to the maximum absorbance of the peptide bond. As there were no aromatic residues in our peptides, the area under the elution peak was used to estimate the quantity of the peptide and correction factors were calculated for each individual stock solution used for the following experiments.

Antibacterial test

Escherichia coli (ER2566 strain) were grown in LB at 37°C. At exponential phase (OD: $0.5<A_{600}<0.6$) they were centrifuged and resuspended in the same volume of a 10mM phosphate buffer, pH 7.0. Peptides were added to the cell cultures in order to reach appropriate concentrations, and incubated for 2h at 37°C without agitation. Cell survival was assessed by making 10 fold dilution series of bacterial suspensions, plating on non-selective LB medium dishes and incubating over-night at 37°C. Duplicates were made of every individual assay. Colonies were counted on dishes representing 20-200 of them. Cell survival ratio was estimated by comparison to a control experiment.

Values resulting from series that didn't represent a visible dilution were not taken into account (Supplemental data). On the other hand, values of series without colonies were taken into account (Supplemental data). Statistical analysis was made by the Mann-Whitney U-test (Supplemental data). P-values < 0.05 were considered significantly. Border line P-values < 0.1 were considered to show a tendency.

Liposome disruption assay

Adapted from [27]. Lipid films composed of 5mg di-oleoyl-phosphatitiylcholine or alternatively 5mg di-oleoyl-phosphatitiylcholine / palmitoyl-oleoyl-phsphatiylglycerol (9:1) were resuspended in a carboxyfluorescein buffer at a self-quenching concentration (50mM). Unilamellar vesicles of 60nm in diameter were obtained by sonication and purified by gel filtration chromatography on Sephadex G-75 in 10mM Hepes buffer at pH 7.4 with 100mM sucrose, 10mM NaCl. Fluorescence (excitation: 450nm, emission: 520nm) of a mix of 2μL of vesicle solution and 200μL of buffer was measured every 1.5s during 1000s. After 30s, 50μL of peptide solution (water/buffer 2:3) was added in order to attain the corresponding final concentration, and at 900s 30μL of Triton X-100 1% was added to measure the maximum fluorescence. Material was generously provided by the Laboratoire de Chimie Physique des Polymères et Membranes (EPFL Switzerland).

Homology modelling

Generously established by the Swiss Institute of Bioinformatics (Switzerland). 3-dimensinal structure of Flo was based on NMR structure of the homologous Napin (PDB entry: 1PNB). Sequence alignment was made, based on this homology. The structure was generated with the programme MODELLER and energy minimisation was made by de programme CHARMM. The model is evaluated by hierarchical clustering of the rmsds values of the α-carbons by MMTSB scripts and calculating of an ANOLEA profile. The structure belonging to the largest cluster and representing the lowest energy in ANOLEA was selected the best candidate. Highly positive energy in the ANOLEA profile were corrected by a making a new alignment.

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SUPPLEMENTAL DATA

Table 1. Individual data points of the effect of all tested peptides on *E. coli* survival after 2h incubation time at 30μM and 300μM peptide concentration.

A) Peptides at 30μM concentration.

Date											
30.09.03	1.5E-03		1.2E+00	1.3E+00							
02.10.03	1.6E-01	2.2E+00 [a]	2.0E+00 [a]	4.9E-01							
09.10.03	8.9E-02	1.8E+00	1.2E+00 1.4E-01	7.8E+00							
21.10.03	3.8E+00 2.7E+00 [a]	1.5E+02	2.2E+00 2.5E+00								
06.11.03	4.7E+00 7.2E-01	9.2E-01	8.7E-01 2.7E-01								
11.11.03	6.0E+01	3.5E+01	1.5E+00		2.2E+00	2.5E+01					
13.11.03	9.9E-01	6.6E+00	7.3E-01 1.4E+00		1.8E+00	3.6E-01					
07.01.04	1.6E-02	2.2E-01	8.7E-03			7.7E-02	1.8E-02	5.8E-06			
08.01.04	5.0E-01 [a]		5.3E-01 [a]		3.8E-01				2.8E-01	5.1E-01	
20.01.04 [c]	1.1E+00	3.0E-01	7.9E-01	7.1E-01		2.1E-01 [a]	8.4E-01	2.6E-01 [a]			
21.01.04	1.5E+00		1.1E+02		9.9E-01	2.5E+00			8.8E-01	3.7E-02	2.7E+00
22.01.04											
average	5.8E+00	2.5E+01	8.7E+00	2.6E+00	1.3E+00	5.6E+00	4.3E-01	1.3E-01	5.8E-01	2.7E-01	2.7E+00
median	9.9E-01	2.0E+00	1.2E+00	1.0E+00	1.4E+00	3.6E-01	4.3E-01	1.3E-01	5.8E-01	2.7E-01	2.7E+00
n	13	8	15	4	4	5	2	2	2	2	1
standard dev.	4.5E+00	1.9E+01	7.6E+00	1.7E+00	4.0E-01	4.8E+00	4.1E-01	1.3E-01	3.0E-01	2.3E-01	

B) Peptides at 300μM concentration.

Date											
30.09.03	1.4E-04		1.5E-03	2.4E-03							
02.10.03	2.7E-11 [b]	7.4E-02	2.7E-09 [b]	2.7E-09 [b]							
09.10.03	5.1E-03	5.6E-01	2.1E-01 1.6E-01	2.6E-02							
21.10.03	9.2E-02 1.1E-01	4.3E+00 [a]	8.7E-01 1.0E-01								
06.11.03	7.3E-03 2.6E-05	1.7E-01	2.8E-09 [b] 7.2E-04								
11.11.03	1.9E-05	8.9E-03 [a]	4.8E-09 [b]		5.0E-06	9.8E-03					
13.11.03	3.4E-02	3.9E-02 [a]	4.3E-09 [b] 8.2E-02		1.2E-04	8.5E-03					
07.01.04	6.2E-04	9.1E-04 [a]	1.8E-08			1.3E-06	2.1E-02	2.4E-02			
08.01.04	3.1E-02		1.8E-07		1.5E-07	3.5E-02 2.5E-02			5.8E-06	5.3E-04	
20.01.04 [c]	6.0E-02	9.4E-02	4.0E-03	5.6E+00		3.5E-02	3.7E+00	6.3E-04 [a]	5.3E-06	7.9E-06	3.7E-06 [b]
21.01.04	2.0E-02		6.5E-07		1.0E-06	4.3E-05					
22.01.04	5.9E-03	5.3E-03	5.0E-10 [b]	5.0E-07 [b]	2.2E-07	3.0E-07 [b]	3.1E-02	3.3E-02 [a]	1.1E-05	6.8E-04	1.0E-06 [b]
average	2.6E-02	5.9E-01	8.9E-02	1.1E+00	2.4E-05	1.4E-02	1.3E+00	1.9E-02	7.3E-06	4.1E-04	2.4E-06
median	6.6E-03	7.4E-02	3.6E-04	2.4E-03	1.0E-06	9.1E-03	3.1E-02	2.4E-02	5.8E-06	5.3E-04	2.4E-06
n	14	9	16	5	5	8	3	3	3	3	2
standard dev.	9.6E-03	4.7E-01	5.4E-02	1.1E+00	2.3E-05	5.4E-03	1.2E+00	9.6E-03	1.7E-06	2.0E-04	1.4E-06

[a] There was no visible dilution effect of bacteria culture on the corresponding Petri-dishes.

[b] All Petri-dishes of the corresponding dilutions were empty.

[c] Phosphate buffer at 50mM was used instead of 10mM.

Table 2. P-values of two by two comparisons with all experiments using the Mann-Whithney U-test.

	Flo	p2	p4	p4G4OR	p4a	p4b	p4bPP21NN	p4c
p4d	0.497	0.064	0.882	1.000	0.053	0.770	0.121	0.121
p4c	0.062	0.064	0.882	1.000	0.245	0.380	0.121	
p4bPP21NN	0.308	0.355	0.456	0.165	0.053	0.242		
p4b	0.721	0.059	0.459	0.450	0.062			
p4a	0.012	0.014	0.965	0.624				
p4G4OR	0.258	0.043	0.920					
p4	0.407	0.089						
p2	0.054							