Amphibian antimicrobial peptide fallaxin analogue FL9 affects virulence gene expression and DNA replication in Staphylococcus aureus

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The Amphibian Antimicrobial Peptide Fallaxin Analogue, FL9, Has Intracellular Targets and Affect *Staphylococcus aureus* Virulence Gene Expression

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Abstract

Several antimicrobial peptides (AMPs) have been isolated from a wide range of organisms and these have shown potential as antimicrobials. However, to utilize AMPs as antimicrobial compounds, it is important to decipher their mode of action (MOA), the bacterial response mechanisms and the activity of the AMP when exposed to different environments. In the present study, the analogue FL9 based on the amphibian AMP Fallaxin, isolated from the frog *Leptodactylus fallax*, was studied to elucidate its MOA and antibacterial activity against the human pathogen, *S. aureus*. The results show that FL9 has a dual mode of action. Around the minimum inhibitory concentration (MIC) FL9 binds chromosomal DNA resulting in inhibition of DNA synthesis and induction of the SOS DNA damage response, whereas at concentrations above MIC, the interaction between *S. aureus* and FL9 leads to poration through membrane disruption. Antibacterial activity of the peptide was maintained at a wide range of NaCl, and $MgCl_2$ concentrations and at alkaline pH, while it was hampered by acidic pH and exposure to serum. We show how natural tolerance mechanisms, including peptide cleavage and addition of positive charge to the cell surface, are important for the bacterial ability to minimize the antimicrobial activity of FL9. In addition, we found that subinhibitory concentrations of FL9 increase the expression of two major virulence factors namely the regulatory *rnaIII* and *hla* encoding α-hemolysin. Collectively, the data show that FL9 affects DNA replication and virulence gene expression and is able to maintain its activity under various environmental conditions.

Introduction

Bacterial infections are re-emerging as a major problem due to development of resistance toward conventional antibiotics and the decline in development of new antimicrobials (Boucher *et al*., 2009). A possible solution to overcome multi-drug resistant bacteria is to use alternative classes of

antimicrobials to treat infectious diseases. One such class is antimicrobial peptides (AMPs), also known as host defense peptides which form an important part of the innate immune system in multicellular organisms (Zasloff, 2002; Pasupuleti *et al*., 2012). AMPs are usually small peptides comprised of less than 40 amino acids divided into four structural classes: α-helical, β-sheet stabilised by two or more disulphide bridges, extended helices and loop structures (Jenssen *et al*., 2006).

It is believed that most AMPs, due to their cationic and amphiphatic nature, selectively kill bacteria by penetrating the anionic cell membrane by membrane disintegration or pore formation (Zasloff, 2002). In addition to membrane disruption, several studies have shown that some AMPs also have the ability to traverse the cytoplasmic membrane and target intracellular molecules such as DNA or RNA (Jenssen *et al*., 2006; Brogden, 2005; Makobongo *et al*., 2012; Peschel & Sahl, 2006). For example, interaction of AMPs with DNA may damage DNA and induce the SOS response (Gunderson & Segall, 2006; Rotem *et al*, 2008; Sarig *et al*., 2011). The SOS regulon comprises genes essential for DNA repair and restart of stalled or collapsed replication forks, and is regulated by the repressor LexA that in response to DNA damage sensed by RecA undergoes autocleavage (Courcelle & Hanawalt, 2003; Cohn *et al*., 2011). In consequence, expression of LexA controlled genes like *recA* may be monitored as reporters of bacterial DNA damage.

However, although AMPs have a highly attractive potential as therapeutics, their use as antimicrobial compounds is associated with several obstacles. One drawback is their sensitivity to various conditions in the human host, e.g. the presence of salt, divalent cations, plasma components, proteases and low or high pH, which can affect their activity (Goldman *et al*., 1997; Lee *et al*., 1997; Minahk & Morero, 2003; Radzishevsky *et al*., 2005; Rozek *et al*., 2003). Furthermore, pathogens have evolved several mechanisms to evade the effects of AMPs. The tolerance

mechanisms include proteases, that degrade the AMPs (Sieprawska-Lupa *et al*., 2004), secretion of proteins that captures AMPs (Jin *et al*., 2004), and reduction of the net anionic charge of the bacterial cell envelope, thus, increasing the electrostatic repulsion of AMPs (Peschel *et al*., 1999; Peschel *et al*., 2001). In addition, during antimicrobial therapy, bacteria may be exposed to sub-MICs of AMPs, which can have unwanted effects such as changes in virulence gene expression (Davies *et al*., 2006). For instance, subinhibitory concentrations of clindamycin and linezolid have the ability to down regulate the expression of *Staphylococcus aureus* (*S. aureus*) exotoxins, including α-hemolysin (*hla*) (Bernardo *et al*., 2004; Herbert *et al*., 2001). In contrast, subinhibitory concentrations of the cell wall acting β-lactam antibiotics have been shown to induce the expression of *hla* (Ohlsen *et al*., 1998).

AMPs constitute the first line of defense against invading pathogens in a wide range of organisms including amphibians and therefore these sources may be used as leads for the development of AMPs as therapeutic agents (Fernandez *et al*., 2009). One such AMP is fallaxin which is a 25-mer AMP amide isolated from the West Indian mountain chicken frog *Leptodactylus fallax*. Fallaxin belongs to the α-helical class of AMPs (Rollins-Smith *et al*., 2005). From a recent structure-activity study of fallaxin, the analog FL9, in which an alanine residue was replaced by a more hydrophobic leucine residue, was identified as having an improved activity toward a range of Gram-positive bacteria including *S. aureus*, compared to the natural AMP (Nielsen *et al*., 2007). Presently we have characterized the activity of FL9 and find that at and above the MIC, the peptide has a dual mode of action, affecting both intracellular targets and the membrane of *S. aureus* whereas at subinhibitory concentrations, it increases expression of the virulence factors *rnaIII* and *hla*. Furthermore, we show that FL9 is active at physiological relevant conditions and several lines of defense exist in *S. aureus* to limit the bactericidal effect of the peptide.

Methods

Peptide synthesis

The FL9 peptide (GVVDILKGLAKDIAGHLASKVMNKL-NH_2) was purchased from GL Biochem (Shanghai) Ltd.

Strains and culture conditions

Strains used in this study are listed in Table 1.The *S. aureus* strains were grown in Tryptone Soy Broth (TSB), at 37°C with shaking unless otherwise stated. When appropriate, antibiotics were added at the following concentrations: 5 μg tetracycline ml^{-1}, 5 μg erythromycin ml^{-1}, and 200 μg spectinomycin ml^{-1}.

Minimum Inhibitory Concentration determination

The minimum inhibitory concentration (MIC) of FL9 was determined using the modified microtiter broth dilution assay for cationic antimicrobial peptides from Hancock (http://cmdr.ubc.ca/bobh/methods/MODIFIEDMIC.html). Briefly, serial 2- fold dilution of FL9 (at 10 times the required test concentration) was made in 0.2% bovine serum albumin and 0.01% acetic acid in polypropylene tubes. Overnight cultures of *S. aureus* 8325-4 and FPR3757 USA300 were diluted 10.000-fold in Mueller Hinton broth to a final concentration of approximately 5×10^5 CFU ml^{-1} and 100 μl was added to each well of a 96-well polypropylene microtiter plate. 11 μl of the 2-fold serial diluted FL9 was added to each well. The plate was incubated overnight and the MIC was read as the lowest concentration of peptide that inhibited visible growth of *S. aureus*. The reported results are from three independent experiments.

Determination of the effect of FL9 on the bacterial membrane - ATP measurements.

Pore formation as caused by peptide addition was determined by measuring ATP leakage from the bacterial cell using a bioluminescence assay as previously described (Thomsen *et al*., 2010). *S. aureus* 8325-4 was grown in TSB at 37°C overnight and then re-inoculated in TSB at 37°C. *S. aureus* was harvested (550 g, 10 min) at mid-exponential phase (OD_{546} of 2.5 ± 0.1), washed once in 50 mM potassium phosphate buffer pH 7.0 and once in 50 mM HEPES buffer pH 7.0. The pellet was resuspended in 50 mM HEPES pH 7.0 to a final OD_{546} of 10. Bacteria were stored on ice and used within 5 h. Bacteria were energized in 50 mM HEPES (pH 7.0) with 0.2% (w/v) glucose and treated with various concentrations of FL9 up to a concentration of 1000 µg/ml. ATP measurements were performed at time-point 0. ATP was determined using a bioluminescence kit (Sigma, FLAA-1KT) and a BioOrbit 1253 luminometer. Total ATP content was determined by rapidly permeabilizing 20 µl cell suspension with 80 µl dimethyl sulfoxide. The cell suspension was diluted in 4.9 ml sterile water, and ATP content was determined in 100 µl of the preparation as described by the manufacturer. To determine the extracellular ATP concentration, the 20 µl cell suspension was mixed with 80 µl sterile water and analyzed as described above. Intracellular ATP concentrations were calculated by using the intracellular volumes of 0.85 μm^3 for *S. aureus*. The number of cells in suspension was determined by plate spreading. The reported results are from two independent experiments.

In vitro* killing kinetics of *S. aureus

S. aureus 8325-4 was grown overnight in TSB medium and diluted 1:50 in TSB medium and allowed to grow for 1 h to an optical density at 600 nm of 0.2. FL9 was added to final concentrations equally to one and five times the MIC value, followed by incubation at 37°C while shaking. A control without FL9 was included. At the specified time points aliquots were diluted

(serial 10-fold dilutions in saline) and plated on TSB agar. CFU were counted after an overnight incubation at 37°C. The reported results are from four independent experiments.

Macromolecular synthesis

Overnight cultures *S. aureus* 8325-4 were diluted 1:50 in TSB and allowed to grow for 1 h (to an optical density at 600 nm of 0.2). 1µCi ml^{-1} (37MBq) of [methyl-^{3}H] thymidine or [5,6-^{3}H]uridine was added to the culture. After 10 min of incubation at 37°C, FL9 was added at 1×MIC and 5×MIC. Samples of 500 µl were removed immediately before addition of FL9 (0 min) and at 5, 10, 20 and 30 min after addition of LP5 and added to 2 volume of 99.9% ice cold EtOH and 0.1 volume of 3 M NaAc, pH 5.5, in order to precipitate macromolecules. After precipitation overnight at -20°C samples are collected by centrifugation (8500g, 10 min) and washed twice in 1 ml of ice cold 70% EtOH. Samples were resuspended in 100 µl of milliQ water and added to 4 ml scintillation vials with EcoscintA liquid scintillation cocktail, and counts were obtained in a Beckman scintillation counter for 5 min for each sample using the tritium program. The reported data are from three independent experiments, showing similar results.

DNA-binding analysis

Gel retardation analysis were performed as previously described (Park *et al*., 1998) by mixing 100 ng of plasmid DNA (pRMC2) (Corrigan & Foster, 2009) isolated from *S. aureus* 8325-4 with increasing concentrations of FL9 in 20 µl binding buffer (5% glycerol, 10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCL and 50 µg bovine serum albumin ml^{-1}). Reaction mixtures were incubated 1 h at room temperature and subjected to 1% agarose gel electrophoresis and visualised using ethidium bromide. The reported data are from three independent experiments, showing similar results.

Effect of FL9 on *S. aureus recA* expression

30 µl of 20 mg ml^{-1} FL9, 0.05 mg ciprofloxacin or H_2O ml^{-1} was tested in an agar diffusion assay as previously described (Nielsen *et al*., 2010). The strain HI2682 (Gottschalk *et al*., 2013) was used to monitor the expression from the *recA* promoter. The reported results are representative of three independent experiments.

MIC determination under environmental conditions

S. aureus 8325-4 was grown in TSB media (85 mM NaCl , pH 7) and diluted 10.000 fold as described above (MIC determinations) in the following media: TSB + NaCl concentrations of 85, 170, 260, 515 and 1030 mM, pH at 3.5, 5.5, 7 and 8.5, or $MgCl_2$ concentrations of 1, 3 and 5 mM. The reported results are from two independent experiments.

Effect of serum on antimicrobial activity

Blood was drawn from healthy chickens into glass tubes without additives and left to coagulate. After coagulation, serum was collected. FL9 was diluted in 0.01% acetic acid + 0.2 % bovine serum albumin and serum was added to achieve a FL9 concentration of 1000 µg ml^{-1} in 0, 10, or 90% serum. The FL9 and serum mixtures were incubated at 37°C and samples were withdrawn at indicated time points. Serum activity of the peptide-serum mixtures were measured by Radial Diffusion Assay (Lehrer *et al*., 1991). *S. aureus* 8325-4 was grown overnight at 37°C in 5 ml of 3% TSB. To obtain cells in the exponential phase, the overnight culture was sub-cultured by 1:50 dilution in fresh TSB and grown for an additional 2 h at 37°C to an optical density at 620 nm of 0.4. The bacteria were centrifuged at 900 g for 10 min at 4°C and washed once in cold 10 ml Tris buffer (10 mM, pH 7.4), and resuspended in 10 ml 10 mM Tris buffer (pH 7.4). The bacterial suspension

was added to warm (40°C) 10 mM Tris buffer (pH 7.4) containing 2% low-electroendomiosis type agarose (Seakem® LE Agarose (Lonza)) to a final concentration of 2×10^5 CFU/ml. After a rapid dispersion of the bacteria, the agar was poured into 20 cm Petri dish to form a uniform layer and was punched with a 4-mm-diameter gel punch to make evenly spaced wells after the agarose had solidified. Following the addition of 10 µl serum samples or FL9 in 0.01% acetic acid + 0.2 % bovine serum albumin at concentrations of 31.25, 62.5, 125, 250, 500, or 1000 µg ml-to each well, the plates were incubated for 3 h at room temperature. An overlay agar composed of double-strength (6%) TSB agar and 10 mM Tris buffer (pH 7.4) was then poured over. After incubation for 18 to 24 h at 37°C, the size of the clear zone surrounding each well was measured. The diameter of the clearing was expressed in units (0.1 mm = 1 U) and was calculated after subtracting the diameter of the central well (4 mm = 40 U). The reported results are from two independent experiments

Impact of the *S. aureus dltA*, *mprF* and *vraF* genes on FL9.

The impact of mutations in the *dltA*, *mprF* and *vraF* genes (Peschel *et al*., 1999; Peschel *et al*., 2001) of SA113 on FL9 were investigated by MIC determinations as described above. The reported data are from four independent experiments. The SA113 Ω*vraF* strain was constructed by transduction using Φ11 phage propagated on NE645 and selected on erythromycin (NARSA). The integration of *bursa aurealis* Tn insertion in SA113 *vra*F was confirmed by PCR using primers ErmB-1 (5´-CGAGTGAAAAAGTACTCAACC-3´) and ErmB-2 (5´-CTTGCTCATAAGTAACGGTAC-3´).

Impact of aureolysin from *S. aureus* on FL9

S. aureus aureolysin was obtained from BioCol GmbH. FL9 (136 μM) was incubated at a peptide-to-enzyme ratio of 300:1 for 4 h at 37°C in Tris buffer. The samples were supplemented with NativePAGE™ Sample Buffer ×4, and proteolysis was heat terminated by 3 min of boiling. Gel electrophoresis was done using NativePAGE™ Novex® 4-12% Bis-Tris gels and NuPAGE® MES SDS Running Buffer at 200 V for 20 min. The gels were subsequently stained for 1 h with SimplyBlue™ SafeStain, destained, and scanned. The reported results are from two independent experiments

Impact of Staphylokinase (SAK) from *S. aureus* on FL9

S. aureus recombinant staphylokinase (SAK) was obtained from PROSPEC (Israel). 2- fold serial dilution of FL9 was made in 0.2% bovine serum albumin and 0.01% acetic acid with or without SAK to a final concentration of SAK in 200 μl of 5 μg ml^{-1}. The FL9-+/- SAK mixtures were incubated 1 h and added to a 96-well polyprostyrene microtiter plate. Overnight cultures of *S. aureus* 8325-4 were diluted 10.000-fold in TSB media and 100 μl was added to each well and MIC was determined.

Effect of FL9 on *S. aureus* virulence gene expression

30 μl of 20 mg FL9 or H_2O (negative control) ml^{-1} was tested in an agar diffusion assay as previously described (Nielsen *et al*., 2010). To monitor promoter activity of *hla* and *rnaIII*, the agarplates contained PC322 or SH101F7 (Chan & Foster, 1998; Horsburgh *et al*., 2002), which harbour a *hla::lacZ* and a *rnaII::lacZ* fusions, respectively.

Northern Blot Analysis

Northern blot analysis was performed as previously described (Thomsen *et al*., 2010). The strains used were *S. aureus* 8325-5 and FRP3757 USA300. Cells were grown in TSB at 37°C with shaking to OD_{600} = 0.7. The cultures were split in two and FL9 at sub-MIC (1/4 MIC) concentration was added to one of the cultures and samples for RNA purification were collected after 20 min exposure. Probes targeting *rnaIII* transcripts was amplified by PCR using the primers: rnaIII fwd. (5'-GGG GAT CAC AGA GAT GTG ATG G-3') and rnaIII rev. (5'-GGG CAT AGC ACT GAG TCC AAG G-3').

Results and Discussion

Determination of MIC of FL9 against *S. aureus*

Minimal inhibitory concentration (MIC) determination was performed on three *S. aureus* strains, two methicillin sensitive (MSSA) and one methicillin resistant (MSSR). The MIC of *S. aureus* SA113 (MSSA) was 64 μg ml^{-1} and for strain USA300 (MRSA) and 8325-4 (MSSA) the MIC was 128 μg ml^{-1}. This corresponds with the MIC values previously found for FL9 against *S. aureus* MSSA and MRSA strains (Nielsen *et al*., 2007).

FL9 performs concentration dependent membrane permeabilization property

Performing dose-dependent time-kill assays, FL9 initially exhibited fast killing activity at both 1×MIC and 5×MIC with a reduction of the CFU counts by 3 and 4 log units, respectively, indicating that FL9 possibly targets the membrane (Fig. 1). Hereafter, the killing gradually slowed down and after 5 hours reached a total reduction of CFU counts by 3 and 6 log units, respectively, showing a dose-dependent bactericidal effect. Many AMPs interact with the bacterial membrane, leading to pore-formation and subsequent leakage of intracellular components (Jenssen *et al*.,

2006). To determine whether FL9 has an impact on the *S. aureus* cytoplasmic membrane, we investigated the membrane integrity by measuring ATP leakage and found that increasing concentrations of FL9 lead to increasing leakage of ATP from the cell (Fig. 2). When FL9 was added at concentrations above 750 μg ml^{-1}, no intracellular ATP was detectable and only extracellular ATP was present indicating that the cytoplasmic membrane was disrupted. However, at lower concentrations of FL9, limited leakage of ATP was observed and intracellular ATP was still present, indicating that FL9 could have other targets.

FL9 interferes with DNA and RNA synthesis and binds DNA *in vitro*

Our data indicated that FL9 targets the *S. aureus* membrane at high concentrations. However, the killing kinetics and the ATP leakage assay suggested that FL9 at lower concentrations has other targets. A dual mode of action was previously suggested for other synthetic peptides which were found to target the cell membrane when applied at high concentrations and to have intracellular targets when used at low concentration (Gottschalk *et al*, 2013; Patrzykat *et al*., 2002; Sahl *et al*., 2005). Therefore, we investigated the possibility of FL9 having intracellular targets by assessing the synthesis of macromolecules in *S. aureus*. The incorporation of the radioactive precursors [methyl-^{3}H] thymidine for DNA synthesis and [5,6-^{3}H] uridine for RNA synthesis, was observed over a time period of 30 min after treatment with 1×MIC and 5×MIC of FL9. Both DNA and RNA synthesis was inhibited within the first 5 min after addition of FL9 at both 1×MIC and 5×MIC (Fig. 3). Some AMPs cause small membrane lesions, which lead to transient leakage of protons and thereby depletion of intracellular ATP, which would affect synthesis of cellular molecules (Huang, 2006). However, from our ATP leakage experiment, it is clear that the intracellular level of ATP does not decrease considerably, until high concentrations of FL9 were employed and increased ATP leakage was observed (Fig. 2).

Some AMPs that inhibit DNA synthesis bind unspecifically to DNA. For example the amphibian AMP, the α-helical AMP Buforin II, can penetrate the cell membrane, without causing disruption of the membrane, and inhibit cellular functions by binding to nucleic acids (Rotem *et al*., 2008; Sarig *et al*., 2011; Gottschalk *et al*., 2013; Park *et al*., 1998; Kobayashi *et al*., 2000). Therefore, to investigate whether the inhibition of DNA synthesis was due to the binding of FL9 to bacterial DNA, a gel retardation assay was performed. The DNA binding ability of FL9 was examined by analysing the electrophoretic mobility of DNA bands at different concentrations of FL9. Gel retardation with plasmid DNA isolated from *S. aureus* demonstrated that in the absence of FL9 pRMC2 migrates as a plasmid (Fig. 4). However, increasing the concentration of FL9 altered the migration of the plasmid, and at high concentrations the plasmid was no longer able to migrate into the gel. The peptide Plectasin was included as a control in the experiments and did not alter the plasmid mobility (data not shown), demonstrating that DNA binding is not a general property of AMPs. Thus, our data suggest that FL9 binds DNA.

FL9 induces the SOS response through the *recA* gene

Other studies have shown that binding of AMPs to DNA can induce the SOS response (Gunderson & Segall, 2006; Su *et al*., 2010). To analyze the effect of FL9 on the SOS response, we examined the expression of a LexA controlled gene, *recA*, using a *S. aureus recA*::*lacZ* fusion and an agar diffusion assay (Nielsen *et al*., 2010). The result clearly demonstrated an increased expression of *recA* in response to FL9 monitored as a blue ring in the agar reporter assay (Fig. 5a). As a positive control the expression of *recA* was induced by the antibiotic ciprofloxacin (Cirz *et al*., 2007) (Fig. 5b). The induction of the SOS response reveals that FL9-DNA binding damage the DNA. The

ability to induce the SOS response has previously been shown for the hexapeptide WRWYCR that exerts its broad bactericidal activity through stalling of bacterial replications forks (Su *et al*., 2010).

Impact of environmental conditions on FL9 activity

The potential use of AMPs as systemic drugs or in the food production is hampered by the reduced activity often seen when the peptides are exposed to environmental stresses as salt, serum and pH changes (Gordon *et al*., 2005). Therefore we exposed FL9 to different stress conditions and evaluated the activity of FL9 against *S. aureus* by MIC determination (Table 2). FL9 activity was highly reduced when pH was lowered, whereas alkaline pH seemed to improve the activity of FL9 by 4-fold. High salt concentrations (up to 1030 mM) had little effect on FL9 activity slightly reducing the MIC by 2-fold at 1030 mM of NaCl. Moreover, increasing concentrations of $MgCl_2$ had no effect on the MIC value for FL9.

In addition, we assessed the activity of FL9 in the presence of 10% and 90% serum and found FL9 activity at both serum concentrations although reduced by 20% and 70%, respectively, as compared to the activity of FL9 without serum (Fig. 6).

Our results illustrate that FL9 maintains growth inhibitory activity under different environmental conditions and to some extent, when exposed to serum. In addition, improved activity was found when FL9 was exposed to pH 8.5. Improved activity at basic pH was also observed for the AMP-mimics belonging to the oligo-acyl-lysyls (OAK) C_{12}K7-α_8 and the OAK $C_{16(\omega 7)}$K-β_{12} with membrane and DNA binding activities, respectively (Sarig *et al*., 2011; Goldfeder *et al*., 2010). At acidic pH, however, the susceptibility of FL9 was hampered. This has also been observed for the membrane-active AMP dermaseptin and the two OAKs (Sarig *et al*., 2011; Goldfeder *et al*., 2010;

Rydlo *et al*., 2006; Yaron *et al*., 2003). The environmental conditions can hamper the effect of AMPs, however, FL9 maintained activity under several conditions relevant for food production, pointing to a potential use of FL9 as lead compound for the future development of antimicrobial compounds used in the food industry.

The impact of FL9 on the tolerance mechanisms of *S. aureus*

S. aureus possess several tolerance mechanisms for protection against AMPs including proteolysis, capture of the AMPs and reduction of the anionic charge of the cell envelope (Peschel & Sahl, 2006). To address the applicability of FL9 as antimicrobial peptide against *S. aureus* we examined if some of the known *S. aureus* tolerance mechanisms protects against the activity of FL9. We found that the presence of the secreted protein SAK, which capture and prevent AMPs from reaching the bacterial cell membrane, did not affect the MIC (data not shown), However, when we investigated whether FL9 was prone to cleavage by the protease aureolysin, we found that FL9 was completely digested *in vitro* (Fig.7). Aureolysin cleavage and inactivation has also previously been shown for the cathelicidin LL-37 (Sieprawska-Lupa *et al*., 2004).

The best characterized tolerance mechanism of *S. aureus* toward AMPs is mediated via Dlt and MprF that enable *S. aureus* to counteract the electrostatic interaction of the cationic AMP to the anionic cell wall by adding positive charges onto the cell surface. Moreover, the VraFG ABC transporter has also been shown to play a role in the tolerance toward AMPs, and is as *dltA* and *mprF* regulated by the GraRS two component system (Falord *et al*., 2011; Li *et al*., 2007). We therefore investigated the roles of DltA, MprF and VraF in the *S. aureus* response to FL9. Using strains mutated in each gene, we found that all of the genes influenced tolerance of *S. aureus* toward FL9 with an 8-fold decrease in the MIC values for the Δ*dltA* and the Δ*mprF* strains and a 4-fold

decrease in the susceptibility of the Ω*vraF* strain compared to wild-type. These results show that the ability to change cell surface charge is an important defence mechanism of *S. aureus* against FL9 activity.

FL9 induces the transcription of *hla* and *rna*III

It has previously been shown that the expression of virulence genes in *S. aureus* is affected by sub-inhibitory concentrations of different antibiotics (Ohlsen *et al*., 1998; Worlitzsch *et al*., 2001; Nielsen *et al*., 2012). Knowledge of how pathogenic bacteria react in the presence of sub-inhibitory concentration of AMPs is important information that so far has not been that thoroughly investigated when evaluating the potential of peptoids as drugs used in the prevention of pathogenic bacteria. Therefore we wondered what effects the presence of low concentrations of FL9 would have on the expression of virulence genes in *S. aureus*. To investigate the influence of subinhibitory concentrations of FL9 on virulence gene expression we used an agar diffusion assay, monitoring the expression of the virulence genes *hla* and *rnaIII* of *S. aureus*. From the agar diffusion assay it is seen that FL9 up-regulate the expression of both virulence genes (Fig. 5 c,d,e,f). Induction of *rnaIII* by FL9 in 8325-4 and USA300 was confirmed by northern blot analysis (data not shown). Thus, failure in the treatment of *S. aureus* infections, caused by sub-inhibitory exposure of FL9, has the potential to lead to enhanced virulence of *S. aureus*.

The present study was set out to investigate the mode of action (MOA) of the Fallaxin analog FL9 and evaluate how various environmental conditions and bacterial tolerance mechanisms affect its activity. If AMPs are going to be used for treatment of bacterial infections or preservatives in the food industry, it is important to understand the impact of the AMPs on the bacteria and know how

the bacteria react, to limit the risk of resistance and maintain activity under the conditions it will be used.

In conclusion, this study shows that an analog of the amphibian AMP fallaxin, FL9, has a dual mode of action, it both have membrane disturbing affects and can penetrate the bacterial membrane and bind DNA, inhibiting DNA synthesis and inducing the SOS response. FL9 maintained activity under a wide range of conditions. However, FL9 is sensitive to most tolerance mechanisms of *S. aureus* and might cause undesirable effects on virulence potential if administered at too low concentrations

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The authors of this study have no conflicts of interest to declare.

References

Bernardo, K., Pakulat, N., Fleer, S., Schnaith, A., Utermohlen, O., Krut, O., Muller, S. & Kronke, M. (2004). Subinhibitory concentrations of linezolid reduce *Staphylococcus aureus* virulence factor expression. *Antimicrob Agents Chemother* **48,** 546-555.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B. & Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis* **48,** 1-12.

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol* **3,** 238-250.

Chan, P. F. & Foster, S. J. (1998). The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4. *Microbiology* **144,** 2469-2479.

Cirz, R. T., Jones, M. B., Gingles, N. A., Minogue, T. D., Jarrahi, B., Peterson, S. N. & Romesberg, F. E. (2007). Complete and SOS-mediated response of *Staphylococcus aureus* to the antibiotic ciprofloxacin. *J Bacteriol* **189,** 531-539.

Cohn, M. T., Kjeldgaard, P., Frees, D., Penadés, J. R. & Ingmer, H. (2011). Clp-dependent proteolysis of the LexA N-terminal domain in *Staphylococcus aureus*. *Microbiology* **157**, 677-684

Corrigan, R. M. & Foster, T. J. (2009). An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. *Plasmid* **61,** 126-129.

Courcelle, J. & Hanawalt, P. C. (2003). RecA-dependent recovery of arrested DNA replication forks. *Annu Rev Genet* **37,** 611-646.

Davies, J., Spiegelman, G. B. & Yin, G. (2006). The world of subinhibitory antibiotic concentrations. *Curr Opin Microbiol* **9,** 445–453

Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H. A., Mongodin, E. F. & others. (2006). Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet* **367,** 731-739.

Falord, M., Mader, U., Hiron, A., Debarbouille, M. & Msadek, T. (2011). Investigation of the *Staphylococcus aureus* GraSR regulon reveals novel links to virulence, stress response and cell wall signal transduction pathways. *PLoS One* **6,** e21323.

Fernandez, D. I., Gehman, J. D. & Separovic, F. (2009). Membrane interactions of antimicrobial peptides from Australian frogs. *Biochim Biophys Acta* **1788,** 1630-1638.

Goldfeder, Y., Zaknoon, F. & Mor, A. (2010). Experimental conditions that enhance potency of an antibacterial oligo-acyl-lysyl. *Antimicrob Agents Chemother* **54,** 2590-2595.

Goldman, M. J., Anderson, G. M., Stolzenberg, E. D., Kari, U. P., Zasloff, M. & Wilson, J. M. (1997). Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **88,** 553-560.

Gordon, Y. J., Romanowski, E. G. & McDermott, A. M. (2005). A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr Eye Res* **30,** 505-515.

Gottschalk, S., Ifrah, D., Lerche, S., Gottlieb, C. T., Cohn, M. T., Hiasa, H., Hansen, P. R., Gram, L., Ingmer, H. & Thomsen, L. E. (2013). The antimicrobial Lysine-Peptoid Hybrid LP5 inhibits DNA replication an induces the SOS response in *Staphylococcus aureus*. *BMC microbiol* **13,** 192

Gunderson, C. W. & Segall, A. M. (2006). DNA repair, a novel antibacterial target: Holliday junction-trapping peptides induce DNA damage and chromosome segregation defects. *Mol Microbiol* **59,** 1129-1148.

Herbert, S., Barry, P. & Novick, R. P. (2001). Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. *Infect Immun* **69,** 2996-3003.

Horsburgh, M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K. & Foster, S. J. (2002). sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* **184,** 5457-5467.

Huang, H. W. (2006). Molecular mechanism of antimicrobial peptides: The origin of cooperativity. *Biochim Biophys Acta* **1758,** 1292-1302

Iordanescu, S. & Surdeanu, M. (1976). Two restriction and modification systems in *Staphylococcus aureus* NCTC8325. *J Gen Microbiol* **96,** 277-281

Jenssen, H., Hamill, P. & Hancock, R. E. (2006). Peptide antimicrobial agents. *Clin Microbiol Rev* **19,** 491-511.

Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J. & Tarkowski, A. (2004). *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J Immunol* **172,** 1169-1176.

Kobayashi, S., Takeshima, K., Park, C. B., Kim, S. C. & Matsuzaki, K. (2000). Interactions of the novel antimicrobial peptide buforin 2 with lipid bilayers: proline as a translocation promoting factor. *Biochemistry* **39,** 8648-8654.

Lee, I. H., Cho, Y. & Lehrer, R. I. (1997). Effects of pH and salinity on the antimicrobial properties of clavanins. *Infect Immun* **65,** 2898-2903.

Lehrer, R. I., Rosenman, M., Harwig, S. S., Jackson, R. & Eisenhauer, P. (1991). Ultrasensitive assays for endogenous antimicrobial polypeptides. *J Immunol Methods* **137,** 167-173.

Li, M., Cha, D. J., Lai, Y., Villaruz, A. E., Sturdevant, D. E. & Otto, M. (2007). The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. *Mol Microbiol* **66,** 1136-1147.

Makobongo, M. O., Gancz, H., Carpenter, B. M., McDaniel, D. P. & Merrell, D. S. (2012). The Oligo-Acyl Lysyl Antimicrobial Peptide C12K-2beta12 Exhibits a Dual Mechanism of Action and Demonstrates Strong In Vivo Efficacy against *Helicobacter pylori*. *Antimicrob Agents Chemother* **56,** 378-390.

Minahk, C. J. & Morero, R. D. (2003). Inhibition of enterocin CRL35 antibiotic activity by mono- and divalent ions. *Lett Appl Microbiol* **37,** 374-379.

Nielsen, A., Nielsen, K. F., Frees, D., Larsen, T. O. & Ingmer, H. (2010). Method for screening compounds that influence virulence gene expression in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **54,** 509-512.

Nielsen, L. N., Roggenbuck, M., Haaber, J., Ifrah, D. & Ingmer, H. (2012). Diverse modulation of *spa* transcription by cell wall active antibiotics in *Staphylococcus aureus*. *BMC Res Notes* **5**, 457.

Nielsen, S. L., Frimodt-Moller, N., Kragelund, B. B. & Hansen, P. R. (2007). Structure-activity study of the antibacterial peptide fallaxin. *Protein Sci* **16,** 1969-1976.

Novick, R. (1967). Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. *Virology* **33,** 155-166.

Ohlsen, K., Ziebuhr, W., Koller, K. P., Hell, W., Wichelhaus, T. A. & Hacker, J. (1998). Effects of subinhibitory concentrations of antibiotics on alpha-toxin (*hla*) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob Agents Chemother* **42,** 2817-2823.

Park, C. B., Kim, H. S. & Kim, S. C. (1998). Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem Biophys Res Commun* **244,** 253-257.

Pasupuleti, M., Schmidtchen, A. & Malmsten, M. (2012). Antimicrobial peptides: key components of the innate immune system. *Crit Rev Biotechnol* **32**, 143–71.

Patrzykat, A., Friedrich, C. L., Zhang, L., Mendoza, V. & Hancock, R. E. (2002). Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. *Antimicrob Agents Chemother* **46,** 605-614.

Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen,W. F., Jung, G., Tarkowski, A. & and others. (2001). *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J Exp Med* **193,** 1067-1076.

Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G. & Gotz, F. (1999). Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J Biol Chem* **274,** 8405-8410.

Peschel, A. & Sahl, H. G. (2006). The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat Rev Microbiol* **4,** 529-536.

Radzishevsky, I. S., Rotem, S., Zaknoon, F., Gaidukov, L., Dagan, A. & Mor, A. (2005). Effects of acyl versus aminoacyl conjugation on the properties of antimicrobial peptides. *Antimicrob Agents Chemother* **49,** 2412-2420.

Rollins-Smith, L. A., King, J. D., Nielsen, P. F., Sonnevend, A. & Conlon, J. M. (2005). An antimicrobial peptide from the skin secretions of the mountain chicken frog *Leptodactylus fallax* (Anura:Leptodactylidae). *Regul Pept* **124,** 173-178.

Rotem, S., Radzishevsky, I. S., Bourdetsky, D., Navon-Venezia, S., Carmeli, Y. & Mor, A. (2008). Analogous oligo-acyl-lysines with distinct antibacterial mechanisms. *FASEB J* **22,** 2652-2661.

Rozek, A., Powers, J. P., Friedrich, C. L. & Hancock, R. E. (2003). Structure-based design of an indolicidin peptide analogue with increased protease stability. *Biochemistry* **42,** 14130-14138.

Rydlo, T., Rotem, S. & Mor, A. (2006). Antibacterial properties of dermaseptin S4 derivatives under extreme incubation conditions. *Antimicrob Agents Chemother* **50,** 490-497

Sahl, H. G., Pag, U., Bonness, S., Wagner, S., Antcheva, N. & Tossi, A. (2005). Mammalian defensins: structures and mechanism of antibiotic activity. *J Leukoc Biol* **77,** 466-475.

Sarig, H., Goldfeder, Y., Rotem, S. & Mor, A. (2011). Mechanisms mediating bactericidal properties and conditions that enhance the potency of a broad-spectrum oligo-acyl-lysyl. *Antimicrob Agents Chemother* **55,** 688-695.

Sieprawska-Lupa, M., Mydel, P., Krawczyk, K., Wojcik, K., Puklo, M., Lupa, B., Suder, P., Silberring, J., Reed, M., Pohl, J. & and others. (2004). Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrob Agents Chemother* **48,** 4673-4679.

Su, L. Y., Willner, D. L. & Segall, A. M. (2010). An antimicrobial peptide that targets DNA repair intermediates in vitro inhibits *Salmonella* growth within murine macrophages. *Antimicrob Agents Chemother* **54,** 1888-1899.

Thomsen, L. E., Gottlieb, C. T., Gottschalk, S., Wodskou, T. T., Kristensen, H. H., Gram, L. & Ingmer, H. (2010). The heme sensing response regulator HssR in *Staphylococcus aureus* but not the homologous RR23 in *Listeria monocytogenes* modulates susceptibility to the antimicrobial peptide plectasin. *BMC Microbiol* **10,** 307.

Worlitzsch, D., Kaygin, H., Steinhuber, A., Dalhoff, A., Botzenhart, K. & Doring, G. (2001). Effects of amoxicillin, gentamicin, and moxifloxacin on the hemolytic activity of *Staphylococcus aureus* in vitro and in vivo. *Antimicrob Agents Chemother* **45,** 196-202.

Yaron, S., Rydlo, T., Shachar, D. & Mor, A. (2003). Activity of dermaseptin K4-S4 against foodborne pathogens. *Peptides* **24,** 1815-1821.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415,** 389-395.

TABLE 1. Bacterial strains

S. aureus strains	Relevant genotype and property	Source/ reference
USA300	Wild type. CA-MRSA clinical isolate	(Diep *et al*., 2006)
NE645	*bursa aurealis* Tn insertion in SAUSA300_0647(*vraF*)	NARSA
8325-4	Wild type	(Novick, 1967)
HI2682	8325-4 *recA*$^{+}$ *recA::lacZ*	(Gottschalk *et al*., 2013)
PC322	8325-4 *hla*$^{+}$ *hla::lacZ*	(Chan & Foster, 1998)
SH101F7	8325-4 *rnaIII*$^{+}$ *rnaIII::lacZ*	(Horsburgh *et al*., 2002)
SA113	Wild type	(Iordanescu & Surdeanu, 1976)
SA113 Δ*dltA*	*dltA*::*spc*	(Peschel *et al*., 1999)
SA113 Δ*mprF*	*mprF*::*erm*	(Peschel *et al*., 2001)
SA113 Ω*vraF*	*bursa aurealis* Tn insertion phage transduced from NE645	This study

TABLE 2. Effects of incubation conditions on MIC of FL9 on *S. aureus* 8325-4

Incubation Conditions	MIC (μg ml^{-1})
NaCl conc (mM)	
85	128
170	128
260	128
515	128
1030	64
pH	
3.5	>512
5.5	>512
7	128
8.5	32
$MgCl_2$ conc (mM)	
1	128
3	128
5	128

Shaded rows represent standard conditions.

FIG. 1. Kinetics of bacterial killing.

Time and concentration dependent killing by *S. aureus* 8325-4 by FL9 at 0 (♦), 1×MIC (128 μg ml^{-1}) (■) and 5×MIC (640 μg ml^{-1}) (▲). Bacteria were sampled at the indicated times and the colony counts determined. The experiment shown is a mean of four independent experiments. CFU, colony-forming units.

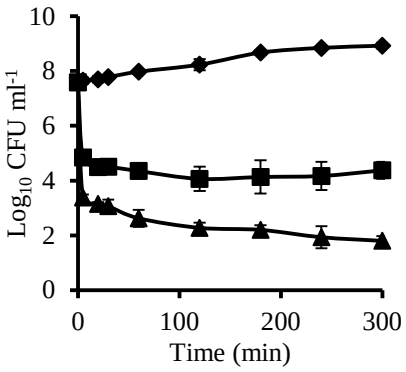

FIG. 2. Measurement of ATP leakage from *S. aureus* 8325-4 after treatment with increasing concentration of FL9.

Measurement of intracellular (IC) and extracellular (EC) concentrations of ATP. The experiment is shown as the mean of two independent experiments.

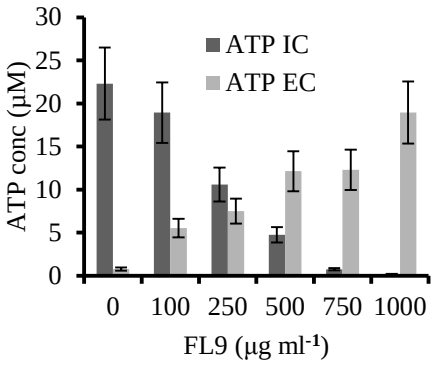

FIG. 3. FL9 inhibit bacterial DNA and RNA synthesis.

Effect of FL9 at 0 (♦), 1×MIC (■) and 5×MIC (▲) on DNA (a) and RNA (b) synthesis of *S. aureus* 8325-4 measured by incorporation of radiolabelled precursors [methyl-^{3}H] thymidine and [5,6-^{3}H] uridine, respectively. The results shown are one representative of three experiments.

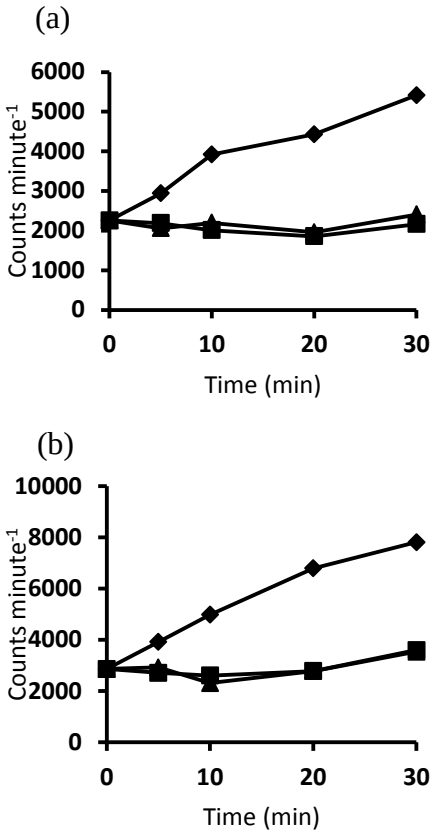

FIG. 4. FL9 binds to *S. aureus* DNA.

Gel retardation with *S. aureus* DNA. Increasing amounts of FL9 were incubated with 100 ng pRMC2 plasmid DNA and run on an agarose gel. Lane 1: negative control containing DNA and binding buffer. Lane 2-8: containing increasing amounts of FL9 (20, 40, 80, 160, 320, 640 and 1280 μg ml^{-1}). The experiment is one representative of three experiments.

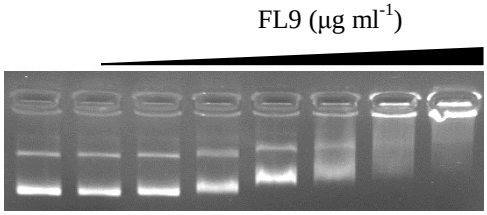

FIG. 5. **FL9 induces *recA, rnaIII* and *hla* expression in *S. aureus* 8325-4.**

(a) FL9 or (b) ciprofloxacin (positive control) was added to wells in TSB agar plates containing the 8325-4 derived *lacZ* reporter strain HI2682 (*recA::lacZ*). (c)-(f) FL9 was added to the wells of a TSA plates containing the 8325-4 derived *lacZ* reporter strains SH101F7 (*rnaIII::lacZ*) (c) or PC322 (*hla::lacZ*) (e). H_2O was used as negative control (d)(f). Incubation time was 18 h. Induction of expression was monitored as blue colonies.

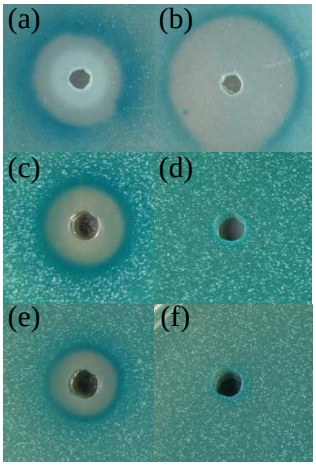

FIG. 6. FL9 activity in the presence of serum.

Activity of FL9 against *S. aureus* 8325-4 in the presence of 0 (♦), 10% (■) and 90% (▲) serum was assessed by radial diffusion assay. Activity of FL9 in the presence of serum was assessed at different time points for 24 h, and the clearing diameter was expressed in units (0.1mm=1U). The experiment shown is a mean of two independent experiments

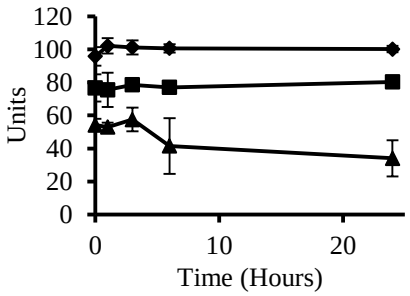

FIG. 7. Aureolysin degrades FL9.

NativePAGE™ Novex® Bis-Tris polyacrylamide gel electrophoresis of FL9 after proteolysis with aureolysin. The proteolysis was performed at a peptide-to-enzyme ratio of 300:1 and at a FL9 concentration of 136 μM and with incubation at 37°C for 4 h. FL9 ÷ and + aureolysin.