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

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The rapid rise in antibiotic-resistant pathogens is causing increased health concerns, and consequently there is an urgent need for novel antimicrobial agents. Antimicrobial peptides (AMPs), which have been isolated from a wide range of organisms, represent a very promising class of novel antimicrobials. In the present study, the analogue FL9, based on the amphibian AMP fallaxin, was studied to elucidate its mode of action and antibacterial activity against the human pathogen *Staphylococcus aureus*. Our data showed that FL9 may have a dual mode of action against *S. aureus*. At concentrations around the MIC, FL9 bound DNA, inhibited DNA synthesis and induced the SOS DNA damage response, whereas at concentrations above the MIC the interaction between *S. aureus* and FL9 led to membrane disruption. The antibacterial activity of the peptide was maintained over a wide range of NaCl and MgCl₂ concentrations and at alkaline pH, while it was compromised by acidic pH and exposure to serum. Furthermore, at subinhibitory concentrations of FL9, *S. aureus* responded by increasing the expression of two major virulence factor genes, namely the regulatory *rnaIII* and *hla*, encoding α-haemolysin.

In addition, the *S. aureus*-encoded natural tolerance mechanisms included peptide cleavage and the addition of positive charge to the cell surface, both of which minimized the antimicrobial activity of FL9. Our results add new information about FL9 and its effect on *S. aureus*, which may aid in the future development of analogues with improved therapeutic potential.

INTRODUCTION

Bacterial infections are re-emerging as a major problem due to the development of resistance towards conventional antibiotics and a decline in the development of new antimicrobials (Boucher *et al.*, 2009). A possible solution to overcome multidrug-resistant bacteria is to use alternative classes of antimicrobials to treat infectious diseases. One such class is antimicrobial peptides (AMPs), also known as host defence 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 fewer than 40 aa divided into four structural classes: α-helical, β-sheet stabilized by two or more disulfide bridges, extended helices and loop structures (Jensen *et al.*, 2006).

It is believed that most AMPs, due to their cationic and amphipathic nature, selectively kill bacteria by penetrating the anionic cell membrane through 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 and RNA (Brogden, 2005; Jensen *et al.*, 2006; Peschel & Sahl, 2006; Makobongo *et al.*, 2012). 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. It is regulated by the repressor LexA, which, in response to DNA damage sensed by RecA, undergoes autocleavage (Courcelle & Hanawalt, 2003; Cohn et al., 2011). In consequence, expression of LexA-controlled genes such as recA may be monitored as reporters of bacterial DNA damage.

However, although AMPs have a highly attractive potential as therapeutics or in preservation of food, their use as antimicrobial compounds is associated with several obstacles. One drawback is their sensitivity to various conditions in the human host or in food, 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; Rozek et al., 2003; Radzishevsky et al., 2005). 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 capture 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, 2001). In addition, there is limited knowledge on how subinhibitory concentrations of AMPs affect pathogens. It has been shown previously that, during antimicrobial therapy, bacteria may be exposed to subinhibitory concentrations of AMPs, which can have unwanted effects such as changes in virulence gene expression (Davies et al., 2006). This effect has been shown when pathogens are exposed to subinhibitory concentrations of antibiotics. For instance, subinhibitory concentrations of clindamycin and linezolid have the ability to downregulate the expression of *Staphylococcus aureus* exotoxins, including ζ-haemolysin (*hla*) (Herbert et al., 2001; Bernardo et al., 2004). 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 defence 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 (Mercer & O’Neil, 2013). One such AMP is fallaxin, a 25-mer AMP amide isolated from the West Indian mountain chicken frog *Leptodactylus fallax*. Fallaxin belongs to the ζ-helical class of AMPs (Rollins-Smith et al., 2005). From a recent structure–activity study of fallaxin, the analogue FL9, in which an alanine residue was replaced by a more hydrophobic leucine residue, was found to have improved activity towards a range of Gram-positive bacteria, including *S. aureus*, compared with the natural AMP (Nielsen et al., 2007). Here, we characterized the activity of FL9 and found that at and above the MIC, the peptide had a dual mode of action, affecting both intracellular targets and the membrane of *S. aureus*, whereas at subinhibitory concentrations it increased expression of the virulence factors genes *raII* and *hla*. Furthermore, we showed that FL9 is active at physiologically relevant conditions and that several lines of defence exist in *S. aureus* to limit the bactericidal effect of the peptide.

**METHODS**

**Strains, peptide and culture conditions.** The *S. aureus* strains used in this study are listed in Table 1. The strains were grown in tryptone soy broth (TSB; Oxoid) 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 (Sigma). The FL9 peptide (GVVDILKGLAKDIAGHLASKVMNL-NH₂) was purchased from Shafer-N. The identity of the peptide was confirmed by liquid chromatography/mass spectrometry.

**MIC determination.** The MIC of FL9 was determined using a modified microtitre broth dilution assay for cationic antimicrobial peptides from the Hancock laboratory (http://cmdr.ubc.ca/bobh/methods/MODIFIEDMIC.html). Briefly, serial twofold dilutions of FL9 (at 10 times the required test concentration) were made in 0.2% BSA (Sigma) and 0.01% acetic acid in polypropylene tubes. Over-night cultures of *S. aureus* 8325-4, USA300 and AK1 were diluted 10⁻⁵-fold in Muller–Hinton broth (Oxoid) to a final concentration of approximately 5 × 10⁸ cfu ml⁻¹, and 100 μl was added to each well of a 96-well polypropylene microtitre plate. A volume of 11 μl of the twofold serially diluted FL9 was added to each well. The plate was incubated overnight and the MIC was determined 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 by peptide addition was determined by measuring ATP leakage from the bacterial cell using a bioluminescence assay, as described previously (Thomsen et al., 2010). *S. aureus* 8325-4 was grown in TSB at 37°C overnight, diluted 1:100 in TSB and grown at 37°C. *S. aureus* was harvested (350g, 10 min) at mid-exponential phase (OD<sub>546</sub> 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<sub>546</sub> of 10. The bacteria were stored on ice and used within 5 h. The 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⁻¹. ATP measurements were performed at time 0. For the time-lapse experiments, 1 × MIC (128 μg ml⁻¹) was added at time 0 and ATP measurements were performed at 0.5, 5, 10, 20, 30 and 60 min after FL9 addition. Total ATP and extracellular ATP were determined using a bioluminescence kit (FLAA-1KT; Sigma) and a BioOrbit 1253 luminometer. Total ATP content was determined by rapidly permeabilizing 20 μl cell suspension with 80 μl DMSO. 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 analysed as described above. Intracellular volumes of *S. aureus* (0.85 μm<sup>3</sup>) were subtracted from the total volume before calculating the extracellular ATP concentration. The intracellular ATP concentration could then be calculated by subtraction of the extracellular ATP concentration from the total ATP. The number of cells in suspension was determined by plate spreading. The reported results are from two independent experiments.
Table 1. S. aureus strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype and property</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA300</td>
<td>WT community-associated MRSA clinical isolate</td>
<td>Diep et al. (2006)</td>
</tr>
<tr>
<td>NE645</td>
<td>Bursa aurealis Tn insertion in SAUSA300_0647(vraF)</td>
<td>NARSA</td>
</tr>
<tr>
<td>8325-4</td>
<td>Wild-type</td>
<td>Novick (1967)</td>
</tr>
<tr>
<td>H12682</td>
<td>8325-4 recA + recA :: lacZ</td>
<td>Gottschalk et al. (2013)</td>
</tr>
<tr>
<td>PC322</td>
<td>8325-4 hla + hla :: lacZ</td>
<td>Chan &amp; Foster (1998)</td>
</tr>
<tr>
<td>SH101F7</td>
<td>8325-4 rnaIII + rnaIII :: lacZ</td>
<td>Horsburgh et al. (2002)</td>
</tr>
<tr>
<td>AK1</td>
<td>8325-4; aur :: ermB (Em&lt;sup&gt;K&lt;/sup&gt;)</td>
<td>Karlsson et al. (2001)</td>
</tr>
<tr>
<td>SA113</td>
<td>Wild-type</td>
<td>Jordonescu &amp; Surdeanu (1976)</td>
</tr>
<tr>
<td>SA113ΔdltA</td>
<td>dltA :: spc</td>
<td>Peschel et al. (1999)</td>
</tr>
<tr>
<td>SA113ΔmprF</td>
<td>mprF :: erm</td>
<td>Peschel et al. (2001)</td>
</tr>
<tr>
<td>SA113ΔvraF</td>
<td>Bursa aurealis Tn insertion phage transduced from NE645</td>
<td>This study</td>
</tr>
</tbody>
</table>

NARSA, Network on Antimicrobial Resistance in Staphylococcus aureus.

In vitro killing kinetics of S. aureus. S. aureus 8325-4 was grown overnight in TSB medium, diluted 1 : 50 in TSB medium and allowed to grow for 1 h to an OD<sub>600</sub> of 0.2. FL9 was added to final concentrations equal to 1× and 5× 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 tryptone soy agar (TSA). The c.f.u. were counted after an overnight incubation at 37 °C. The limit of detection in the assay was 10 c.f.u. The reported results are from four independent experiments.

DNA synthesis. Overnight cultures of S. aureus 8325-4 were diluted 1 : 50 in TSB and allowed to grow for 1 h (to an OD<sub>600</sub> of 0.2). To the culture was then added 37 MBq (1 μCi ml<sup>−1</sup>) [methyl-<sup>3</sup>H]-thymidine. After 10 min of incubation at 37 °C, FL9 was added at 1× 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 FL9. These samples were added to 2 vols 99.9 % ice-cold ethanol and 0.1 vol. 3 M sodium acetate (pH 5.5) to precipitate the DNA molecules. After precipitation overnight at −20 °C, samples were collected by centrifugation (8500 g, 10 min) and washed twice in 1 ml ice-cold 70 % ethanol. Samples were resuspended in 100 μl Milli-Q 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. Error bars represent 1 SD from three independent experiments.

DNA-binding analysis. Gel retardation analysis were performed as described previously (Park et al., 1998) by mixing 100 ng plasmid DNA (pRM2C2) (Corrigan & Foster, 2009) isolated from S. aureus 8325-4 with increasing concentrations of FL9 (net charge +2) (20, 40, 80, 160, 320, 640 and 1280 μg ml<sup>−1</sup>) or plectasin (net charge +3) (20, 40, 80, 160, 320, 640 and 1280 μg ml<sup>−1</sup>) (negative control) in 20 μl binding buffer [5 % glycerol, 10 mM Tris/HCl (pH 8), 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl and 50 μg BSA ml<sup>−1</sup>] (positive control) or H<sub>2</sub>O (negative control) was added to the wells and the plates were incubated at 37 °C for 18 h. The reported results are representative of three independent experiments, which all showed similar results.

Effect of FL9 on S. aureus recA expression. The expression of recA was tested in an agar diffusion assay as described previously (Nielsen et al., 2010). The strain H12682 was used to monitor expression from the recA promoter. Briefly, 1 ml 10<sup>−3</sup>-diluted overnight culture was placed in a Petri dish to which 25 ml TSA (≈ 40 °C) containing 150 μg X-Gal (5-bromo-4-chloro-3-indoly-l-β-D-galactopyranoside) ml<sup>−1</sup> was added and mixed by careful whirling. The plates were dried and wells were formed with a sterile drill. A volume of 30 μl FL9 (20 mg ml<sup>−1</sup>), ciprofloxacin (0.05 mg ml<sup>−1</sup>, positive control) or H<sub>2</sub>O (negative control) was added to the wells and the plates were incubated at 37 °C for 18 h. The reported results are representative of three independent experiments, which all showed similar results.

β-Galactosidase assay. β-Galactosidase activity was measured from the SOS-responsive recA−lacZ fusion in H12682. Cells were grown in TSB at 37 °C to an OD<sub>600</sub> of 0.25. FL9 was added at subinhibitory concentrations (0.25× MIC; 32 μg ml<sup>−1</sup>) and measurements were performed every 30 min and compared to a control sample without FL9. Assays were performed as recommended by Sambrook and Russel (2001). The reported results are one representative of two independent experiments, showing similar fold changes of regulation.

MIC determination under environmental conditions. S. aureus 8325-4 was grown in TSB medium (85 mM NaCl, pH 7) and diluted 10<sup>−4</sup>-fold as described above (MIC determinations) in the following medium: TSB+NaCl concentrations of 85, 170, 260, 515 and 1030 mM, pH of 3.5, 5.5, 7, and 8.5, or MgCl<sub>2</sub> 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, the serum was collected. FL9 was diluted in 0.01 % acetic acid + 0.2 % BSA and serum was added to achieve an FL9 concentration of 1000 μg ml<sup>−1</sup> in 0, 10 or 90 % serum. The FL9 and serum mixtures were incubated at 37 °C and samples were withdrawn at the indicated time points. The serum activity of the peptide/serum mixtures was measured by a radial diffusion assay (Lehre et al., 1991). S. aureus 8325-4 was grown overnight at 37 °C in 5 ml 3 % TSB. To obtain cells in the exponential phase, the overnight culture was subcultured by 1 : 50 dilution in fresh TSB and grown for an additional 2 h at 37 °C to an OD<sub>600</sub> of 0.4. The bacteria were centrifuged at 900 g for 10 min at 4 °C, washed once in cold 10 ml Tris/HCl buffer (10 mM, pH 7.4) and resuspended in 10 ml 10 mM Tris/HCl buffer (pH 7.4). The bacterial suspension was added to warm (40 °C) 10 mM Tris/HCl buffer (pH 7.4) containing 2 % low-electro-endosmosis-type agarose (Seakem LE Agarose; Lonza) to a final concentration of 2×10<sup>9</sup> c.f.u. ml<sup>−1</sup>. After rapid dispersion of the bacteria, the agar was poured into a 20 cm Petri dish to form a
uniform layer and was breached with a 4 mm-diameter gel punch to make evenly spaced wells after the agarase had solidified. Following the addition of 10 µl serum samples or FL9 in 0.01 % acetic acid +0.2 % BSA at concentrations of 31.25, 62.5, 125, 250, 500 or 1000 µg ml⁻¹ to each well, the plates were incubated for 3 h at room temperature. An overlay agar composed of double-strength (6 %) TSA and 10 mM Tris/HCl buffer (pH 7.4) was then poured over. After incubation for 18–24 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. The drawing of blood from healthy chickens was conducted with permission from the National Animal Experiments Inspectorate (http://www.dyrforforsorgstilsynet.dk) in accordance with Danish law, licence number 2012-15-2934-0039.

**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, 2001) of SA113 on FL9 was investigated by MIC determinations as described above. The reported data are from four independent experiments. The SA113 *ΩvraF* strain was constructed by transduction using *Φ11* phage propagated on NE645 (Network on Antimicrobial Resistance in *Staphylococcus aureus*) and selected on erythromycin. The integration of the *bursa aurealis Tn* insertion in SA113 *vraF* was confirmed by PCR using primers ErmB-1 (5′-CGAGTGAAGAAGTACTCAACCG-3′) and ErmB-2 (5′-CTTGC TCATAAAGGCTGTTAC-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 : enzyme ratio of 300:1 for 4 h at 37 °C in Tris/HCl (pH 7.4) buffer. The samples were supplemented with NativePAGE Sample Buffer (¼) (Thermo Fisher Scientific), 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 (Thermo Fisher Scientific) at 200 V for 20 min. The gels were subsequently stained for 1 h with SimplyBlue SafeStain (Thermo Fisher Scientific), destained and scanned. The reported results are from two independent experiments.

**Impact of staphylocinase (SAK) from S. aureus on FL9.** *S. aureus* recombinant SAK was obtained from PROSPEC. A twofold serial dilution of FL9 was made in 0.2 % BSA and 0.01 % acetic acid with or without SAK to a final concentration of SAK in 200 µl of 3 µg ml⁻¹. The FL9 ± SAK mixtures were incubated for 1 h and added to a 96-well polystyrene microtitre plate. The MIC was determined in the SAK/FL9 mixture as described above.

**Effect of FL9 on S. aureus virulence gene expression.** Virulence gene expression was tested in an agar diffusion assay as described previously (Nielsen *et al.*, 2007). To monitor the promoter activity of *hla* and *rnavI*, the agar plates contained PC322 or SH101F7 (Chan & Foster, 1998; Horsburgh *et al.*, 2002), which harbour an *hla*: lacZ and an *rnavI*: lacZ fusion, respectively. Briefly, 1 ml 10⁻³-diluted overnight culture was placed in a Petri dish to which 25 ml TSA (~ 40 °C) containing 150 µg X-Gal ml⁻¹ was added and mixed by careful swirling. The plates were dried and wells were formed with a sterile drill. A volume of 30 µl FL9 (20 mg ml⁻¹) or H₂O (negative control) was added to the wells and the plates were incubated at 37 °C for 18 h.

**Northern blot analysis.** Northern blot analysis was performed as described previously (Thomsen *et al.*, 2010). The strains used were *S. aureus* 8325-4 and USA300. Cells were grown in TSB at 37 °C with shaking to an OD₆₀₀ of 0.7. The cultures were split in two, and FL9 at a subinhibitory concentration (0.25 × MIC) was added to one of the cultures; samples for RNA purification were collected after 20 min exposure. The cells were quickly cooled and lysed mechanically using a FastPrep machine (Bio101; Qiogene), and RNA was isolated using an RNasy kit (Qiagen) according to the manufacturer’s instructions. The RNA was transferred to a nylon membrane (Boehringer Mannheim) by capillary blotting, and hybridization was performed using gene-specific probes that had been labelled with [³²P]dCTP using Ready-to-Go DNA-labelling beads from Amersham Biosciences. Probes targeting *rnavI* transcripts were amplified by PCR using the primers: rnavI forward (5′-GGGGATCACAGAGATGTGATGG-3′) and rnavI reverse (5′-GGGCGATAAGCCTGATCAAGGG-3′).

**RESULTS AND DISCUSSION**

**Determination of MIC of FL9 against S. aureus**

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

**FL9 performs concentration-dependent membrane permeabilization**

In dose-dependent time–kill assays, FL9 initially exhibited fast killing activity at both 1× and 5× MIC with a reduction in the c.f.u. count by 3 and 4 logs, respectively (Fig. 1). Hereafter, the killing gradually slowed down and after 5 h reached a total reduction of c.f.u. count by 3 and 2 logs, respectively. The MIC with a 90 % killing of S. aureus 8325-4 by FL9 at 0 (●), 1× MIC (■, 128 µg ml⁻¹) and 5× MIC (▲, 640 µg ml⁻¹) was obtained from BioCol GmbH. FL9 (136 µM) was incubated at a peptide : enzyme ratio of 300:1 for 4 h at 37 °C in Tris/HCl (pH 7.4) buffer. The samples were supplemented with NativePAGE Sample Buffer (¼) (Thermo Fisher Scientific), 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 (Thermo Fisher Scientific) at 200 V for 20 min. The gels were subsequently stained for 1 h with SimplyBlue SafeStain (Thermo Fisher Scientific), destained and scanned. The reported results are from two independent experiments.

**Fig. 1.** Kinetics of bacterial killing. Time- and concentration-dependent killing of *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 c.f.u. determined. Results are shown as means ± 1 SD from four independent experiments.
6 logs, respectively, showing a dose-dependent bactericidal effect. At 1 × MIC, we observed that growth was arrested but the bacteria were not killed. 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 had an impact on the S. aureus cytoplasmic membrane, we investigated membrane integrity by measuring ATP leakage. We found that increasing concentrations of FL9 led to increasing leakage of ATP from the cell (Fig. 2a). 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. A time-lapse experiment using 1 × MIC (128 µg ml⁻¹) showed that FL9 did not lead to increased extracellular ATP over time (Fig. 2b).

When compared with the killing kinetics, both experiments show a rapid effect, causing ATP leakage and a drop in c.f.u. immediately after FL9 was added to the bacteria. However, over time, FL9 affected neither ATP leakage nor the number of c.f.u. (Figs 1 and 2b).

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

Although AMPs have been reported previously to target the membrane, we cannot exclude the possibility that ATP leakage is the consequence of cellular perturbations. A dual mode of action was suggested previously for other synthetic peptides that were found to target the cell membrane when applied at high concentrations, and to have intracellular targets when used at low concentration (Patrzykat et al., 2002; Sahl et al., 2005; Gottschalk et al., 2013). Therefore, we investigated the possibility of FL9 having intracellular targets by assessing the synthesis of DNA molecules in S. aureus. Incorporation of the radioactive precursor [methyl-³H]thymidine in DNA synthesis was observed over a time period of 30 min after treatment with 1 × MIC of FL9. DNA synthesis appeared to be inhibited within the first 5 min after addition of FL9 (Fig. 3). Some AMPs cause small membrane lesions, which lead to transient leakage of protons and thereby depletion of intracellular ATP, which would affect the synthesis of cellular molecules (Huang 2006). However, from our ATP leakage experiment, it was clear that the intracellular level of ATP did not decrease considerably until high concentrations of FL9 were employed and increased ATP leakage was observed, showing that the decrease in DNA synthesis was probably not caused by leakage of protons (Fig. 2).

**Fig. 2.** Measurement of ATP leakage from S. aureus 8325-4 after treatment with FL9. Measurement of intracellular (IC) and extracellular (EC) concentrations of ATP after treatment with increasing concentration of FL9 (a) and over time (b) when exposed to 1 × MIC (128 µg ml⁻¹) at time 0. Columns represent the mean of two independent experiments. Bars denote maximum and minimum values.

**Fig. 3.** FL9 inhibits bacterial DNA synthesis. Effect of FL9 at 0 MIC (・) and 1 × MIC, 128 µg ml⁻¹ (●) on DNA synthesis of S. aureus 8325-4 measured by incorporation of the radiolabelled precursor [methyl-³H]thymidine. Results are shown as means ± 1 SD from three independent experiments.
Some AMPs that inhibit DNA synthesis bind non-specifically to DNA. For example, the amphibian AMP, the α-helical AMP buforin II, can penetrate the cell membrane without causing its disruption, and inhibits cellular functions by binding to nucleic acids (Park et al., 1998; Kobayashi et al., 2000; Rotem et al., 2008; Sarig et al., 2011; Gottschalk et al., 2013). Therefore, to investigate whether the inhibition of DNA synthesis could be 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, the plasmid pRMC2 migrated as expected (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 (Fig. 4), 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 analyse 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). When exposed to H2O we found no blue ring of bacteria, confirming that it was FL9 that had induced recA expression (Fig. 5c). This induction of recA was confirmed by measuring beta-galactosidase expressed from the recA::lacZ fusion. The data revealed a growth-phase-dependent induction (Fig. 5d). Most likely, the greater induction observed in agar plates, compared with the activity recorded in the liquid β-galactosidase assay, was caused by cellular accumulation of the enzyme over time in the agar plate system. The induction of the SOS response supports our hypothesis that FL9 might traverse the membrane and bind DNA, as DNA interference can

![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. Lanes: 1 and 8, negative control containing DNA and binding buffer; 2–7, increasing amounts of FL9; 9–14, increasing amounts of plectasin (negative control for peptide–DNA binding).](http://jmm.microbiologyresearch.org)

![Fig. 5. FL9 induces recA expression in S. aureus 8325-4. (a–c) FL9 (a) or ciprofloxacin (b; positive control) was added to wells in TSA plates containing the 8325-4-derived lacZ reporter strain HI2682 (recA::lacZ). H₂O was used as negative control (c). The incubation time was 18 h. Induction of expression was monitored as blue colonies. (d) β-Galactosidase expression from the recA::lacZ strain was measured with 0.25 × MIC of FL9 (32 µg ml⁻¹) (■) and without FL9 (▲). The reported results are one representative of two independent experiments, showing similar fold changes of regulation.](http://jmm.microbiologyresearch.org)
induce the SOS response. Induction of the SOS response is known to lead to growth arrest (Smith & Walker, 1998), and our results correspond with the growth arrest observed in Fig. 1. The ability to induce the SOS response has been shown previously for the hexapeptide WRWYCR, which exerts its broad bactericidal activity through stalling of bacterial replication forks (Su et al., 2010).

**Impact of environmental conditions on FL9 activity**

The potential use of AMPs as systemic drugs or in food production is hampered by the reduced activity often seen when the peptides are exposed to environmental stresses such 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). Alkaline pH seemed to improve the activity of FL9 fourfold, whereas FL9 had no activity at a pH below 7. High salt concentrations (up to 1030 mM) had little effect on FL9 activity, slightly reducing the MIC twofold at 1030 mM NaCl. Moreover, increasing pH to 8.5. Improved activity at a basic pH was also observed for AMP mimics belonging to the oligo-acyl-lysyls (OAK) C12K7-C8 and the OAK C16(α7)K-β12 with membrane and DNA-binding activities, respectively (Peschel et al., 1999; 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 (Yaron et al., 2003; Rydlo et al., 2006; Goldfeder et al., 2010; Sarig et al., 2011). Environmental conditions can hamper the effect of AMPs; however, FL9 maintained its 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.

**Impact of FL9 on the tolerance mechanisms of *S. aureus***

*S. aureus* possesses several tolerance mechanisms for protection against AMPs including proteolysis, capture of 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 whether some of the known *S. aureus* tolerance mechanisms protect against the activity of FL9. A large number of *S. aureus* strains are able to secrete the protein SAK, which captures AMPs and prevents them from reaching the cell membrane (Bokarewa et al., 2006). To investigate whether SAK can affect the MIC of FL9, we exposed *S. aureus* 8325-4 (SAK negative) to both FL9 and FL9 + SAK. We found the MIC values to be 128 μg ml⁻¹ when the bacteria were exposed to both FL9 alone and FL9 and SAK in combination. This result indicated that this secreted protein is not able to protect *S. aureus* from FL9. When we investigated whether FL9 was prone to cleavage by proteases, we found that FL9 was completely digested *in vitro* when aureolysin was added to the peptide (data not shown). Aureolysin cleavage and inactivation has also been shown previously for the cathelicidin LL-37 (Sieprawska-Lupa et al., 2004). However, when we examined an aureolysin deletion mutant (AK1) exposed to FL9, we observed that the MIC of the mutant was equal to that of the WT, suggesting that *S. aureus* 8325-4 does not secrete aureolysin at levels sufficient to protect the bacteria from FL9. This discrepancy may be explained by a stringent cell-density-dependent control of expression of aureolysin. Previous results have shown that most WT *S. aureus* strains produce very weak aureolysin activity and that the level of secreted aureolysin is growth phase dependent (Dubin, 2002; Sieprawska-Lupa et al., 2004).

An alternative mechanism is the presence of a large number of proteins that establish a positive membrane charge to protect against AMPs; however, FL9 maintained its 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.

### Table 2. Effects of incubation conditions on the MIC of FL9 against *S. aureus* 8325-4

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>MIC (μg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl concentration (mM)</td>
<td>128</td>
</tr>
<tr>
<td>85</td>
<td>128</td>
</tr>
<tr>
<td>170</td>
<td>128</td>
</tr>
<tr>
<td>260</td>
<td>128</td>
</tr>
<tr>
<td>515</td>
<td>128</td>
</tr>
<tr>
<td>1030</td>
<td>64</td>
</tr>
<tr>
<td>pH</td>
<td>128</td>
</tr>
<tr>
<td>3.5</td>
<td>128</td>
</tr>
<tr>
<td>5.5</td>
<td>128</td>
</tr>
<tr>
<td>7</td>
<td>128</td>
</tr>
<tr>
<td>8.5</td>
<td>32</td>
</tr>
<tr>
<td>MgCl₂ concentration (mM)</td>
<td>128</td>
</tr>
<tr>
<td>1</td>
<td>128</td>
</tr>
<tr>
<td>3</td>
<td>128</td>
</tr>
<tr>
<td>5</td>
<td>128</td>
</tr>
</tbody>
</table>

Italic indicates standard conditions.
of AMPs. Moreover, the VraFG ABC transporter has also been shown to play a role in tolerance towards AMPs. This system is important for *S. aureus* in sensing the presence of AMPs and signalling this to the GraRS two-component system, leading to increased expression of the dlt operon and mprF (Li et al., 2007; Falord et al., 2011). 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 these genes influenced tolerance of *S. aureus* towards FL9. We observed an eightfold decrease in the MIC values for the ΔdltA and ΔmprF strains and a fourfold decrease in the susceptibility of the ΔvraF strain compared with WT (data not shown). These results showed 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 rnaIII**

It has been shown previously that the expression of virulence genes in *S. aureus* is affected by subinhibitory 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 AMPs is important information that to date has not been thoroughly investigated when evaluating the potential of peptides as drugs used in the inhibition 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*. The α-haemolysin hla is one of the most prominent *S. aureus* cytotoxins and RNAIII is the key effector of the agr system, controlling the switch between the expression of surface proteins and excreted toxins. From the agar diffusion assay, it was seen that FL9 upregulated the expression of both virulence genes (Fig. 6a–c). Induction of rnaIII by FL9 in 8325-4 and USA300 was confirmed by Northern blot analysis (Fig. 6d, e). Thus, failure in the treatment of *S. aureus* infections, caused by subinhibitory exposure to FL9, can affect the virulence potential of *S. aureus*.

The present study set out to investigate the mode of action of the fallaxin analogue 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 as preservatives in the food industry, it is important to understand their impact on the bacteria and to know how the bacteria will react, in order to limit the risk of resistance and to maintain activity under the conditions in which they will be used.

In conclusion, this study showed that an analogue of the amphibian AMP fallaxin, FL9, has a dual mode of action: it has membrane-disturbing effects and can also affect intracellular processes and inhibit DNA synthesis, inducing the SOS response. However, FL9 is sensitive to most tolerance mechanisms of *S. aureus* and might cause undesirable effects on virulence potential if administered at too low a concentration. These results showed that FL9 in its current form is useful neither for the treatment of *S. aureus* infections nor for food preservation. However, our results also revealed that FL9 maintained its activity under a wide range of conditions, showing the potential of using a modified structure of FL9, in particular as a food additive. Importantly, obtaining knowledge on how AMPs function can be used for future improvement of synthetic AMPs.

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