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Induced resistance to the antimicrobial peptide lactoferricin B in *Staphylococcus aureus*

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Abstract **This study was designed to investigate inducible intrinsic resistance against lactoferricin B in *Staphylococcus aureus*. Serial passage of seven *S. aureus* strains in medium with increasing concentrations of peptide resulted in an induced resistance at various levels in all strains. The induced resistance was unstable and decreased relatively rapidly during passages in peptide free medium but the minimum inhibitory concentration remained elevated after thirty passages. Cross-resistance to penicillin G and low-level cross-resistance to the antimicrobial peptides indolicidin and Ala^{3,13,18}-magainin was observed. No cross-resistance was observed to the human cathelicidin LL-37. In conclusion, this study shows that *S. aureus* has intrinsic resistance mechanisms against antimicrobial peptides that can be induced upon exposure, and that this may confer low-level cross-resistance to other antimicrobial peptides.**

Keywords: Lactoferricin; *Staphylococcus aureus*; Resistance; Antimicrobial peptide

1. Introduction

The development of resistance to multiple antimicrobial agents in several important bacterial pathogens has lead to the search for new antimicrobial agents with novel bacterial targets. Cationic antimicrobial peptides have been introduced as a new source of such potential antimicrobial agents [1,2]. Antimicrobial peptides are part of the innate immune system in multicellular organisms, including humans, through the direct killing of microbes and/or immunomodulatory functions such as upregulation and downregulation of genes, enhanced antibody response, chemotactic activity and modulation of inflammation [3,4].

The antibacterial mode of action of antimicrobial peptides has been extensively studied and shows a wide range of effects from interaction with the membrane to specific effects on intracellular targets (see reviews [5–7]). Currently, different antimicrobial peptides are in late clinical trials as treatment of various infections [8,9].

In *Staphylococcus aureus* several different resistance mechanisms to antimicrobial peptides have been described: (I) modification or blocking of teichoic acid [10,11], (II) alterations in the cytoplasmic membrane [12,13], (III) involvement of proteases [14,15], (IV) defective electron transport chain [16–18] and (V) involvement of bacterial stress response system [19]. Development of resistance by the use of antimicrobial peptides as antimicrobial agents is largely unknown. A worst case scenario is development of cross-resistance to human antimicrobial peptides [20]. It is more difficult to develop resistance to antimicrobial peptides in vitro than to for instance norfloxacin or gentamicin [21,22] and no experimental evidence for cross-resistance has so far been reported.

This study was aimed to test the hypothesis that *S. aureus* possess intrinsic resistance mechanisms that are inducible under exposure to antimicrobial peptides. Lfcin B consists of amino acid 17–41 from the N-terminal of bovine lactoferrin, and is generated by pepsin cleavage [23]. It has activity against a wide range of microorganisms, including viruses, protozoa, fungi, Gram-negative and Gram-positive bacteria [23–28]. Lfcin B is in preclinical trials for antifungal infections [9] and Lfcin B and other mammalian lactoferricin peptides have been used as templates for the development of more active antimicrobial peptides [29].

2. Materials and methods

2.1. Peptides and antibiotics

Lactoferricin B 17-41 (Lfcin B) was prepared by pepsin digestion of bovine lactoferrin at the Centre for Food Technology. (Queensland, Australia). Indolicidin and LL-37 were synthesised at the University of Tromsø, Department of Biochemistry as previously described [30]. Ala^{3,13,18}-magainin, chloramphenicol, dicloxacillin sodium monohydrate, erythromycin, fusidic acid, rifampicin and clindamycin hydrochloride were purchased from Sigma–Aldrich Norway (Oslo, Norway). Penicillin G was obtained from Panpharma SA (France) and gentamicin from Invitrogen Ltd (Paisley UK). Bacitracin and bacitracin-Zn was obtained from Alpharma (Oslo, Norway).

2.2. Bacterial strains

S. aureus ATCC 25923 was used as a reference strain. Six clinical *S. aureus* strains were obtained from patient samples of pus sent to the Department of Medical Microbiology, University Hospital of North Norway, as previously described [14]. 2% Bacto peptone water (BPW) (Difco, Detroit, USA) was used as growth medium for all strains. For LL-37 anion-exchanged MH-broth was used for the MIC testing.

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2.3. Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined using a standard broth microdilution technique as previously described [10]. The MIC was determined as the lowest concentration where no visible growth occurred. For determination of the MIC in the presence of protease inhibitors, the protease inhibitor cocktail set II (Calbiochem CA, USA), was added to the assay according to Ulvatne et al. [14]. Resistant bacteria were taken directly from the frozen culture to minimise a possible loss of induced resistance by incubation without the presence of Lfcin B. To exclude differences in the MIC due to testing directly from frozen culture the wild-type ATCC 25923 strain was tested directly from frozen culture and from overnight culture. No difference in MIC was observed between the two testing methods. All assays were performed in duplicate and on at least two separate occasions. The MIC assay was performed with more titration steps than just double dilutions to more accurately determine the MIC. The MIC values are presented as the median value. Induced resistance and cross-resistance was defined as bacteria with MIC values at least two or more titration steps higher than the MIC of the wild-type bacteria.

2.4. Serial passage

Serial passage was initiated from overnight cultures grown to exponential growth phase and added to medium containing Lfcin B (final bacterial concentration of approximately 1.0×10^6 CFU/ml). The bacteria were incubated overnight at 37 °C and an aliquot was transferred to a new tube with a higher concentration of Lfcin B. Concentrations used were 10, 16, 20, 25, 30, 60, 80, 100, and 200 μg/ml in the primary induction. The four resistant strains with the highest MIC after the primary induction were further passed in medium with increasing concentrations of Lfcin B (500 and 1000 μg/ml) (strains designated -h). To investigate if the resistance could be rapidly induced, the ATCC 25923 strain was passed through four passages with Lfcin B (0–100–200–500–1000 μg/ml). The remaining culture at each step was harvested by centrifugation at $3000 \times g$. The pellet was resuspended in brain heart infusion broth (Oxoid, Hampshire, England) supplemented with either glycerol (Merck, Darmstadt, Germany) or dimethyl sulphoxide (Sigma–Aldrich) and frozen immediately at −70 °C. The MIC was determined at each step as described above.

Based upon the results during the primary induction of resistance, five induced strains were selected, and the stability of the induced resistance was determined by serial passages in medium without Lfcin B.

2.5. Measurement of protease activity

Extracellular protease activity of the bacteria was determined as previously described [31,32].

2.6. Growth characteristics on solid agar

Both the wild-type and induced bacteria were spread on human blood agar plates (Oxoid) and PDM Antibiotic sensitivity medium II (AB Biodisk, Sweden) and incubated at 37 °C overnight. Colony morphology, pigmentation and hemolysis were assessed.

2.7. Electron microscopy and immunogold labelling

Electron microscopy and immunogold labelling was performed on the ATCC 25923 wild-type strain, ATCC 25923-i, ATCC 25923-h, the wild-type clinical strain 39909 and 39909-i as described previously [33]. The bacteria were exposed to 20 μg/ml Lfcin B for 30 min at 37 °C.

3. Results

3.1. Induction of resistance

All the *S. aureus* strains had an MIC between 20 and 30 μg/ml before the induction (Fig. 1 and Table 1). The primary induction resulted in induced resistance at various levels in all strains tested. (Fig. 1 and Table 1, strains designated -i).

Four resistant strains were further passed in medium with increasing concentrations of Lfcin B up to 1000 μg/ml (strains designated -h, Table 1). In ATCC 25923-h and 63978-h, the MIC increased further from 100 to 600 μg/ml and from 95 to 200 μg/ml, respectively. In strains 39909-h and 63643-h the MIC decreased during further passages from 290 to 210 μg/ml and from 180 to 120 μg/ml, respectively.

To investigate if resistance could be rapidly induced, the ATCC 25923 strain was exposed to four passages in medium with Lfcin B (Table 1; strain designated ATCC 25923-r). Under this induction the MIC increased from 30 to 1000 μg/ml.

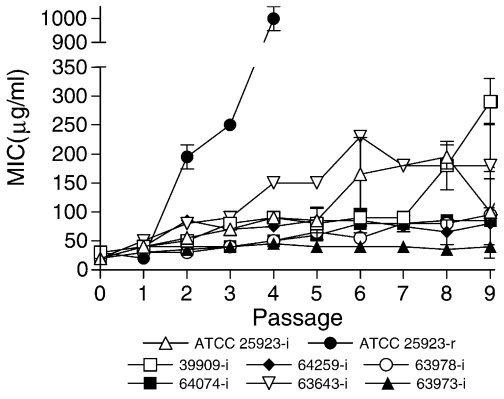

Fig. 1. Induction of resistance to Lfcin B. The bars indicate ±S.D.

Table 1
MIC to Lfcin B, indolicidin (Indo), ala[3,13,18]-magainin (Ala-M), LL-37 and penicillin G (PenG) before and after induction of resistance

Strain	Antimicrobial agent, MIC (μg/ml)				
	Lfcin B	Indo	Ala-M	LL-37	PenG
ATCC 25923	20	14	10	20	<0.06
ATCC 25923-i	**100**	20	17.5	18	<0.06
ATCC 25923-h	**600**	*	*	*	*
ATCC 25923-r	**1000**	*	*	*	*
63978	20	12	10	20	16
63978-i	**95**	20	15	16	**64**
63978-h	**200**	*	*	*	*
64074	20	12	7.5	*	<0.06
64074-i	**85**	**24**	**15**	*	<0.06
39909	30	12	7.5	14	24
39909-i	**290**	**28**	**15**	16	**112**
39909-h	**210**	*	*	*	*
64259	30	14	7.5	*	<0.06
64259-i	**80**	24	**15**	*	<0.06
63973	20	14	9	*	0.375
63973-i	**40**	20	15	*	**8**
63643	20	12	7.5	20	<0.06
63643-i	**180**	**24**	**15**	16	0.125
63643-h	**120**	*	*	*	*

The MIC values are presented as median values. MIC values that are two or more times higher and at least two or more titration steps higher than the wild-type bacteria are indicated in bold.
*, Not tested; -i, induced bacteria after the primary induction of 9 passages; -h, -i strains further induced in medium with Lfcin B up to 1000 μg/ml; -r, rapidly induced bacteria with four concentrations of Lfcin B.

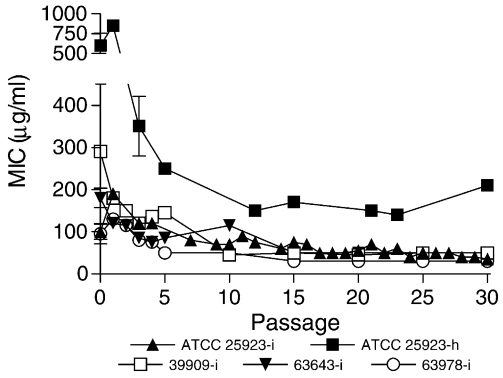

Fig. 2. Stability of induced resistance to Lfcin B in four resistant strains after the primary induction and the ATCC 25923-h strain. The bars indicate ±S.D.

3.2. Stability of the induced resistance

The stability of the induced resistance was tested by serial passage of four resistant strains and the ATCC 25923-h strain in medium without Lfcin B for thirty passages (Fig. 2). After 5 passages, the MIC of the resistant clinical strains and ATCC 25923-h decreased by 2–3 fold. In the ATCC 25923-i strain the MIC was unchanged after 5 passages. With further passages the MIC decreased in all strains. In the ATCC 25923-h strain the MIC stabilised at a high level, 4–10 fold higher than the wild-type, while in the other strains the MIC stabilised at a lower level, but still elevated compared to wild-type bacteria.

3.3. Growth characteristics on solid agar and involvement of proteases

We have previously described that proteases are involved in the susceptibility to Lfcin B in both *Escherichia coli* and *S. aureus* [14]. To investigate if the induced resistance could be due to an upregulation of a protease, the MIC was determined in the presence of protease inhibitors in four i-strains and the ATCC 25923-h strain. The MIC was reduced in all strains when the protease inhibitors were present, but there were no significant differences between wild-type and resistant bacteria (data not shown). Protease activity in the bacterial supernatant was also measured before and after induction. The extracellular protease activity was reduced in all but one i-strain; however the difference in activity was not significant between the wild-type and resistant bacteria (data not shown). Both the wild-type

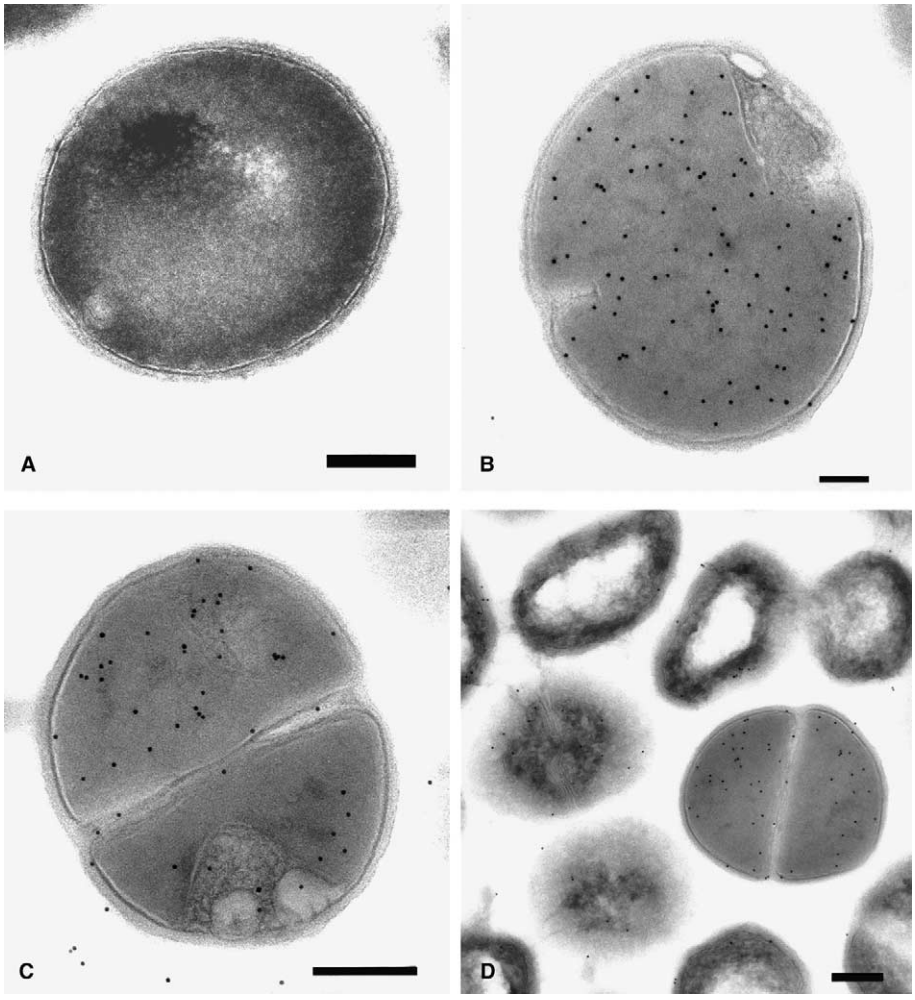

Fig. 3. Electron micrographs of the wild-type and the resistant bacteria. (A) *S. aureus* ATCC 25923 not exposed to Lfcin B. Bar: 200 nm. (B) ATCC 25923 exposed to 20 µg/ml Lfcin B for 30 min. Bar: 100 nm. (C) ATCC 25923-h exposed to 20 µg/ml Lfcin B for 30 min. Bar: 200 nm. (D) Representative electron micrograph of ATCC 25923-h bacterial cells exposed to 20 µg/ml Lfcin B for 30 min. Bar: 200 nm.

and resistant bacteria showed the same characteristics when grown on different solid agar. No difference was observed with regard to colony morphology, pigmentation or hemolysis (data not shown).

3.4. Electron microscopy and immunogold labelling

Electron microscopy and immunogold labelling were performed with the ATCC 25923 wild-type strain, ATCC 25923-i, ATCC 25923-h, the wild-type clinical strain 39909 and 39909-i, to study morphological changes and to determine the location of Lfcin B. The bacteria were exposed to 20 μg/ml Lfcin B for 30 min. Lfcin B crossed the cytoplasmic membrane and entered the cytoplasm of both the wild-type and resistant bacteria (Fig. 3). Morphological changes like formation of mesosomes, separation of the membrane from the cell wall and condensation of DNA was observed in both the wild-type and resistant bacteria exposed to Lfcin B.

3.5. Cross-resistance

Resistant i-strains were tested for cross-resistance to three other antimicrobial peptides and several antimicrobial agents (see Table 1). Low-level cross-resistance to indolicidin and $Ala^{3,13,18}$-magainin was observed in three and four i-strains, respectively. Also, the MIC to these two antimicrobial peptides was elevated in all i-strains. No cross-resistance was observed to the human cathelicidin LL-37. Cross-resistance to penicillin G was observed in three strains. Two of these strains (63978-i and 39909-i) were resistant to penicillin G before the induction, but a further increase in MIC was observed after the induction. Wild type strain 63973 was sensitive to penicillin G while 63973-i became resistant after the induction. No cross-resistance was observed with the other antimicrobial agents tested.

4. Discussion

Since the discovery of antimicrobial peptides there has been an increasing interest in the potential of these peptides as antimicrobial agents for therapeutic use. Along with this interest, the concern about the consequences of possible development of resistance to antimicrobial peptides has emerged [20]. The results presented here indicate that resistance to Lfcin B is possible to induce in *S. aureus*, and this resistance also affects the susceptibility to other antimicrobial peptides and agents.

The resistance observed is rapidly inducible, strain dependent and unstable over time. Induction of resistance may be a result of several metabolic and/or genetic changes in the bacterial population. These changes may involve several resistance mechanisms. The mode of action of Lfcin B suggests a multi-hit mode of action involving both membrane and intracellular targets [10,33–36]. Modification of the initial binding sites of the bacteria [10,11] and/or alterations in the cytoplasmic membrane [12,13] would prevent the peptide from reaching its target. However, the electron microscopy shows that Lfcin B is located in the cytoplasm of both wild-type and resistant strains. Hence, no significant exclusion of peptide may explain the induced resistance observed here.

No difference in the susceptibility to Lfcin B in the presence of protease inhibitors was observed between the wild-type and induced strains and no increase in extracellular proteolytic activity was observed in the same strains. The electron microscopy images also indicate that no increased proteolytic degradation is taking place in the resistant strains. The induction of resistance in *S. aureus* is most likely not due to the activity of proteolytic degradation alone.

It could be speculated that since Lfcin B has a multi-hit mode of action the development of resistance could occur in several steps related to the different actions of Lfcin B on the bacteria. Bacteria exposed to a rapid induction resulted in a high MIC equal the concentration used during the induction (strain ATCC 25923-r) while further induction of the -i strains resulted in lower MIC values than the final concentration used in the induction (-h strains). There might be one rapid and one adaptive resistance mechanism. The rapid resistance mechanism might be through activation of a stress response in the bacteria. The involvement of a stress response protein in the resistance to Lfcin B and cathepsin G has previously been shown [14,19], arguing for an activation of the bacterial stress system as a possible resistance mechanism. Also, suggesting different resistance mechanisms is the observation that there are differences in the stability of the induced resistance. The induced resistance in the ATCC 25923-h strain remained stable at a high level while the induced resistance arising from the primary induction almost normalised without selective pressure.

A defective electron transport chain, a characteristic of *S. aureus* small colony variants (SCV), has been described previously as a mechanism of resistance to antimicrobial peptides including Lfcin B [16–18]. It could be speculated that Lfcin B during the passages induces an unstable SCV phenotype which reverts without the selective pressure of the peptide. However, no changes in colony morphology, hemolysis or pigment was observed between the induced strains and the wild-type bacteria when grown on different agar suggesting that no major metabolic change, characteristic for SCVs, has occurred.

Low-level cross-resistance to the antimicrobial peptides indolicidin and $Ala^{3,10,18}$-magainin, and cross-resistance to penicillin G indicates that the resistance mechanism in *S. aureus* to Lfcin B affects the susceptibility both to other antimicrobial peptides and to conventional antimicrobial agents. The cross-resistance to penicillin G was only observed in 3 out of the 7 strains tested (Table 1), suggesting that the effect could be strain dependent. Lfcin B inhibits autolysin (Atl) induced lysis [16] which is required for the lytic activity of penicillin G [37]. It could be speculated that Lfcin B induces changes in the Atl function or regulation of the *atl* gene resulting in cross-resistance to penicillin G. Autolysis is also inhibited by heat shock [38], and previous observations have shown that the heat shock induced protein DegP/HtrA is involved in the susceptibility to Lfcin B in *E. coli* [14]. Heat shock proteins could therefore be involved in the resistance to Lfcin B and penicillin G. Bovine lactoferrin, from which Lfcin B is derived, inhibits β-lactamase production [39] and Lfcin B might also possess this property.

The mechanism(s) for the low-level cross-resistance to the antimicrobial peptides indolicidin and the magainin-2 analogue also remains unclear. Due to the low-level of cross-resistance, the mechanism(s) for this might involve the cytoplasmic membrane, which is a likely common target for the antimicrobial peptides. However, both indolicidin and Lfcin B inhibits macromolecular synthesis [36,40], and a common intracellular resistance mechanism could also be possible.

In conclusion, this study shows that resistance to the antimicrobial peptide Lfcin B can be readily induced in *S. aureus,* and

that the resistance mechanism(s) involved also results in low-level cross-resistance to two other antimicrobial peptides and cross-resistance to penicillin G. The mechanism(s) for the induced resistance is currently not elucidated, but the results imply that the resistance mechanisms may be as diverse as the mode of action. Further experiments are ongoing to investigate the resistance mechanism, and these studies may identify possible targets for new antimicrobial agents.

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