Inhibition of virulence factor expression in *Staphylococcus aureus* by the *Staphylococcus epidermidis* agr pheromone and derivatives

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Abstract The agr quorum-sensing system in *Staphylococci* controls the production of surface proteins and exoproteins. In the pathogenic species *Staphylococcus aureus*, these proteins include many virulence factors. The extracellular signal of the quorum-sensing system is a thiolactone-containing peptide pheromone, whose sequence varies among the different staphylococcal strains. We demonstrate that a synthetic *Staphylococcus epidermidis* pheromone is a competent inhibitor of the *Staphylococcus aureus* agr system. Derivatives of the pheromone, in which the N-terminus or the cyclic bond structure was changed, were synthesized and their biological activity was determined. The presence of a correct N-terminus and a thiolactone were absolute prerequisites for an agr-activating effect in *S. epidermidis*, whereas inhibition of the *S. aureus* agr system was less dependent on the original structure. Our results show that effective quorum-sensing blockers that suppress the expression of virulence factors in *S. aureus* can be designed based on the *S. epidermidis* pheromone.

Key words: Agr; Quorum-sensing; Pheromone; *Staphylococcus aureus*; *Staphylococcus epidermidis*

1. Introduction

Bacteria have the ability to signal and sense the state of population density in order to respond to changing physiological needs under different growth conditions. This phenomenon is commonly called quorum-sensing [1]. Recently, the systems involved in quorum-sensing of Gram-negative and Gram-positive bacteria have been proposed as promising targets for anti-microbial therapy. In pathogenic bacteria, many of the extracellular virulence factors are regulated by such systems. Unlike conventional antibiotics, drugs interfering with quorum-sensing systems would have the advantage of specifically suppressing the expression of virulence factors without killing the bacteria, thus minimizing the selection for resistant strains [2].

Staphylococcus aureus is a pathogen which can cause serious, severe infections such as endocarditis, septicemia, pneumonia and toxic shock syndrome [3]. Furthermore, it is the primary cause of nosocomial infections. There is an urgent need for new effective antibiotics against *S. aureus* since most clinical strains are now multi-resistant. Appearance of intermediate vancomycin-resistance in *S. aureus* has already been observed in the USA and in Japan [4,5] and there are no further antibiotics for the treatment of multi-resistant *Staphylococci*.

Many virulence factors of *S. aureus*, including α-toxin, β-toxin, δ-toxin, serine protease, DNase, fibrinolysin, enterotoxin B and toxic shock syndrome toxin-1, are controlled by the agr system [6]. The *agr* locus of *S. aureus*, about 3.5 kb in size, comprises the *agrA*, *agrC*, *agrD* and *agrB* genes, which are co-transcribed (forming the mRNA RNAII), and the gene for a regulatory RNA molecule, RNAIII. The RNAIII DNA region also encodes the gene for the δ-toxin (*hld*). RNAIII controls the expression of target genes by an unknown mechanism. The *agr* genes are transcribed from the P2 promoter and the RNAIII molecule is synthesized from the P3 promoter [7].

The roles of AgrB and AgrD have recently become more clear. A small peptide is excized from the AgrD protein, modified and secreted as the agr pheromone peptide into the surrounding medium. This peptide represents the autoinductive signal of the agr system. The pheromone activates the AgrC/AgrA two-component regulatory system that in turn activates transcription of the *agrBDCA* and RNAIII genes [8,9]. The staphylococcal agr pheromone peptides contain an intramolecular thiolester between a conserved central cysteine and the C-terminal carboxy group. We have demonstrated this for the pheromone peptide of *Staphylococcus epidermidis* [10]. AgrB seems to be involved in the maturation and secretion process of the modified peptide.

Here, we report on the biological effects of the *S. epidermidis* agr pheromone. Derivatives of the pheromone were synthesized to evaluate the influence of changes at the N-terminus and of the bond type in the cyclic structure. We show that the *S. epidermidis* pheromone is a potent inhibitor of the *S. aureus* agr system and of the agr-controlled expression of virulence factors in *S. aureus*. By derivatization of the pheromone, we were able to construct a compound that inhibits the *S. aureus* agr system as well as the natural pheromone, but does not activate the *S. epidermidis* agr system.

2. Materials and methods

2.1. Bacterial strains

S. epidermidis Tü3298 (DSM 3095 [11]) was the wild-type test strain and the host for the promoter test plasmid pRB594P3. *S. aureus* strains tested for δ-toxin production were *S. aureus* Newman (NCTC 8178), 8325-4 (derived from NCTC 8325, [12]), SA113 (ATCC 35556: derived from NCTC 8325), ATCC 12600, RN4220 (derived from 8325-4, [13]), ATCC 33591 and three further strains from our collection (60/055, 6538, 502A). *S. aureus* RN6390 [14] is a prototypic strain from which the following mutant strains are derived: *S. aureus* RN6911 ('agr$^-$') is an isogenic mutant carrying an agr::getM mutation, 'sar$^-$' (strain ACL 136) carries a sar::Tn917LTV1

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Abbreviations: TSB, tryptic soy broth; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; Fmoc, fluorenylmethoxycarbonyl; Mmt, *p*-methoxytrityl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl

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mutation and 'agr$^-$/sar$^-$' (strain ACL 135) carries both a sar:: Tn917LTV1 and an agr::tetM mutation [15].

2.2. Plasmids

The promoter test plasmid pRB594 is a derivative of pRB373 [16]. It contains the promoterless pUB112 *cat* gene [17] adjacent to a multiple cloning site and carries the erythromycin-resistance gene *ermB* from transposon Tn551. Plasmid pRB594P3 was constructed by insertion of a *Bam*HI-digested PCR product of the *agr* P3 region of *S. epidermidis* ATCC 14990 into the *Bam*HI site of the multiple cloning site [10].

2.3. General methods

S. epidermidis cells were grown in TSB or BM ('basic medium': 1% tryptone (Difco), 0.5% yeast extract (Gibco BRL), 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose). Staphylococcal cells were disrupted in 20 mM Tris-HCl (pH 7.8) by glass beads as described [18]. Cell debris were removed by centrifugation (10 min, $5000\times g$). Membrane fractions were prepared by additional ultracentrifugation of the crude cell extract at $105\,000\times g$ for 1 h. Surface-associated proteins were isolated by boiling cells at 100°C for 5 min and centrifugation (10 min, $5000\times g$). Surface proteins were isolated by incubating cells with lysostaphin for 10 min at 37°C and centrifugation (10 min, $5000\times g$). Chromosomal staphylococcal DNA was prepared according to the method of Mamur [19].

Proteins were separated by tricine-SDS-PAGE according to Schägger and von Jagow [20] using Bio-Rad Protean IIxi chambers and a separation length of 16 cm.

2.4. DNA sequence analysis

DNA was sequenced by cycle sequencing on a DNA sequencer 4000 L (LI-COR, Lincoln, NE, USA) using the Thermo Sequenase fluorescent-labelled prime cycle sequencing kit (Amersham, Little Chalfont, UK). All fluorescent-labelled primers were purchased from MWG-Biotech (Ebersberg, Germany).

2.5. Electrospray mass spectrometry (ESI-MS)

ESI-MS was performed on an API III TAGA Triple Quadrupole (Perkin Elmer Sciex, Thornhill, Ont., Canada). Samples were dissolved in acetonitrile/water (1:1, v/v) and introduced into the ion source at a constant flow rate of 70 μl/min. The orifice voltage was set at 80 V.

2.6. Preparative and analytical high performance liquid chromatography (HPLC)

Crude peptides were isolated on a Waters 600 Multi Solvent Delivery System equipped with a Lambda Max Model 481 as detector. A semi-preparative column (Nucleosil C18, 4×250 mm; 5 μm; Grom, Herrenberg, Germany) was eluted at a flow rate of 3.5 ml/min with a linear gradient (10–100% B in A in 45 min; solvent A: 0.1% trifluoroacetic acid (TFA) in water; solvent B: 0.1% TFA in acetonitrile). The detection wavelength was 214 nm.

The concentration of purified peptides, redissolved in dimethylsulfoxide (DMSO), was determined using analytical HPLC on a Kontron HPLC system with Kroma System 2000 software. An analytical column (Spherisorb ODS2 2×100 mm; 5 μm; Grom, Herrenberg, Germany) was eluted at a flow rate of 250 μl/min with a linear gradient (0–100% B in A in 30 min; solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in acetonitrile). The detection wavelength was 214 nm. A known amount of the (unmodified) peptide DSVCASYF was used as a reference.

The amount of δ-toxin was quantified using the same system. A Pharmacia Resource PHE 1 ml column was eluted with 1.5 column volumes of a linear gradient (0–100% of B in A; A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile). The *S. epidermidis* δ-toxin was eluted using the same conditions on an ÄKTA explorer 100 system (Amersham Pharmacia Biotech, Freiburg, Germany). The isolated δ-toxin was chemically analyzed by ESI-MS.

2.7. Synthesis of peptides

Peptides were synthesized using the Fmoc/tBu strategy on Trityl-resin (PepChem: Clausen and Goldammer, Tübingen, Germany). The sequence of the peptide was DSVXASYF, with cysteine (C), serine (S) or 1,3-diaminopropionic acid (Dpr) in the X position. The corresponding protected amino acids for the synthesis of cyclic peptides were Fmoc-Cys(Mmt)-OH, Fmoc-Ser(Trt)-OH (both cleavable with TFA:TIS in dichloromethane [21]) and Fmoc-Dpr(Dde)-OH (cleavable with hydrazine [22]). The cyclic peptides were synthesized and purified according to Otto et al. [10]. The purity of peptides (>90%) was controlled by RP-C18 chromatography and ESI-MS.

2.8. Chloramphenicol acetyltransferase (CAT) assay

CAT activity was determined according to the method of Shaw [23]. The assay mixture contained 100 mM Tris-HCl (pH 7.8), 0.1 mM acetyl-coenzyme A and 0.4 mg 5,5′-dithiobis-2-nitrobenzoic acid (DTNB)/ml. Assays were performed in 96 well microtiter plates using a SpectraMax 340 microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA) with SpectraMaxPro software. Cell extract (5 μl) and 5 mM chloramphenicol in 100% ethanol (5 μl) (or 5 μl 100% ethanol in controls) were added to 90 μl of the assay mixture. Cell extracts were diluted 1:10 or 1:100 with 20 mM Tris-HCl (pH 7.8) when necessary. Absorption at 412 nm was measured every 15 s for 20 min. The linear part of the resulting curve was used to determine the CAT activity (absorption coefficient $\varepsilon = 13\,600$ l/M for DTNB). For calculation of the specific activity, protein contents of the cell extracts were determined using the Bio-Rad DC protein assay for detergent-containing samples (Bio-Rad Laboratories GmbH, Munich, Germany).

2.9. Immunoblots

SDS-polyacrylamide gels were blotted onto nitrocellulose membranes (Schleicher and Schuell BA 83) using the semi-dry blotting technique. Blots were blocked overnight with 5% skim milk. The first antibody was applied for 2 h at a concentration of 1:20 000 (anti-α-toxin) or 1:40 000 (anti-protein A). After washing, the blots were incubated with anti-IgG-coupled HRP from Amersham Pharmacia (1:5000) for 1 h. All dilutions were made in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Signals were detected with the ECL detection system (Amersham Pharmacia Biotech, Freiburg, Germany).

2.10. Test of synergistic hemolysis

Blood plates were prepared with sheep blood agar base (Oxoid) to which 5% defibrinated sheep blood was added. Samples were spotted onto filters, which were dried and then laid on agar plates and incubated at 37°C for at least 24 h.

3. Results

3.1. Synthesis and activity of S. epidermidis pheromone derivatives

We investigated the biological activity of the *S. epidermidis* agr pheromone. Previously, we have demonstrated that it contains an intramolecular thiolester between the central cysteine and the C-terminal carboxy group within the primary sequence DSVCASYF [10]. We have also previously shown that the derivatives a and b, which represent peptides that are shortened or elongated, respectively, by one amino acid of the N-terminal linear peptidyl tail, and which are derived from the sequence of the AgrD prepeptide, do not activate the *S. epidermidis* agr system (for structural representation of the derivatives see Fig. 1), [10].

To investigate the influence of the thiolactone bond on the biological activity of the pheromone peptide, the following derivatives of the peptide were synthesized. The strategy was to substitute the cysteine by serine (Ser) or Dpr. The original pheromone peptide (compound c_S) was stable under acidic conditions [10]. However, the peptide was more sensitive to an elevated pH and the ring opened. The serine lactone peptide (compound c_O) derivative was extremely sensitive to nucleophiles and electrophiles, which caused the ring to open. In contrast, the amide bond of the Dpr derivative (compound c_{NH}) resulted in a significantly stabilized cyclic peptide. Like the N-terminal derivatives, the lactone and lactam derivatives

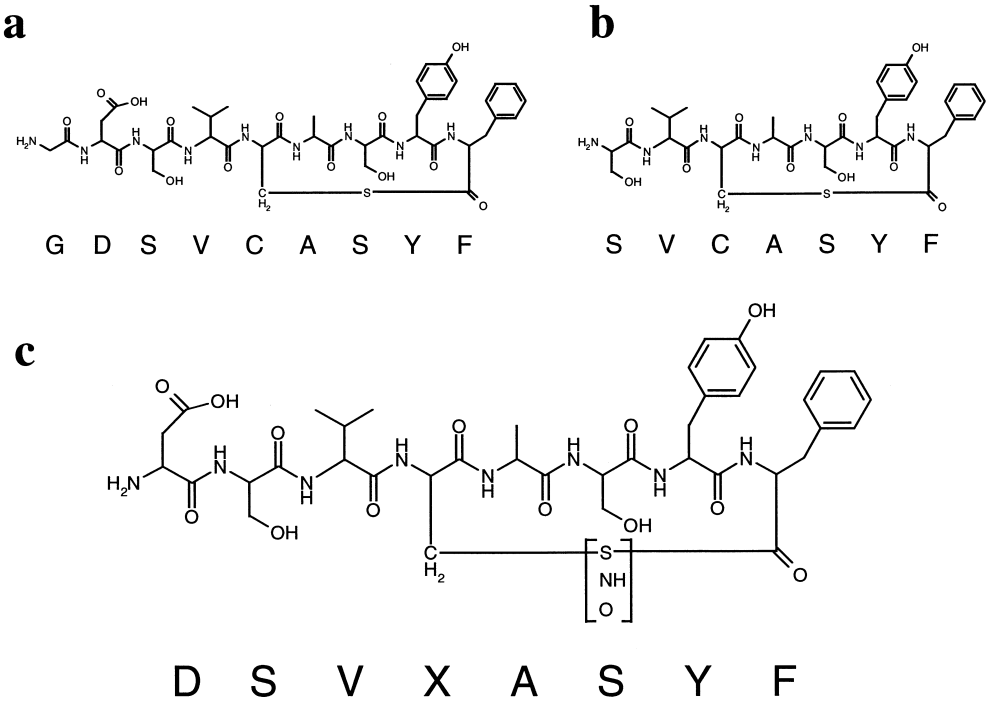

Fig. 1. Structures of the compounds used in this study. (a) Thiolactone-containing *S. epidermidis* pheromone harboring an additional glycine at the N-terminus (compound a); (b) thiolactone-containing *S. epidermidis* pheromone lacking the original aspartate residue at the N-terminus (compound b); (c) *S. epidermidis* pheromone with the original thiolactone structure (-S-, compound c_S), replaced by a lactone (-O-, compound c_O) or a lactam (-NH-, compound c_{NH}).

also were not able to activate the *S. epidermidis* agr system (data not shown), which was demonstrated by their failure to activate CAT expression in our promoter test vector, which contains the *S. epidermidis* agr P3 promoter cloned in front of the *cat* gene [10] and which is activated in response to extracellularly present pheromone via the AgrC/AgrA two-component system.

These results show that the activation of the agr system, which is mediated by the interaction of the agr pheromone and the AgrC sensor kinase is extremely specific and dependent on a correct N-terminus and a thiolactone structure.

3.2. Linear derivatives

Balaban et al. [24] purified an octapeptide from the supernatant of an *S. aureus* mutant strain. The octapeptide contained an unresolved amino acid at a position corresponding to that of cysteine in the agr pheromone peptide. These authors demonstrated that a synthetic peptide containing a tryptophan residue at this position inhibits the agr system of *S. aureus*. We therefore speculated that Balaban et al. had originally purified a mutated pheromone (from a mutant strain) and that the introduced tryptophan side chain could substitute for the thiolactone structure. To determine whether the side chain of another amino acid in the center of the *S. epidermidis* pheromone peptide could simulate a ring structure and lead to restored biological activity, we exchanged the central cysteine of the *S. epidermidis* pheromone peptide for every classic proteinogenic amino acid. We have previously shown that a ring structure is necessary for biological activity [10]. However, only the linear peptide with the central cysteine and without the thiolester bridge has been used as a control. None of the linear peptides synthesized had any effect on the agr system in our tests, neither an activating effect on *S. epidermidis* nor an inhibiting effect on *S. aureus* (data not shown).

3.3. Inhibition of the S. aureus agr system

We tested the *S. epidermidis* pheromone for its activity on the *S. aureus* agr system. The effect on the *S. aureus* agr system was determined by HPLC measurement of the δ-toxin, which is the translation product of a part of RNAIII. A Resource PHE column and a water/acetonitrile gradient allowed many samples to be passed through without the column becoming blocked and offered the advantage that most proteins and peptides passed through the column, whereas the extremely amphiphilic α-helical δ-toxin eluted as a distinct

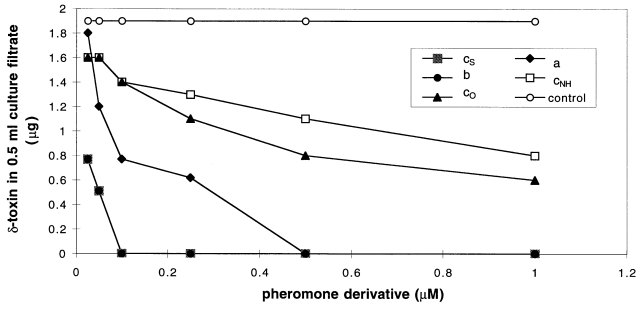

Fig. 2. Concentration-dependent suppression of δ-toxin production in *S. aureus* Newman by the addition of synthetic *S. epidermidis* pheromone or one of its derivatives. Media were inoculated with a 1/100 volume of an overnight preculture of *S. aureus* Newman. At the same time, the *S. epidermidis* pheromone or one of its derivatives was added at the given concentration. The peptides were dissolved in DMSO, the control received only DMSO. After 8 h of growth, the amount of δ-toxin produced was determined by HPLC. The optical density (578 nm) at the harvest time was 2.9 for all cultures. Abbreviations of the compounds refer to Fig. 1.

peak. Using the supernatant of the *S. epidermidis* test strain Tü3298, this peak was identified as the δ-toxin by mass spectrometry and by testing for synergistic hemolysis (data not shown).

The HPLC δ-toxin assay was used to screen first for *S. aureus* strains that produced δ-toxin, which indicates that the agr system is active in these strains. The δ-toxin-negative strain *S. aureus* RN4220 was used as a control. Of eight strains tested, four produced δ-toxin (strains *S. aureus* 60/055, Newman, 8325-4 and ATCC 33591). DNA sequencing of *agrD* showed that the pheromone peptide of each of the positive strains belonged to the *S. aureus* agr pheromone subgroup I (pheromone peptide sequence: YSTCDFIM). For the agr inhibition tests, *S. aureus* strains Newman and 8325-4 were chosen.

Results are shown in Fig. 2 for the inhibition of *S. aureus* Newman. They were identical for *S. aureus* 8325-4. The *S. epidermidis* pheromone was able to completely suppress the production of δ-toxin over an 8 h incubation period already at a concentration of 100 nM. The derivative b was equally active, whereas the derivative a and the lactone and lactam derivatives showed a reduced inhibiting activity. Still all of the tested derivatives were able to inhibit the *S. aureus* agr system, showing that, compared to the activating activity in *S. epidermidis*, the inhibiting activity is less dependent on the original structure of the *S. epidermidis* pheromone.

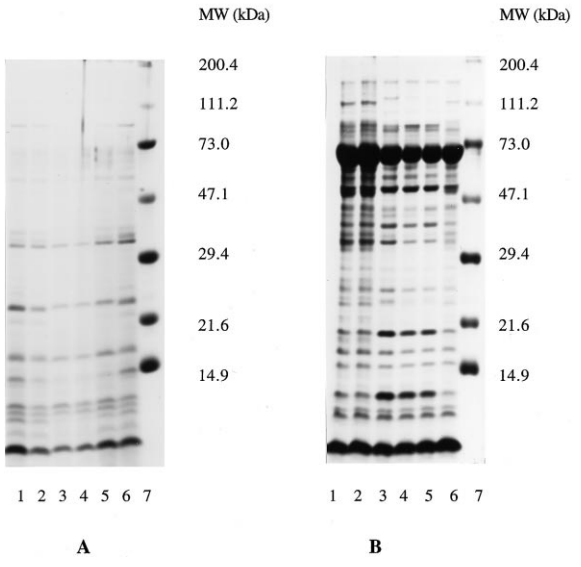

Fig. 3. Influence of the *S. epidermidis* pheromone and its derivatives on the production of exoproteins and surface-associated proteins in *S. aureus* Newman. (A) Exoproteins: basic medium was inoculated with 1/100 volume of a preculture of *S. aureus* Newman. At the same time, the pheromone or one of its derivatives was added at a concentration of 1 μM. The peptides were dissolved in DMSO, a control received only DMSO. Culture filtrates (20 μl) of the cultures harvested after 16 h of growth were applied to tricine SDS-PAGE and stained with Coomassie blue. (B) Surface-associated proteins: pellets of the centrifuged 16 h cultures were dissolved in SDS-PAGE loading buffer containing 1% SDS, boiled at 100°C for 5 min and applied to tricine SDS-PAGE after removal of insoluble material by centrifugation. Lanes in (A) and (B) (abbreviations of compounds refer to Fig. 1): (1) compound c_{NH} (amide derivative); (2) compound c_O (oxygen ester derivative); (3) compound b; (4) compound c_S (synthetic *S. epidermidis* pheromone); (5) compound a; (6) control (with DMSO); (7) molecular weight standards.

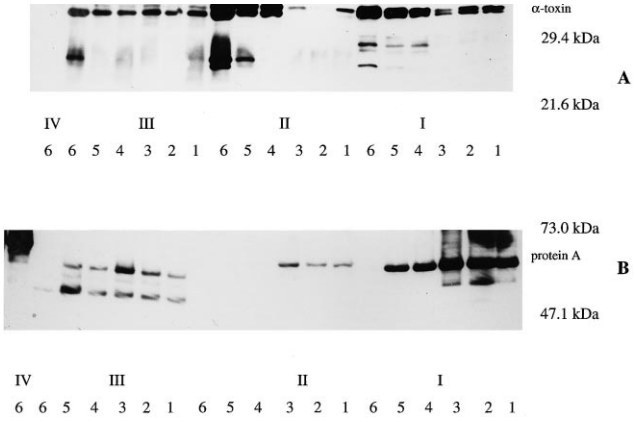

Fig. 4. Influence of the *S. epidermidis* pheromone and its derivatives on α-toxin production and protein A production in *S. aureus* strains. (A) Immunoblot with anti-α-toxin antiserum. Basic medium was inoculated with 1/100 volume of a preculture of the respective *S. aureus* strains. At the same time, the pheromone or one of its derivatives was added at a concentration of 1 μM. The peptides were dissolved in DMSO, a control received only DMSO. The culture filtrate (20 μl) of the cultures harvested after 16 h of growth was applied to tricine SDS-PAGE. Immunoblotting and development are described in Section 2. (B) Immunoblot with anti-protein A antiserum. For protein A detection, the cell pellets of the 16 h cultures (see above) were treated with lysostaphin to release the covalently attached protein A from the cell surface. After centrifugation, the supernatant was applied to tricine SDS-PAGE. Immunoblotting and development are described in Section 2. (A and B) lanes (abbreviations of compounds refer to Fig. 1): I: *S. aureus* Newman; II: *S. aureus* RN6390; III: *S. aureus* sar$^-$; IV: *S. aureus* agr$^-$; (1) compound a; (2) compound c_S (synthetic *S. epidermidis* pheromone); (3) compound b; (4) compound c_O (oxygen ester derivative); (5) compound c_{NH} (amide derivative); (6) control (with DMSO).

In none of our experiments using *S. epidermidis* pheromone or its derivatives, addition of these substances to *S. aureus* cultures changed the optical density of the *S. aureus* cultures after the incubation period.

3.4. Phenotypic characterization of the inhibiting effect on the S. aureus agr system

The *S. aureus* agr system regulates the production of certain exoproteins and surface-associated proteins. In most cases, the exoproteins are up-regulated and the surface-associated proteins are down-regulated [25]. The effect of the synthetic cyclic peptides on the production pattern of surface-associated proteins and exoproteins was investigated. The synthetic cyclic peptides were added at a concentration of 1 μM to a culture that was inoculated by 1/100 volume of a preculture. The cultures were incubated for 16 h. A reduction of exoprotein production was observed. It was most pronounced with the peptides that were also effective inhibitors of δ-toxin production, i.e. the natural pheromone peptide and the modified peptides b and a (Fig. 3A). Certain surface-associated proteins were synthesized in higher amounts after addition of these peptides (Fig. 3B). The same effects were also observed with the lactone- and lactam-containing peptides, but the effects were less pronounced, which is in keeping with the results of the δ-toxin test.

Immunoblot analysis of surface protein samples of *S. aureus* Newman with antiserum raised against protein A, as an example of a surface protein known to be down-regulated by the agr system, showed the expected increased levels of pro-

tein A production in the samples to which the thiolactone-containing derivatives of the *S. epidermidis* pheromone were added (Fig. 4B). In the control samples, no protein A was detected. The addition of the lactone- and lactam-containing cyclic peptides led to a detectable protein A production, but to a lesser extent than that of the thiolactone-containing peptides. Immunoblot analysis of the exoprotein samples with antiserum raised against α-toxin, known to be up-regulated by the agr system, gave the expected opposite results (Fig. 4A). The same results were obtained with *S. aureus* RN6390, although the protein A expression in this strain was generally lower.

The sar system has been shown to activate the agr system in *S. aureus* [26]. We therefore also tested the influence of our derivatives on sar$^-$, agr$^-$ and agr$^-$/sar$^-$ mutant strains to investigate if the biological effects of our pheromone derivatives are exclusively mediated by their action on the agr system and if these can be maintained in the absence of a functional global regulator of superior hierarchy. In the sar$^-$ strain, the effects were similar as in the wild-type but less pronounced, most likely because of the lacking direct effect of sar on the expression of α-toxin and protein A, which has been reported [26,27]. Interestingly, in the sar$^-$ strain, both protein A and α-toxin appear mostly as proteolytic degradation products. This is probably due to the overexpression of proteases in sar$^-$ strains, which has been demonstrated by Chan et al. [28]. The agr$^-$ strain showed the expected high expression levels of protein A and a low expression of α-toxin (Fig. 4). Addition of the pheromone or its derivatives to this strain or to an agr$^-$/sar$^-$ strain did not alter the protein A or α-toxin expression, as expected (data not shown). The lack of any effect in an agr$^-$ background demonstrates that our derivatives exclusively act via the agr system.

The decreased production of the δ-toxin and the α-toxin, one of the most important staphylococcal virulence factors [3], was also shown by the analysis of synergistic hemolysis [29] on sheep blood agar plates. The control sample exhibited pronounced synergistic hemolysis, the samples treated with the pheromone or its derivatives showed a strongly reduced or no synergistic hemolysis.

4. Discussion

4.1. Influence of derivatization on biological activity, importance of the thiolactone structure

Of all derivatives tested in this study, only the natural *S. epidermidis* pheromone peptide with the correct N-terminus and a cyclic thiolester could activate the *S. epidermidis* agr system. We have previously reported that the length of the N-terminal linear peptidyl tail is crucial for this activating effect. We now provided evidence that substitution of the thiolester by an (oxygen-) ester or an amide also does not lead to an activating peptide. This suggests that the interaction of the peptide with the AgrC sensor kinase is extremely specific and dependent on the unusual thiolactone structure. On the other hand, the cyclic peptides all showed a more or less pronounced inhibitory effect on the agr system of *S. aureus*. Similar results have been achieved by Mayville at al. with derivatives of the *S. aureus* AgrDII pheromone. These authors speculate that the indispensability of the thiolactone structure for the activating, but not inhibiting, ability suggests a possible covalent modification reaction of the AgrC sensor kinase by the agr pheromone, probably by a trans-acylation reaction [30]. There is no direct evidence for such a modification reaction so far, however, our results with the *S. epidermidis* pheromone and its cyclic bond type derivatives would be in keeping with their hypothesis.

The introduction of an amide instead of the original thiolactone resulted in an increased chemical stability, but a strongly reduced biological activity in the *S. aureus* inhibition tests. The thiolactone-containing peptides suppressed the *S. aureus* agr system more efficiently in the 16 h cultures, as shown by α-toxin, protein A and general protein levels, as well as in the tests for synergistic hemolysis. This demonstrates that despite the unusual reactive thiolester structure, these compounds have an unexpectedly high stability in bacterial cultures.

This is the first study that provides evidence that the *S. epidermidis* agr pheromone peptide acts as an inhibitor of the *S. aureus* agr system and the expression of virulence factors in *S. aureus*. It is tempting to speculate that *S. epidermidis* exploits this effect to compete with *S. aureus* in certain environments in the human body. *S. epidermidis* is the predominant species on the human skin and in catheter-related infections [3]. Presumably, in cases where there is a high population density of *S. epidermidis* cells, the highly active agr system leads to an increased production of *S. epidermidis* pheromone, which inhibits virulence factor expression of competing *S. aureus* cells and thereby provides a selection advantage for *S. epidermidis*.

4.2. Development of quorum-sensing blockers of the S. aureus agr system

Quorum-sensing blockers have been proposed as new promising agents for anti-microbial therapy. They offer the advantage of a reduced risk for the development of resistance against the antibiotic because they only suppress the expression of virulence factors and do not act as bacteriolytic or bacteriostatic compounds. Thus, the selection of resistant bacteria is assumed to be minimized [2].

For many bacteria, including pathogens, quorum-sensing systems have been characterized and, in some cases, the structure of the pheromones is known. In Gram-negative bacteria, these are *N*-acylhomoserine lactones, whereas in Gram-positive bacteria, the pheromones are peptides that are often post-translationally modified [1,31]. Effective synthetic quorum-sensing blockers for Gram-positive human pathogens have not yet been developed. There are examples of anti-infective compounds that inhibit two-component signal transduction. However, all these compounds also inhibit the bacterial growth and therefore do not provide the advantage of a reduced risk for the development of resistance.

Mayville et al. recently synthesized thiolactone-containing *S. aureus* agr pheromone peptides and derivatives. They could show an inhibiting activity of synthetic group II pheromone towards *S. aureus* cells of a different subgroup. This has also been demonstrated in vivo, when *S. aureus* cells were injected into mice together with the inhibiting pheromone, which led to suppression of the development of skin abscesses [30]. However, as the natural *S. aureus* population is assumed to be composed of members of different subgroups, treatment of *S. aureus* infections with one of these pheromones should bear the risk that the agr system including the expression of virulence factors is suppressed in some of the bacterial cells, but

activated in others, which belong to the subgroup of the applied pheromone.

We therefore tried to use the *S. epidermidis* pheromone as a basis for the development of *S. aureus* agr quorum-sensing blockers, as this pheromone is unlikely to activate virulence factor expression in *S. aureus* pheromone subgroups of a known or yet to be identified sequence, because, as we and Mayville et al. [30] could show, the agr-activating effect is highly dependent on the original pheromone structure.

In our experiments, the synthetic *S. epidermidis* pheromone and its derivatives inhibited the expression of the agr system of *S. aureus* with a varying efficiency, as was demonstrated by the suppression of δ-toxin production and by testing for synergistic hemolysis. In a 16 h culture, the substances caused a reduction of the production of α-toxin, which is one of the most important virulence factors of *S. aureus*, whereas an increase in the production of the surface-associated protein A was observed. Since there was no effect on bacterial growth, the *S. epidermidis* pheromone and its derivatives meet the above mentioned requirements of quorum-sensing blockers.

S. epidermidis is an opportunistic pathogen and the production of virulence factors in this strain is likely to be regulated also by the agr system. Therefore, it was desirable to find a derivative of the pheromone that does not activate the *S. epidermidis* agr system, but is still an effective inhibitor of the *S. aureus* agr system. The thiolactone-containing derivative b, which lacks the N-terminal aspartate residue of the natural pheromone, showed exactly these characteristics. This derivative may therefore be a promising candidate as a quorum-sensing blocker for anti-infective therapy against *S. aureus*. The results also show that in general, the derivatization of pheromones can lead to compounds with altered agr-activating or agr-inhibiting effects.

With none of our synthesized linear derivatives, we achieved any activating or inhibiting biological effect. We therefore conclude that for the systematic construction of biologically active derivatives based on the agr pheromone, a cyclic structure is an important prerequisite.

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