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Pheromone Cross-Inhibition between *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Cross-inhibition by quorum-sensing pheromones between *Staphylococcus aureus* and *Staphylococcus epidermidis* was investigated using all known *S. aureus agr* pheromone subgroups. All *S. aureus* subgroups were sensitive towards the *S. epidermidis* pheromone, with the exception of the recently identified subgroup 4. The subgroup 4 pheromone was also the only *S. aureus* pheromone able to inhibit the *S. epidermidis agr* response. The close relation of subgroup 4 to subgroup 1 suggests that subgroup 4 might have evolved from subgroup 1 by mutation under the selective pressure of competition with *S. epidermidis*. The competition between *S. aureus* and *S. epidermidis* by means of quorum-sensing cross talk seems to be generally in favor of *S. epidermidis*, which might explain the predominance of *S. epidermidis* on the skin and in infections on indwelling medical devices.

Quorum-sensing systems, which sense and signal the state of cell density, are of high importance for the survival of bacteria, as they enable them to respond to changing environmental conditions (14). The *agr* system of staphylococci is a quorum-sensing system which controls the expression of exoproteins and surface proteins in a growth phase-dependent manner (10). The extracellular signal, which is used by the staphylococcal *agr* system, is a small peptide pheromone that harbors an unusual posttranslational modification (2). For *Staphylococcus aureus* and *Staphylococcus epidermidis*, it has been shown that this posttranslational modification is an intramolecular thioester, which links the thiol group of a central cysteine to the C-terminal carboxy group (5, 8). Interestingly, the primary sequence of the pheromones of different species and also of different pheromone subgroups within one species varies completely. Only the central cysteine and its distance to the C terminus are conserved (5, 8). For three *S. aureus* subgroups it has been demonstrated that the corresponding pheromones can inhibit the *agr* response of foreign subgroups (1). We have shown that this is also the case between different staphylococcal species, as the *S. epidermidis* pheromone has proven to be an efficient inhibitor of the *agr* response of *S. aureus* strain Newman (9). *agr* controls the expression of several important virulence factors in *S. aureus*, such as alpha-toxin, beta-toxin, delta-toxin, serine protease, DNase, fibrinolysin, enterotoxin B, and toxic shock syndrome toxin 1 (12). Suppression of the *agr* response in *S. aureus* by the above-mentioned pheromones also suppresses the expression of various virulence factors in in vitro studies (5, 9). Furthermore, the development of *S. aureus*-induced lesions in mice was efficiently suppressed when the infecting strain was injected subcutaneously together with the inhibiting pheromone of a foreign subgroup (5). Therefore, *agr* pheromones and their derivatives have been proposed as new antistaphylococcal drugs, especially in the treatment of infections by *S. aureus* and *S. epidermidis*, which rank among the most important pathogens in nosocomial infections (11).

S. aureus causes many acute severe infections, such as impetigo, wound infections, or in toxic shock syndrome, whereas chronic infections tend to be caused in a higher relative proportion by *S. epidermidis* (13). The prevalence of *S. epidermidis* in many nosocomial infections, among them infections on indwelling medical devices, raises the question about the advantage that *S. epidermidis* possesses in these situations compared to *S. aureus*. Among the infections predominantly caused by *S. epidermidis* one can often find the involvement of biofilms (15), which constitute a high-density population. This led us to the assumption that the more frequent participation of *S. epidermidis* in these infections might be due to interspecies concurrence based on cell density control mechanisms. It has been proposed that the inhibiting properties of the staphylococcal pheromones serve as weapons in a struggle between different staphylococcal strains (1). To address the question of interspecies concurrence, we added synthetic natural *S. epidermidis* pheromone to *S. aureus* strains of subgroups 1 to 4 and synthetic pheromones of *S. aureus* subgroups to *S. epidermidis*. *S. aureus* subgroup 4 has only recently been discovered independently by us and by G. Lina (G. Lina, personal communication). In a previous study, we investigated 15 *S. epidermidis* strains by sequencing the DNA coding for the AgrD prepheromone and found only a single *S. epidermidis* pheromone sequence, DSVCASYF (8), suggesting that there is only one *S. epidermidis agr* pheromone group or that this one is by far the most frequent. All pheromones and pheromone derivatives were prepared by solid-phase synthesis as previously described (8) and are shown in Fig. 1. The staphylococcal strains used were *S. epidermidis* ATCC 14990, *S. aureus* strains A950227 (subgroup 1), A950085 (subgroup 2), A920226 (subgroup 3), A970377 (subgroup 4), A970392 (subgroup 4), A850484 (subgroup 4), and 1527/97 (subgroup 4). All *S. aureus* strains are clinical isolates. Strain 1527/97 was kindly provided by W. Witte, Robert-Koch Institut, Berlin, Germany, and classified as subgroup 4 strain in our laboratory by DNA sequencing; the other *S. aureus* strains were kindly provided by G. Lina, Centre

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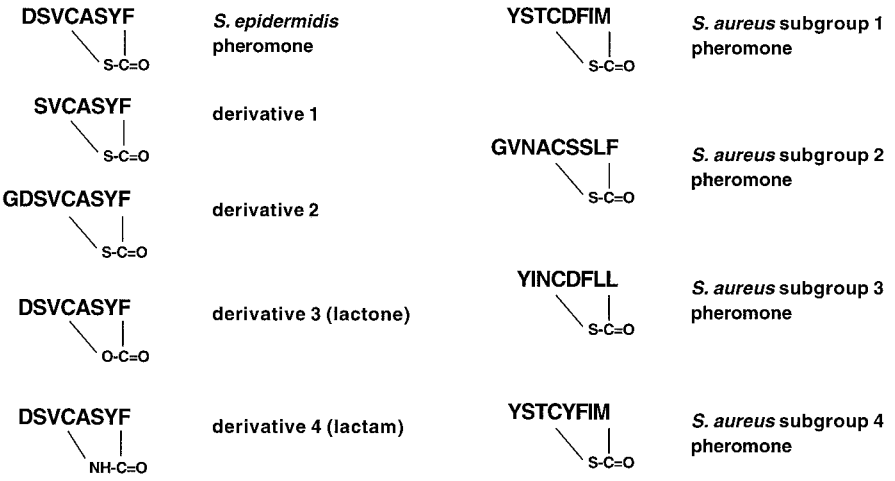

FIG. 1. Synthetic *agr* pheromone and derivatives used in this study.

Hospitalier et Universitaire de Lyon, Lyon, France, and were classified by G. Lina.

Inhibition of the *agr* system was monitored as we have previously described (7). Briefly, delta-toxin expression was determined by a high-performance liquid chromatography assay using a Pharmacia Resource PHE column and a water-acetonitrile gradient with 0.1% trifluoroacetic acid. Main cultures that were inoculated 1:100 from precultures were grown for 8 h, with pheromone addition at the time of inoculation. Afterwards, the samples were centrifuged and the supernatant was injected onto the column. Delta-toxin is encoded within the gene for the regulatory RNAIII, which is the intracellular effector of the *agr* system (6). Its expression is therefore a means to measure the activity of the *agr* system.

As shown in Fig. 2A, using pheromone concentrations ranging from 25 nM to 1 μM, the synthetic peptide corresponding to the natural *S. epidermidis* pheromone was very active against *S. aureus* subgroup 3. It had considerable activity against subgroups 1 and 2, but it was inactive against subgroup 4. On the other hand, *S. epidermidis* was insensitive to *S. aureus* pheromones from subgroups 1 to 3 and showed moderate sensitivity against *S. aureus* pheromone of subgroup 4 (Fig. 2B). This sensitivity was lower than that of the *S. aureus* subgroups 1 to 3 towards the *S. epidermidis* pheromone. At the very high pheromone concentration of 10 μM, the *S. epidermidis* pheromone completely inhibited delta-toxin expression in *S. aureus* subgroups 1, 2, and 3 but showed no effect on subgroup 4. At this concentration, the pheromone of *S. aureus* subgroup 4 was able to entirely suppress delta-toxin expression by *S. epidermidis*, whereas the pheromones of *S. aureus* subgroups 1 to 3 still did not show any inhibiting effect (data not shown).

These difference are most likely best explained by the more or less tight interaction of the pheromones with their receptor, the histidine kinase membrane enzyme AgrC. The third extracellular loop of this enzyme has been demonstrated to interact with the pheromone (4). It remains unclear if this interaction is a covalent one (by a *trans*-acylation reaction), as has been proposed (5). The charge of the pheromones does not correlate with the observed inhibiting properties, as the *S. epidermidis* pheromone and *S. aureus* pheromones of subgroups 1

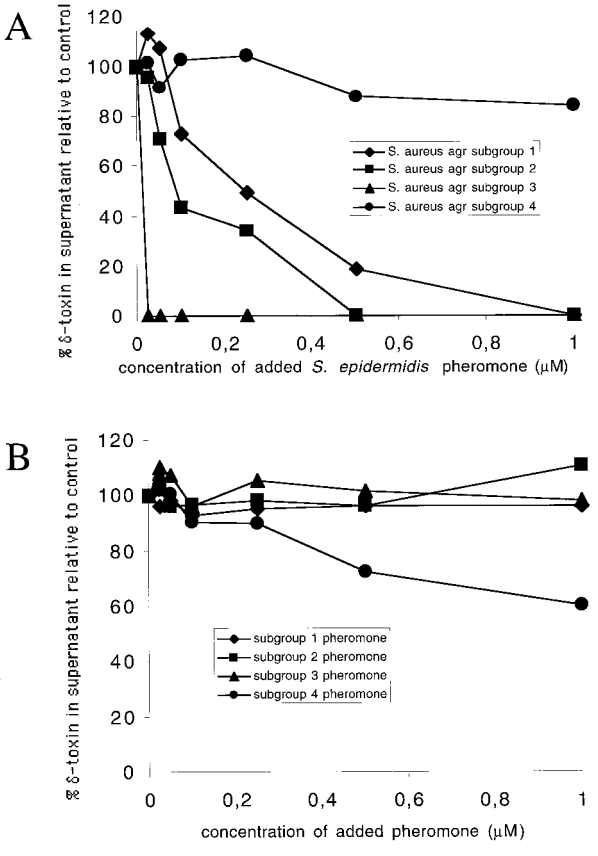

FIG. 2. Cross-inhibition of *S. epidermidis* and *S. aureus* by *agr* pheromones. The amount of delta-toxin in 500 μl of supernatant was determined by high-performance liquid chromatography after 8 h of growth and under the influence of different concentrations of added pheromone or pheromone derivatives. Pheromones were diluted in dimethyl sulfoxide; the control received only dimethyl sulfoxide. Cultures were grown in basic medium (1% tryptone [Difco], 0.5% yeast extract [Gibco BRL], 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose) with shaking at 37°C. (A) Effect of the *S. epidermidis* pheromone on delta-toxin production of *S. aureus agr* subgroups. (B) Effect of the pheromones of different *S. aureus agr* subgroups on delta-toxin production of *S. epidermidis*.

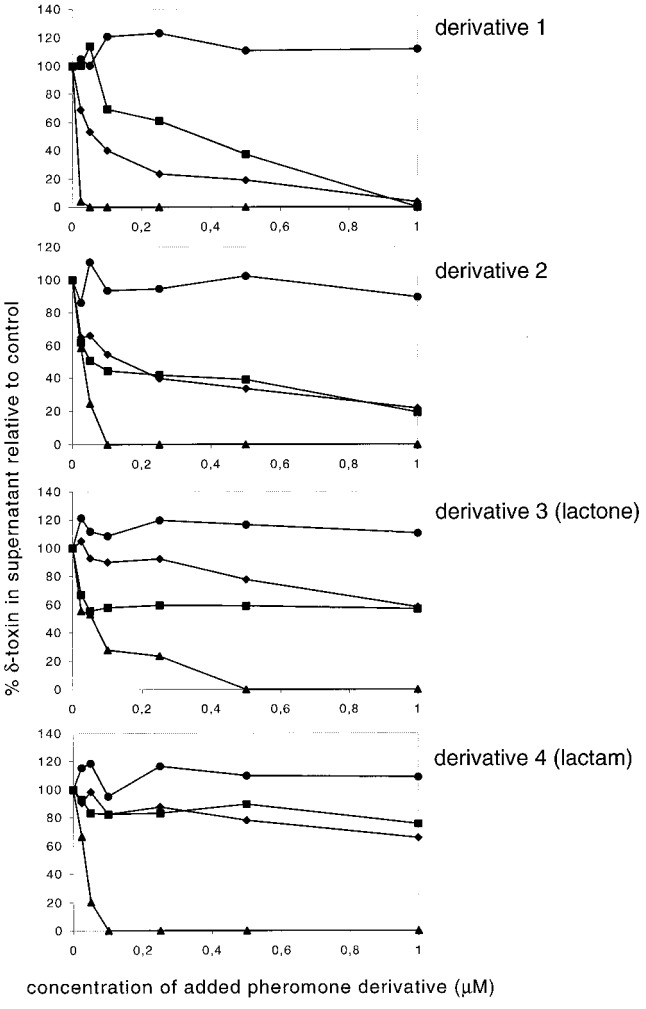

FIG. 3. Inhibition of the *agr* response in *S. aureus agr* subgroups by *S. epidermidis* pheromone derivatives. The amount of delta-toxin in the supernatants of *S. aureus agr* subgroup strains after addition of the *S. epidermidis* pheromone derivatives was determined as outlined in the legend for Fig. 2. Symbols used for *S. aureus agr* subgroups are the same as in Fig. 2A. Structures of the derivatives are shown in Fig. 1.

and 3 have a net charge of zero, whereas the other tested pheromones harbor one positive charge. Therefore, other structural properties might be responsible for the differing interaction of the various pheromones with AgrC.

The most interesting result is the complete inactivity of the *S. epidermidis* pheromone against *S. aureus* subgroup 4 and the fact that only the pheromone of *S. aureus* subgroup 4 showed activity against *S. epidermidis*. We have tested three more subgroup 4 strains, which also proved to be completely insensitive to the *S. epidermidis* pheromone (data not shown).

Recently it has been observed that subgroup 4 strains are often involved in infections leading to scalded skin syndrome (G. Lina, personal communication). This means that these strains live on the skin, where they come into close contact and concurrence with *S. epidermidis*, which normally is the predominant strain on the skin (3). The subgroup 4 pheromone differs from that of subgroup 1 only by one amino acid (primary sequence YSTCYFIM instead of YSTCDFIM). It is therefore tempting to speculate that subgroup 4 might have evolved from subgroup 1 in order to be able to compete with *S. epidermidis*.

Activity of *S. epidermidis* pheromone derivatives against *S. aureus agr* subgroups. Among the tested *S. epidermidis* pheromone derivatives are lactone and lactam derivatives (derivatives 3 and 4) and derivatives with different lengths of the N-terminal tail, adjacent to the thiolactone-bearing ring structure (derivatives 1 and 2). Derivative 2 was slightly less active than the natural pheromone against subgroups 1, 2, and 3. This is in accordance with earlier data by which we could also show a reduced activity against *S. aureus* Newman (9), which belongs to subgroup 1. Derivative 1 exhibited an activity similar to that of the natural pheromone against subgroups 1 and 3 but was less active against subgroup 2. Both derivatives were inactive against subgroup 4. The derivatives in which the thiolactone structure was replaced by a lactone (derivative 3) or a lactam (derivative 4) showed a slightly further reduced activity against all subgroups, as already reported for subgroup 1 (9), but again no activity against subgroup 4 (Fig. 3).

In summary, the *S. epidermidis* pheromone seems to be a more potent inhibitor of the *S. aureus agr* system, compared to the activity of *S. aureus* pheromones against *S. epidermidis*. The predominance of *S. epidermidis* on the skin and in chronic infections, for example, on indwelling medical devices, might be due to this advantage. As a normal resident of the skin's microflora, *S. epidermidis* might contribute to the body's barrier to colonization by the pathogenic *S. aureus* via quorum-sensing cross-inhibition. An interesting exception is *S. aureus* subgroup 4, which seems to have escaped from this unfortunate situation by mutation, probably because of close contact with *S. epidermidis* on the skin.

As far as *agr* pheromones are concerned in terms of potential therapeutics, our results show that an *agr* pheromone or a derivative may have strongly varying activity against different staphylococcal strains. It is therefore not easy to evaluate their therapeutic use in a patient who normally carries a lot of different staphylococcal strains. Furthermore, selection of resistant strains may quickly occur, as might have occurred already during the competition between staphylococcal strains during evolution, as our results with subgroup 4 suggest.

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