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Construction and Characterization of an *agr* Deletion Mutant of *Staphylococcus epidermidis*

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The physiological significance of the accessory gene regulator (*agr*) system of *Staphylococcus epidermidis* was investigated by construction of an *agr* deletion mutant via allelic replacement with a spectinomycin resistance cassette. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the protein pattern was strongly altered in the mutant; the amounts of most surface proteins were higher, whereas the amounts of most exoproteins were lower. The *agr* system of *S. epidermidis* thus appears to have an important impact on growth phase-dependent protein synthesis as has been shown for *Staphylococcus aureus*. The activity of the exoenzymes lipase and protease, assumed to be involved in staphylococcal pathogenicity, was investigated by agar diffusion assays and SDS-PAGE activity staining. A general reduction of these enzyme activities in the *agr* mutant was found. The difference in overall lipase activity was small, but zymographic analysis suggested a clear defect in lipase processing in the *agr* mutant.

In recent years *Staphylococcus epidermidis* has emerged as one of the most important pathogens in nosocomial infections (11). Effective antibiotic treatment of *S. epidermidis* is difficult because of the slime capsule which surrounds biofilm-forming colonies of this bacterium and which can barely be penetrated by many antibiotics. The situation has become even more severe because of the appearance of multiresistant and vancomycin-resistant *S. epidermidis* strains (26). Although these problems have been recognized for a number of years, the identification of *S. epidermidis* virulence factors and the investigation of their regulation has not kept pace with the research done in *Staphylococcus aureus*. Virtually nothing is known about the regulation of potential virulence determinants in *S. epidermidis*.

The *S. aureus* accessory gene regulator (*agr*) system is responsible for the growth-phase-dependent regulation of virulence factors and has been extensively investigated (14, 17). We have recently identified the *S. epidermidis agr* system, whose gene structure and sequence is very similar to that of *S. aureus* and which may therefore play a role comparable to that in *S. aureus*. The *agr* systems of *S. aureus* and of *S. epidermidis*, approximately 3.5 kb in size, comprise the *agrA*, *agrC*, *agrD*, and *agrB* genes, which are cotranscribed (RNAII), and the gene for the effector molecule of the *agr* system, RNAIII, which also encodes the gene for delta-toxin (*hld*). RNAIII controls expression of target genes in an unknown manner (20, 22, 24). The *agr* system is activated during the transition from the exponential growth phase to the stationary phase by an autoregulatory mechanism involving a modified pheromone peptide (14, 22).

The *agr* system in *S. aureus* downregulates the synthesis of many surface proteins, and upregulates the synthesis of many exoproteins at the onset of the stationary growth phase. Both groups of proteins mainly comprise factors that contribute to the pathogenic potential of *S. aureus*. To date, *agr*-regulated targets of *S. epidermidis*, which are likely to include virulence determinants, have not yet been identified.

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The most important virulence factor of *S. epidermidis* is assumed to be biofilm formation on indwelling medical devices (reviewed in references 7 and 8). In association with sepsis or wound infection of immunocompromised patients (6; A. Berges, J. Gutierrez-Cebollada, J. M. Garces, and O. Pallas, Letter, Enferm. Infecc. Microbiol. Clin. 9:383–384, 1991), some other determinants might also contribute to the virulence of *S. epidermidis*. These determinants include proteases, delta-toxin, lipases, and unknown bacterial components.

Here we report the construction of an *agr* deletion mutant of an *S. epidermidis* wild-type strain and the effects of the *agr* deletion on protein synthesis in general and virulence factor production in particular. The expression of two important virulence-determining exoproteins, lipase and protease, was analyzed in detail.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. epidermidis* cells were grown in B medium (1% tryptone [Difco], 0.5% yeast extract [Gibco BRL], 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose). Antibiotics were used at the following concentrations: chloramphenicol, 10 μg/ml; spectinomycin, 150 μg/ml; and ampicillin, 100 μg/ml. Cultures were generally incubated at 37°C with shaking at 140 rpm, unless otherwise noted.

Molecular cloning techniques, transformation, and DNA sequencing. DNA manipulation, isolation of plasmid DNA, and transformation of *E. coli* were performed by using standard procedures (29). Staphylococcal plasmid DNA was prepared by using the Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany). The manufacturer's instructions were followed except that the cells were incubated for 15 min at 37°C at 37°C in 4 ml of P1 buffer containing 25 μg of lysostaphin (Sigma, St. Louis, Mo.) per ml before buffer P2 was added. Chromosomal DNA was isolated according to the procedure of Marmur (16). Enzymes for molecular cloning were obtained from Boehringer Mannheim (Mannheim, Germany), Gibco BRL, and Amersham Pharmacia Biotech (Freiburg, Germany); incubation conditions were as recommended by the suppliers. PCR was performed with *Vent* polymerase (New England Biolabs) as recommended by the manufacturer. The primers for PCR were as follows: cvIaBam, GGAAAAGGGCAAGGATCCACTAGCGTTTAG; cvIbSph, GAAGAAAAGCCAATGGCATGCGCTTTACGAAC; cvIIaSal, CAAGCCGTGAGTCGACCCCAAGCTCACGG; and cvIIbHind, GTAGTTACCATGAAAGCTTAGCCCGTA. Restriction sites are underlined. PCR primers were purchased from Interactiva (Ulm, Germany) or from MWG-Biotech (Ebersberg, Germany).

DNA was sequenced by using fluorescent-labeled primers and a LI-COR sequencer (MWG-Biotech). The nucleotide sequences were analyzed by using the program MacDNASIS Pro (Hitachi Software Engineering, San Bruno, Calif.).

Construction of plasmid pBTΔagr and homologous recombination. In order to delete the *agr* genes in *S. epidermidis* Tü3298, DNA fragments of 821 bp (with PCR primers cvIaBam and cvIbSph) and 1,235 bp (with PCR primers cvIIaSal and cvIIbHind) upstream and downstream of the *agr* region were amplified by

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Reference or source	Description
Strains		
S. epidermidis Tü3298	2	*agr* wild-type strain
S. epidermidis TüF38	This study	Isogenic mutant of Tü3298 with an *agr*::*spc* mutation
S. aureus RN6390	26	Laboratory strain that maintains its hemolytic properties when propagated on sheep erythrocytes and has a genetic background similar to that of 8325-4
S. aureus RN6911	17	Isogenic mutant of RN6390 with an *agr*::*get*M mutation
Plasmids		
pBT2	6	Temperature-sensitive vector for allelic replacement by homologous recombination
pBTΔagr	This study	Constructed vector for homologous recombination of the *agr* system in *S. epidermidis*; harbors *spc* as resistance selection gene
pIC156	20	Plasmid harboring the *spc* gene for spectinomycin resistance

PCR and digested with *Bam*HI/*Sph*I and *Sal*I/*Hin*dIII, respectively. The two DNA fragments were inserted into the polylinker region of the temperature-sensitive shuttle vector pBT2 together with a 1,277-bp *Sph*I/*Sal*I-digested fragment encoding the spectinomycin adenyltransferase gene (*spc*) from Tn*554* (18), as shown in Fig. 1. The fidelity of the sequence of PCR-amplified regions was proven by nucleotide sequencing. *S. epidermidis* Tü3298 was transformed by electroporation with the resulting plasmid pBTΔagr (2). The recombination procedure has been described recently in detail (4). The proper integration of *spc* was verified by direct sequencing of the chromosomal DNA at the borders of the PCR-derived regions (25).

Lipase assay. Lipase activity was determined by an agar plate assay with Ca^{2+}-containing tributyrylglycerol-basic agar (Merck) containing 1% Tween 20. Overnight precultures of *S. epidermidis* and *S. aureus* strains grown in B medium at 37°C were diluted 1:100 in B medium and incubated for 12 h at 37°C with shaking at 140 rpm. Then, 10-ml supernatants of 12-h bacterial cultures were lyophylized; the lyophilysate was dissolved in 2 ml of 20 mM Tris-HCl (pH 8.0) and passed through 0.22-μm-pore-size filters. Next, 25 μl was applied four times onto filters, which were air dried and placed on Tween 20 agar plates (19). Plates were then incubated at 37°C for 24 h.

Protease assay. Protease activity was determined by an agar plate assay. The test agar contained 1% skim milk, 1% tryptone (Difco), 0.5% yeast extract (Gibco BRL), 0.5% NaCl, and 1.5% agar. Bacterial strains were grown overnight on the agar plates at 37°C.

Protein isolation and SDS-PAGE. Exoproteins of 12-h bacterial cultures were isolated by precipitation with a 1/9 volume of trichloroacetic acid. Pellets were dissolved in 7 M urea–100 mM Tris-HCl (pH 8.0). Surface proteins were isolated by incubating the cells with 25 μg of lysostaphin/ml (Sigma, St. Louis, Mo.) for 15 min at 37°C and subsequently centrifuging at 19,000 × *g* for 10 min. Surface-associated proteins were isolated by boiling cells at 100°C for 5 min and subsequently centrifuging them at 19,000 × *g* for 10 min. Proteins were separated by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Schägger and von Jagow (30) using a Bio-Rad Protean II XI chamber and a separation length of 16 cm. Molecular mass standards were purchased from Gibco BRL.

Zymographic analysis of protease and lipase activity. SDS-PAGE was performed as outlined above, but under nonreducing conditions. After electrophoresis, gels were washed twice in 20% isopropanol for 15 min and then twice in water for 30 min. Gels were then laid on 1% agarose plates containing 50 mM Tris-HCl (pH 7.8), 1 mM $CaCl_2$, and 1% casein (according to Hammersten; for protease detection) or 1% Tween 20 (for lipase detection) and incubated at 37°C overnight.

Delta-toxin detection by HPLC. The amount of delta-toxin in culture filtrates was determined by analytical high-performance liquid chromatography (HPLC) on a Kontron HPLC system with Kroma System 2000 software as described previously (23). A Pharmacia Resource PHE 1-ml column was eluted with 15 column volumes of a linear gradient (0 to 100% of B in A [A, 0.1% trifluoroacetic acid in water; B, 0.1% trifluoroacetic acid in acetonitrile]). The detection wavelength was 280 nm.

RESULTS

Deletion of the *agr* system of *S. epidermidis*. We have previously published the sequence of the *agr* genes of *S. epidermidis*. The genes have high sequence similarity to those of the *agr* system of *S. aureus* and *Staphylococcus lugdunensis* (22). To investigate its function in *S. epidermidis*, we deleted the *agr* genes in *S. epidermidis* Tü3298 by homologous recombination. First, two DNA fragments upstream and downstream of the *agr* region were amplified by PCR (Fig. 1). The two amplified DNA fragments and an appropriate antibiotic resistance marker (spectinomycin adenyltransferase gene [*spc*] from Tn*554* [18]) were cloned into the polylinker region of the temperature-sensitive shuttle plasmid pBT2 (4), yielding plasmid pBTΔagr. This plasmid was sequenced in order to prove (i) that the two PCR fragments did not harbor any polymerase-introduced mutation and (ii) that the orientation of the antibiotic resistance marker was correct. *S. epidermidis* Tü3298 was transformed with plasmid pBTΔagr by electroporation (2). Transformants were selected for chloramphenicol and spectinomycin resistance. The procedure used for homologous recombination was that described by Brückner et al. (4), and we then screened for spectinomycin-resistant and chloramphenicol-sensitive clones.

Insertion of the antibiotic resistance marker into the *agr* locus of *S. epidermidis* Tü3298 was confirmed by direct sequencing of chromosomal DNA (25) of the *agr* deletion mutant. The resulting strain in which the complete *agr* system is deleted was named *S. epidermidis* TüF38.

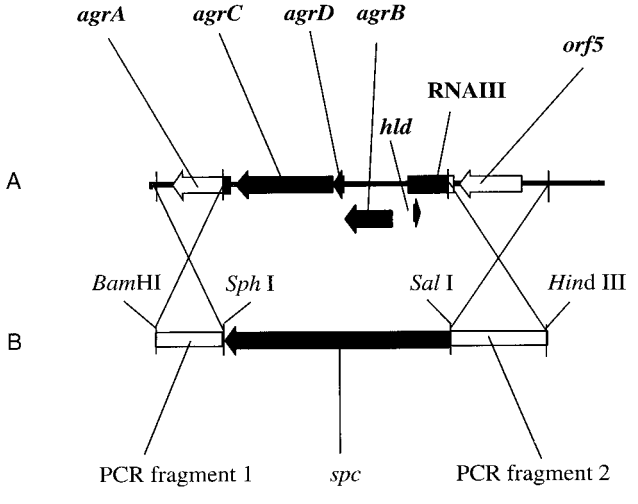

FIG. 1. Physical map of the *agr* system of *S. epidermidis* (A) and construction of pBTΔ*agr* (B). Plasmid pBTΔ*agr* was constructed for homologous recombination of the *agr* system of *S. epidermidis* by the insertion of a spectinomycin adenyltransferase gene (*spc*) and two PCR-amplified *agr*-flanking regions into plasmid pBT2. Open reading frames are depicted by arrows, which indicate their orientation. Recognition sites for restriction enzymes, resistance markers, and PCR-amplified fragments are also shown. The crosses indicate the sites of homologous recombination.

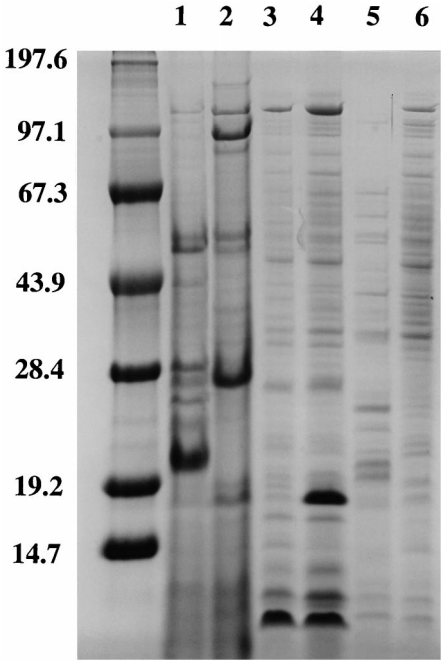

FIG. 2. Protein profiles of the *S. epidermidis* wild-type Tü3298 and the isogenic *agr* deletion strain TüF38. SDS-polyacrylamide (10%, wt/vol) gels of exoproteins (lane 1, *agr*$^+$; lane 2, *agr* mutant), surface-associated proteins prepared by boiling with SDS (lane 3, *agr*$^+$; lane 4, *agr* mutant), and surface proteins prepared by treatment with lysostaphin (lane 5, *agr*$^+$; lane 6, *agr* mutant) are shown. Aliquots were collected after 12 h of growth, and protein samples were prepared as described in Materials and Methods. The proteins were stained with Coomassie blue R250; the molecular mass protein standards are in the left lane and are indicated in kilodaltons.

Since the delta-toxin is encoded within the RNAIII-encoding region (12, 22) and RNAIII is the effector molecule of the *agr* system (20), expression of delta-toxin can be used to demonstrate the functionality of the *agr* system. Therefore, the failure of delta-toxin production was used as a further confirmation that the allelic replacement was successful. No delta-toxin could be detected by analytical HPLC in the supernatant of the *agr* deletion mutant *S. epidermidis* TüF38, indicating that the replacement had occurred and the *agr* system was not functional anymore (data not shown).

Production of exoproteins, surface-associated proteins, and surface proteins. The general influence of the *agr* system of *S. epidermidis* on protein synthesis was investigated by SDS-PAGE analysis of exoprotein and surface protein samples from stationary-phase cultures. The *agr* deletion in *S. epidermidis* TüF38 resulted in a pleiotropic alteration of the pattern of exoproteins, surface-associated proteins (prepared by boiling with SDS), and surface proteins (prepared by treatment with lysostaphin) on SDS-polyacrylamide gels, compared to the parental strain *S. epidermidis* Tü3298 (Fig. 2). Treatment with SDS releases noncovalently attached proteins from the cell surface, including, for example, many autolysins (13, 32). Lysostaphin releases covalently linked proteins from the cell by cleaving within the pentapeptide crossbridges of the staphylococcal peptidoglycan (31).

In the mutant, the amounts of several exoproteins were lower, but a few proteins were also expressed at higher amounts. Some of them might represent degradation products of surface proteins released to the surrounding fluid. Several exoproteins that are expressed at very high levels in the wild type seem to be completely repressed in the mutant. Most remarkable in this respect is a protein of about 21 kDa which is extremely abundant in the supernatant of the wild type but absent from the supernatant of the mutant.

Generally, the amounts of many surface proteins and surface-associated proteins were higher in the mutant than in the wild type. The most striking differences were observed for two proteins of about 120 and 18 kDa in the samples containing surface-associated proteins which appeared in much higher amounts in the mutant. The samples containing lysostaphin-released proteins showed a generally stronger expression of higher-molecular-weight proteins in the mutant, whereas at lower molecular weights we could also detect proteins that were expressed in higher amounts in the wild type. This is probably not due to proteolysis since the proteins appear as distinct bands over the entire molecular weight range.

Regulation of specific exoenzyme activities. *S. epidermidis* does not produce exotoxins as does *S. aureus*. Also, little is known about the specific exoenzyme activities of *S. epidermidis*. We focused on lipase and protease activity because it is known that lipase and protease are important virulence factors of *S. aureus* (9, 10). This might also be the case in *S. epidermidis*. We tested the activities of lipase and protease in the supernatant of the *S. epidermidis* wild type and the *agr* mutant strain by

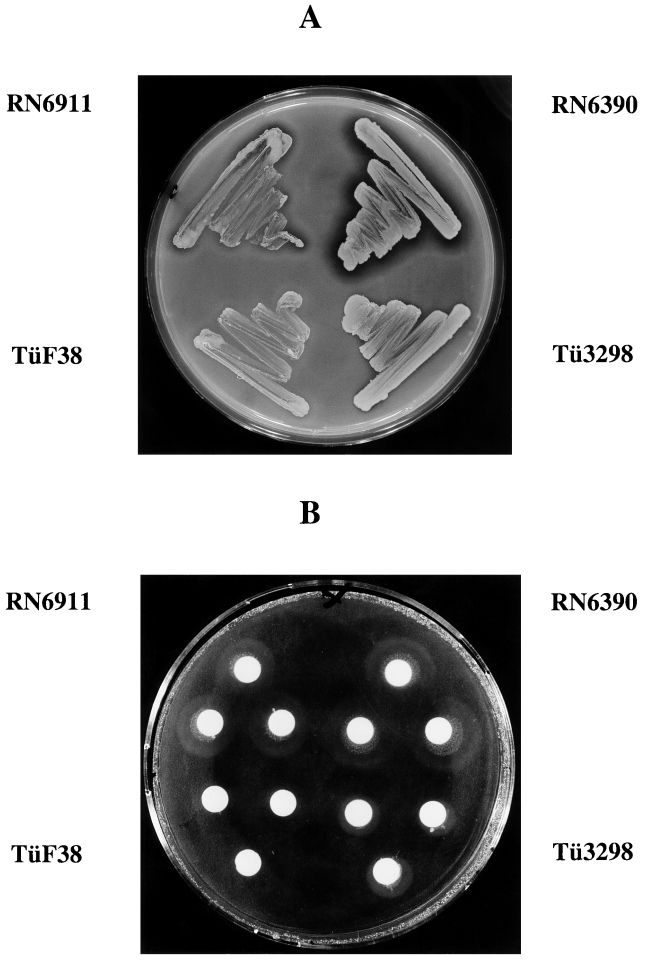

FIG. 3. Lipase and protease activity on agar plates. (A) Protease activity. Bacterial strains were grown overnight on skim milk agar plates at 37°C. (B) Lipase activity. A total of 25 μl of supernatants, 5× concentrated by lyophylization, were applied four times onto filters, air dried, and laid on Tween 20 agar plates, which were incubated 24 h at 37°C.

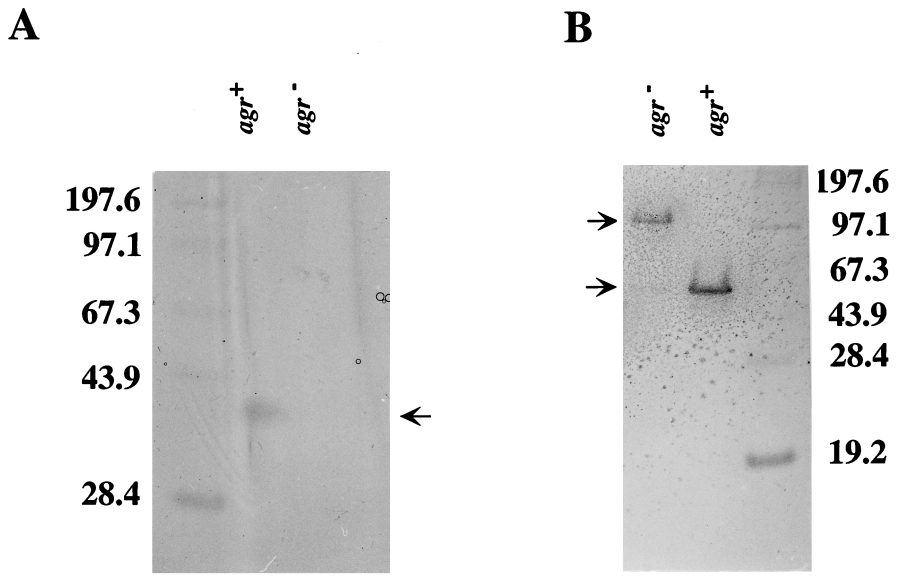

FIG. 4. Zymographic analysis of lipase and protease activity. Exoprotein samples of *S. epidermidis agr*$^+$ (Tü3298) and *agr* mutant (TüF38) strains were prepared as described in Materials and Methods and run on SDS-polyacrylamide (10%, wt/vol) protease (A) and lipase (B) test gels. The molecular mass standard is shown on the right in panel A and on the left in panel B; the molecular masses are indicated in kilodaltons.

agar plate assays. We also compared the activities of *S. epidermidis* to the activities of an *S. aureus agr*$^+$ strain (RN6390) and an *agr* mutant strain (RN6911). On protease test plates (Fig. 3A), *S. aureus* showed a generally higher protease activity than *S. epidermidis*, and the *agr*$^+$ strain exhibited clearly higher activity than the *agr* mutant strain in both species. On lipase test plates, effects could hardly be seen in *S. epidermidis* because of the low lipase activity of the test strain. Therefore, supernatants were concentrated by repetitively applying aliquots to filters, which were air dried between each application and then laid on test plates. Activities in *S. aureus* were generally higher than in *S. epidermidis*, but in both cases the *agr*$^+$ strains showed larger lysis zones than the *agr* mutant strains (Fig. 3B).

A more detailed view of the differing lipase and protease activities was achieved by zymographic analysis (Fig. 4).

Protease test gels (Fig. 4A) revealed a single strong proteolytic band at about 34 kDa, which was clearly more pronounced in the *agr*$^+$ strain than in the *agr* mutant strain. The molecular weight of this protease corresponds exactly to that of the published extracellular metalloprotease of *S. epidermidis* (34). Our assumption that the proteolytic activity we found is due to this known protease was further supported by the fact that it could be completely abolished by the inclusion of EDTA in the test plates (not shown).

Lipase test plates (Fig. 4B) showed a distinct lipolytic band at about 50 kDa in the sample of the *S. epidermidis agr*$^+$ and at about 100 kDa in the sample of the *S. epidermidis agr* mutant strain. These values correspond well to the reported apparent molecular sizes of the pro-form and the mature form of staphylococcal lipases (10). Thus, in the *agr*$^+$ strain, only the mature form seems to be present, whereas in the *agr* mutant strain, no processing of the pro-form appears to occur.

DISCUSSION

In *S. aureus*, many virulence factors are regulated by a global regulatory quorum-sensing system called *agr* for accessory gene regulator (20, 24). We have previously shown that the *agr* system is present in *S. epidermidis*, that its genes show strong sequence similarity to those in *S. aureus*, and that the expression of the *agr* system in *S. epidermidis* is growth phase dependent (22).

The aims of the present study were to investigate which phenotypic factors of *S. epidermidis* are regulated by the *agr* system and to determine what influence *agr* has on the expression of *S. epidermidis* virulence factors. We constructed an *agr* mutant (TüF38) in which the entire *agr* gene cluster was replaced by a spectinomycin resistance cassette. Correct insertion was proven by direct sequencing of the flanking regions; loss of functionality of the *agr* system was additionally shown by the inability of the mutant to synthesize delta-toxin. The *agr* system of *S. aureus* affects the synthesis of many exoproteins (e.g., toxic shock syndrome toxin 1, alpha-toxin, and tissue-degrading enzymes) and surface proteins (e.g., protein A, coagulase, and fibronectin-binding proteins) in a growth phase-dependent manner (12, 15, 24, 27). Samples prepared from stationary-phase cultures of the *S. epidermidis* TüF38 *agr* deletion mutant showed a pronounced alteration in the production pattern on the SDS-polyacrylamide gels of exoproteins, surface-associated proteins, and surface proteins compared to that of the isogenic strain *S. epidermidis* Tü3298. As in *S. aureus*, the *agr* system in *S. epidermidis* appears to be responsible for the upregulation of many exoproteins and the downregulation of many surface-bound and surface-associated proteins in the stationary growth phase, although a small number of proteins seem to be under the opposite control.

Lipase and protease activities are involved in tissue damage and the inflammatory host response (9, 10). Proteases can also play a role in the degradation of host peptide signaling factors, such as neutrophile defensins (33), platelet microbiocidal proteins (35), and antibodies (e.g., degradation of immunoglobulin G by V8 serine protease of *S. aureus* [33]). These two degradative exoenzymes clearly contribute to the destruction of tissue proteins and enhanced invasiveness (9, 10).

In our assays, a clear reduction of protease activity in the *S. epidermidis agr* mutant strain was detected, which by zymographic analysis could be attributed exclusively to the reduced expression of a strong proteolytic activity with an apparent

molecular mass of about 34 kDa. The accordance of molecular size and the inhibition of the proteolytic activity by EDTA strongly suggest that this activity is due to the published metalloprotease of *S. epidermidis* (34).

Reduced lipolytic activity of the *agr* mutant seems to be mainly due to the strongly reduced processing of lipase to the mature form. This might be due to reduced production of the processing protease. The lipolytic activity in the *agr* mutant is still relatively high because the pro-form of staphylococcal lipases exhibits considerable activity (10). In *S. hyicus*, growth phase-dependent processing has been demonstrated to be performed by a 34-kDa metalloprotease. It is homologous to the *S. epidermidis* 34-kDa metalloprotease (34), which as discussed above is most likely the protease we detected in the zymographic analysis. This protease is therefore very likely the one that cleaves the lipase pro-form. The extreme inhibition of the processing of the lipase pro-form in the *agr* mutant accordingly is in agreement with the fact that the putative processing protease was only detectable in the wildtype, but not in the *agr* mutant. The *agr* system thus seems to be responsible for the appearance of mature lipase in stationary growth phase, which is managed by upregulation of the expression of the processing protease. Whether the lipase expression is also regulated on a transcriptional level still remains to be shown. However, although the relation between the activity of the pro-form and the mature form of the lipase is not known, the high activity of the pro-form in the *agr* mutant does not suggest that there is a strong regulation on the transcriptional level or even any regulation at all.

The virulence of *S. epidermidis* so far has mainly been attributed to its ability to form biofilms on indwelling medical devices (8). We have also found an impact of the *agr* system on this virulence factor and will report on this issue elsewhere.

Taken together, our results suggest that the general role of *agr* in *S. epidermidis* is the same as in *S. aureus*: in an early stage of infection, when the cell density is still low and planktonic cells are present, binding proteins are synthesized to allow colonization of host tissues. When cell clusters are formed on host tissue or on the skin, and the cell density is high, activation of the *agr* quorum-sensing system allows the cells to respond to the changed physiological needs by synthesizing tissue-degrading proteins and other exoproteins. The virulence of *S. aureus* is mainly caused by *agr*-regulated proteins, and deletion of the *agr* system in *S. aureus* may lead to mutants with decreased virulence (1, 3, 5, 21). Animal models will show if global regulators as *agr* have a similar impact on the virulence of *S. epidermidis*.

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