Impact of the *agr* Quorum-Sensing System on Adherence to Polystyrene in *Staphylococcus aureus*

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Biofilm formation by *Staphylococcus aureus* is a serious problem in nosocomial infections. There are great differences in the capacity of *S. aureus* to express biofilms, but the reasons are unknown. In all, 105 *S. aureus* strains were tested for a correlation between the agr quorum-sensing system phenotype and the ability of *S. aureus* to adhere to polystyrene. Some 78% of agr-negative, but only 6% of agr-positive, strains formed a biofilm, demonstrating a profound impact of agr on biofilm formation. This result was confirmed with defined *agr* mutants and by inhibition of agr with quorum-sensing blockers. The observed effect was not due to differential expression of the autolysin Atl or of the exopolysaccharide polysaccharide intercellular adhesin but seemed to be caused, at least in part, by the surfactant properties of δ-toxin. The detected biofilm-enhancing effect of *S. aureus* quorum-sensing blockers call into question the proposed therapeutic use of such substances.

Staphylococcus aureus, a significant human pathogen that causes nosocomial and community-acquired infection, has become resistant to most available antibiotics. In nosocomial infections, the virulence of *S. aureus* is often due to its ability to form biofilm on indwelling medical devices [1]. Biofilm formation is assumed to develop in 2 different stages: primary attachment of the bacteria onto polymer surfaces and cell-cell proliferation to form multilayered clusters. In primary attachment, several factors might be involved (e.g., adhesins and cell wall components) [2]. Proliferation of cells to clusters and further to biofilm layers is mediated by the production of polysaccharide intercellular adhesin (PIA) or poly-*N*-succinyl-β-1,6-glucosamine, both of which are synthesized by the gene products of the *ica* locus [3, 4]. In catheter-associated infections, antibiotic therapy is nearly impossible, because antibiotics can only barely penetrate the slime capsule of the bacteria [1, 5, 6]. Such infections might contribute to the chronic persistence of the bacteria in the human body, causing the requirement for long-term therapy. As a consequence, the search for new agents to combat infections of multiresistant and biofilm-forming bacteria is underway.

Cell-cell communication via quorum-sensing systems affects the expression of virulence factors in many bacteria [7, 8]. Thus, the targeting of quorum-sensing systems with so-called quorum-sensing blockers has been suggested as an alternative means of treating bacterial infections [9]. Because quorum-sensing blockers act only as suppressors of virulence and do not kill bacteria, the development of resistant strains by natural selection is minimized.

The only quorum-sensing system known in staphylococci is the *agr* locus. The *agr* system of *S. aureus* consists of 4 genes *(agrA, agrC, agrD,* and *agrB)* that are cotranscribed (RNAII) and the gene for the effector molecule of the agr system, RNAIII, which also encodes the gene for δ-toxin *(hld)* [10, 11]. The agr system is a true quorum-sensing system that is activated during the transition from the exponential growth phase to the stationary growth phase by an autoregulatory mechanism involving a modified pheromone peptide that signals the state of cell density [8]. Derivatives of the pheromones inhibit the agr system response [12, 13] and efficiently block the development of *S. aureus*–induced lesions in mice [12]. These derivatives have been proposed as quorum-sensing blockers for the treatment of staphylococcal infection.

Materials and Methods

Transposon mutagenesis. *S. aureus* 601055 was transformed by electroporation with the plasmid pTV1ts, which carries the *Enterococcus faecalis* transposon Tn917. The transposon mutagenesis procedure used in this study has been described in detail elsewhere [14]. Transposon-harboring clones were screened for hemolysis on sheep blood agar plates; clones with reduced hemolysis were then assayed for δ-toxin production by analytical high-performance liquid chromatography (HPLC), as described elsewhere [15]. The integration locus of transposon Tn917 in mutant 6 (mut6) was verified by direct sequencing of the chromosomal DNA downstream and upstream of the *erm* resistance gene naturally associated with Tn917. The transposon in mut6 had integrated into the *agrC* gene at the codon encoding amino acid residue 180 (arginine), resulting in a nonfunctional agr system.

Biofilm assay. Biofilm formation of *S. aureus* cells on polystyrene was quantified by using the microtiter plate assay first de-

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scribed by Christensen et al. [16] and modified as noted. Microtiter plates (96 U-bottom polystyrene wells; Greiner, Frickenhausen, Germany) were incubated at 37°C for 24 h without shaking. Biofilm formation was made visible by staining *S. aureus* cells with 0.1% safranin (Serva Feinbiochemica, Heidelberg, Germany) for 30 s. *S. aureus* biofilm formation in microtiter wells was quantified by microtiter plate reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA) with SpectraMaxPro software. Absorbance was measured at 490 nm. For the investigation of the influence of δ-toxin on biofilm formation, purified δ-toxin was added to the biofilm assay at final concentrations of 0.1, 1, and 10 μg/mL at the time of inoculation. Dilutions were made in water.

agr *inhibition assays.* *S. epidermidis* pheromone peptides (the natural thiolactone-containing pheromone peptide, its lactone and lactam derivatives, and 2 thiolactone-containing peptides with an enlarged or shortened *N*-terminal tail, as described by Otto et al. [13, 17], in stock solutions in dimethylsulfoxide [DMSO]) were added to 1:100 dilutions of overnight-grown precultures. The corresponding amounts of DMSO were added to control samples. Aliquots (200 μL) of the diluted *S. aureus* suspensions were dispensed into the microtiter plate wells.

Bacteriolytic enzyme activity analysis. Tryptic soy broth supplemented with 0.5% glucose was inoculated with 1:100 volume precultures, and the cultures were incubated until the stationary phase was reached (12 h). The cell pellets were washed in Tris-buffered saline (TBS; 20 m*M* Tris-HCl buffer and 100 m*M* NaCl, pH 8.0), were resuspended in 1% SDS, and were boiled for 5 min at 100°C, to release cell wall–associated proteins. Lyophilized *S. carnosus* cells were added to the resolving gel of a 10% Tricine SDS-polyacrylamide gel; after electrophoresis, the bacteriolytic enzyme activities (clearing zones) were made visible by staining the gels with methylene blue (0.1% in water) [18].

Immunoblot analyses of PIA production. To quantify the PIA production of *agr*-positive (*agr*$^+$) and *agr* strains, equal amounts of *S. aureus* cells were used. PIA was isolated by extraction of the cells with 0.5 *M* EDTA (pH 8.0) for 5 min at 100°C. The supernatants were then incubated ⩾24 h with Pronase E (20 mg/mL; Sigma-Aldrich Chemie, Deisenhofen, Germany) to cleave protein A. Two 3-μL aliquots of the samples were applied to a nitrocellulose membrane, which then was air dried and blocked with 0.5% skim milk in TBS buffer (150 m*M* NaCl and 10 m*M* Tris-HCl [pH 7.4]) overnight. The nitrocellulose membrane then was incubated for 2 h with αPIA-antiserum (1:5000) and subsequently for 2 h with an αIgG alkaline-phosphatase conjugate (1:5000; Sigma-Aldrich Chemie). Spots were detected by addition of 5-bromo-4-chloro-3-indolyl phosphate (Carl Roth, Karlsruhe, Germany) and nitroblue tetrazolium (Sigma-Aldrich Chemie).

Analytical and preparative purification of δ-toxin. Twelve-hour cultures grown in basic medium (1% tryptone [Difco, Detroit], 0.5% yeast extract [Gibco BRL, Gaithersburg, MD], 0.5% NaCl, 0.1% K_2HPO_4, and 0.1% glucose) were analyzed for production of δ-toxin, as described elsewhere [15]. Larger amounts of δ-toxin were purified by precipitation of culture filtrates of *S. aureus* RN6390 with 1:10 vol of 100% trichloroacetic acid. The precipitate was dissolved in 8 *M* urea after centrifugation at 3000 *g* for 15 min. The resulting solution was injected onto a column (HR 16/10 (filled with SOURCE PHE material); Amersham Pharmacia Biotech, Freiburg, Germany). δ-toxin was eluted with a gradient of 20 column volumes (buffer A, 0.1% trifluoroacetic acid in 10% acetonitrile; buffer B, 0.1% trifluoroacetic acid in 90% acetonitrile) at a flow rate of 4 mL/min. Chromatography was performed on an ÄKTA explorer 100 system (Amersham Pharmacia Biotech). δ-toxin–containing fractions were lyophilized, and the lyophilisate was dissolved in 50% ethanol.

Results

The impact of the *agr* genotype on biofilm formation was investigated first by comparison of wild-type *agr*$^+$ strains with constructed isogenic *agr* strains and second in a study of 105 *S. aureus* strains. In the latter study, we examined a correlation between the adhesion phenotype and *agr* expression. Biofilm formation was assayed by the microtiter plate assay first described by Christensen et al. [16], which measures adherence to polystyrene surface.

agr *mutants show enhanced biofilm formation.* To investigate the biofilm phenotype of constructed *agr* mutants, the laboratory strain *S. aureus* RN6390 was compared with the *agr* deletion strain *S. aureus* RN6911. Furthermore, a transposon-insertion mutant (*S. aureus* mut6) of a clinical strain, in which the *agr* system was inactivated by insertion of the transposon in the *agrC* gene, was compared with the corresponding wild-type strain (*S. aureus* 601055). This mutant was constructed to include a true clinical isolate in our investigation; strain RN6390, in contrast, went through several mutagenesis procedures [10]. As shown in figure 1*A,* biofilm formation was strongly enhanced in the *S. aureus agr* mutants RN6911 and mut6, compared with the isogenic *agr*$^+$ wild-type strains RN6390 and 601055. This suggests that an *agr*$^-$ genotype contributes to a distinct enhancement of biofilm formation on polystyrene.

Correlation between agr *functionality and biofilm formation in* S. aureus. To investigate whether an *agr*-negative (*agr*$^-$) genotype is also statistically correlated with a pronounced biofilm in *S. aureus,* we screened 105 *S. aureus* wild-type strains for biofilm formation and agr functionality. agr functionality was assayed by determining δ-toxin production by HPLC [15]. We measured the production of δ-toxin as a convenient way and alternative to Northern blotting of RNAIII, to analyze agr functionality. We are aware that point mutations within the δ-toxin gene might lead to the possible, yet rare, situation of an *agr*$^+$ but δ-toxin–negative (δ-toxin$^-$) strain. However, Northern analysis of RNAIII can also lead to misclassification of strains: mutations in RNAIII might classify strains as *agr*$^+$, which show an agr phenotype. Because of these possible misclassifications, we included a large number of strains in our investigation. Of the 105 tested strains, 27 (26%) were δ-toxin$^-$ and, therefore, disturbed in the functionality of the agr system (table 1). Biofilm formation of the strains that did not produce δ-toxin (i.e., *agr* strains) was strongly pronounced. Statistical analysis showed a strong correlation between an agr phenotype and adherence. Determined values were $\chi^2 = 54.834$ and $P < .001$. Thus, many

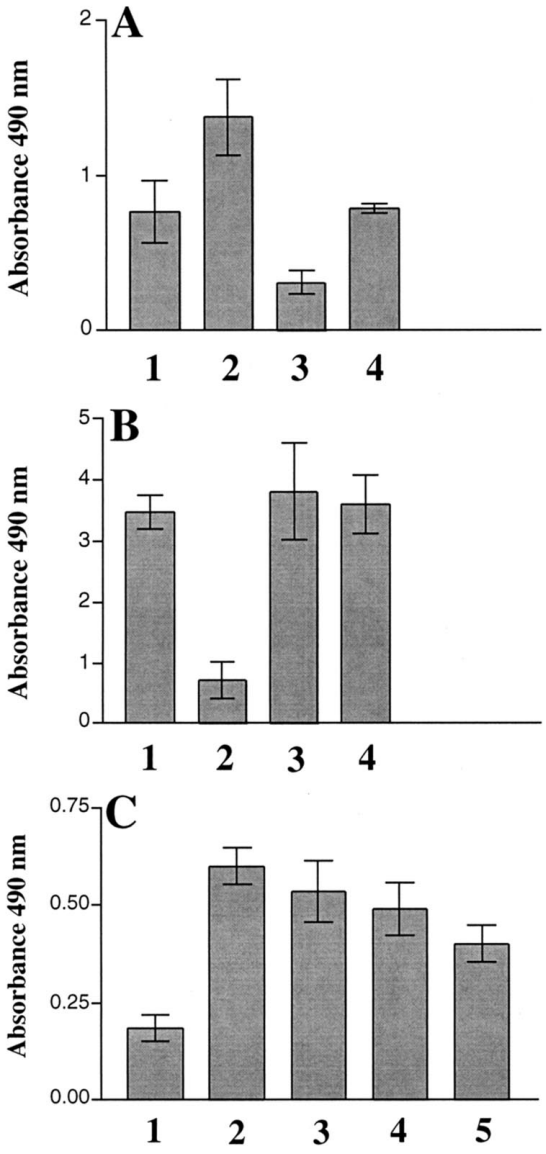

Figure 1. *A,* Biofilm formation of *Staphylococcus aureus* on polystyrene. *Lane 1, S. aureus* RN6390 (*agr*-positive [*agr*$^+$]); *lane 2, S. aureus* RN6911 *(agr); lane 3, S. aureus* 601055 (*agr*$^+$); *lane 4, S. aureus* mut6 *(agr).* All were grown as described in Materials and Methods. Data are mean of 16 microtiter plate wells. *B,* Biofilm formation of *S. aureus* after addition of *S. epidermidis* pheromone peptide. Microtiter plate assay for biofilm formation was done as described in Materials and Methods, using *S. aureus* RN6390 (*agr*$^+$) as indicator strain and *S. aureus* RN6911 as *agr* control strain. Data are mean of 8 microtiter plate wells. *Lane 1, S. aureus* RN6390 (*agr*$^+$) with pheromone; *lane 2, S. aureus* RN6390 (*agr*$^+$) control; *lane 3, S. aureus* RN6911 *(agr)* with pheromone; *lane 4, S. aureus* RN6911 *(agr)* control. *C,* Biofilm formation of *S. aureus* RN6911 *(agr)* after addition of δ-toxin. Microtiter plate assay for biofilm formation was done as described in Materials and Methods, using *S. aureus* RN6911 *agr* strain. Data are mean of 8 microtiter plate wells. *Lane 1, S. aureus* RN6390 (*agr*$^+$); *lane 2, S. aureus* RN6911 *(agr)* control without δ-toxin; *lanes 3–5, S. aureus* RN6911 *(agr)* with addition of δ-toxin (0.1, 1, and 10 μg/mL, respectively). *A–C,* Absorbance measured at 490 nm. Error bars show SD.

S. aureus strains appeared to be naturally occurring *agr* mutants. A distinct correlation was seen between an *agr* background and enhanced biofilm formation.

Expression of Atl and PIA is not under the control of agr. Two factors that mediate biofilm formation on polystyrene have been characterized intensively: AtlE and PIA [19, 20]. To elucidate whether the production of Atl in *S. aureus agr* strains (RN6911 and mut6) differs from that of their isogenic *agr*$^+$ parental strains (RN6390 and 601055), we measured the bacteriolytic enzyme activity of the strains. As shown in figure 2, several autolytic proteins were detected (Atl is marked by an arrow). Further autolytic activities might be due to degradation products of Atl or to different autolysins. The *agr* strains produced the same amount of autolysins or even less than the *agr*$^+$ strains. Therefore, autolysin production cannot be responsible for the increased biofilm formation of *agr* strains.

The production of the intercellular adhesin PIA is necessary for the formation of multilayered cell clusters. A stronger cell-cell clustering caused by overproduction of PIA might also enhance the observed biofilm phenotype in *agr* mutants. To analyze whether the production of PIA is affected by the agr system, we isolated and quantified the PIA from *agr* (RN6911 and mut6) and *agr*$^+$ (RN6390 and 601055) *S. aureus* strains by immuno–dot-blot analyses. The amount of PIA produced by *agr*$^+$ and *agr* strains did not differ (figure 3).

Because the difference in biofilm formation between *agr*$^+$ and *agr* strains could not be attributed to different amounts of Atl or PIA produced, agr is likely to influence biofilm formation via other, possibly hitherto unknown factors, or via factors that have not yet been associated with biofilm formation. One such factor might be the δ-toxin, which is encoded within the part of the *agr* locus, which codes for RNAIII. The strong surfactant properties of δ-toxin [21] might prevent hydrophobic interactions of the cell surface with polystyrene and therefore account for the absence of adherence in *agr*$^+$ strains. To test this hypothesis, we purified δ-toxin from the *agr*$^+$ test strain *S. aureus* RN6390 and added it to biofilm assays, using the corresponding isogenic *agr* strain *S. aureus* RN6911.

In a first experiment, time-dependent biofilm formation of *S. aureus* RN6390 and *S. aureus* RN6911 was compared (figure 4). Differences in biofilm formation were found to develop at the onset of stationary growth phase. At 3–6 h of growth in the microtiter plates, the difference was most pronounced. We determined the amount of δ-toxin in the supernatant of *S. au-*

Table 1. Correlation between *agr* functionality and biofilm formation in *Staphylococcus aureus.*

	Biofilm phenotype	
Strains tested	Positive	Negative
δ-Toxin negative (n = 27)	21 (78)	6 (22)
δ-Toxin positive (n = 78)	5 (6)	73 (94)
Total (N = 105)	26 (25)	79 (75)

NOTE. Data are no. (%) of strains.

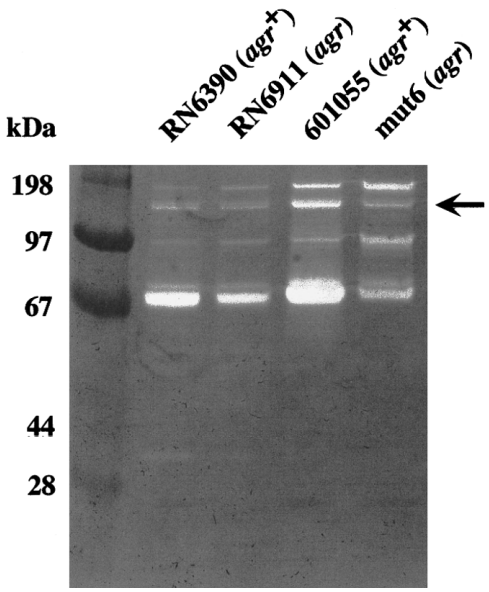

Figure 2. Bacteriolytic enzyme profile of *Staphylococcus aureus* wild-type and *agr* strains. Zymographic analysis of surface-attached proteins isolated from cultures grown to stationary phase was done as described in Materials and Methods. *Arrow,* Lytic bands corresponding to Atl protein. *Left lane,* Molecular mass standard. agr^+, *agr*-positive; mut6, mutant 6.

reus RN6390 during this time of growth in microtiter plates under the same conditions. The δ-toxin concentration was ~1 μg/mL. We used 3 different concentrations of δ-toxin, ranging from 1 log below and above the determined concentration (0.1, 1, and 10 μg/mL). We found that increasing concentrations of added δ-toxin indeed decreased the attachment of the *agr* strain *S. aureus* RN6911 to polystyrene (figure 1*C*). δ-toxin therefore seems to be at least one factor that contributes to the *agr*-dependent differing biofilm formation that we observed.

Quorum-sensing blockers of the agr *system of* S. aureus *artificially enhance biofilm formation.* The use of cross-inhibiting pheromones for the treatment of staphylococcal infections has been proposed, because agr pheromones of one agr subgroup exert an inhibitory effect on other *S. aureus* agr subgroups and because the *S. epidermidis* pheromone inhibits the agr system of *S. aureus* [13, 22]. To evaluate the usefulness of such substances against *S. aureus* infections in which biofilm formation is involved, we artificially inhibited the agr system of *S. aureus* RN6390 with the *S. epidermidis* synthetic peptide pheromone and its derivatives. We previously showed that the *S. epidermidis* pheromone inhibits the *S. aureus* agr system in *S. aureus* Newman and that derivatives of the pheromone exhibit a similar or reduced inhibitory effect [13]. The influence of these substances on the *agr* expression of *S. aureus* RN6390 was tested by the δ-toxin assay and was very similar to the results obtained in *S. aureus* Newman (data not shown). Biofilm formation of *S. aureus* RN6390 increased after addition of the *S. epidermidis* pheromone (DSVCASYF harboring a thiolactone structure between the central cysteine and the C-terminal carboxy group), compared with the control (figure 1*B*). All the derivatives used showed a similar biofilm-enhancing effect at final concentrations of 100 n*M* and 1 μ*M* (data not shown).

Discussion

In this study, we examined the impact of the global regulator agr on biofilm formation in *S. aureus*. With agr^+ (RN6390 and 601055) and constructed *agr* (RN6911 and mut6) *S. aureus* strains, we demonstrated that an agr^- genotype leads to a pronounced attachment to polystyrene, compared with that of the isogenic agr^+ wild type. Furthermore, an investigation with >100 *S. aureus* strains statistically confirmed a strong correlation between an agr^- genotype and enhanced biofilm formation. The influence of the global regulator agr on biofilm formation in staphylococci therefore contrasts with the regulation of biofilm expression by global regulators in the gram-negative species *Pseudomonas aeruginosa* and *Pantoea stewartii* subsp. *stewartii* [23, 24]. In these species, the quorum-sensing systems investigated seem to be responsible for the efficient expression of factors involved in biofilm formation.

The correlation between agr and biofilm formation might provide an answer to the question that has long puzzled researchers of staphylococci: why do some strains form a large amount of biofilm and others do not? Furthermore, as the

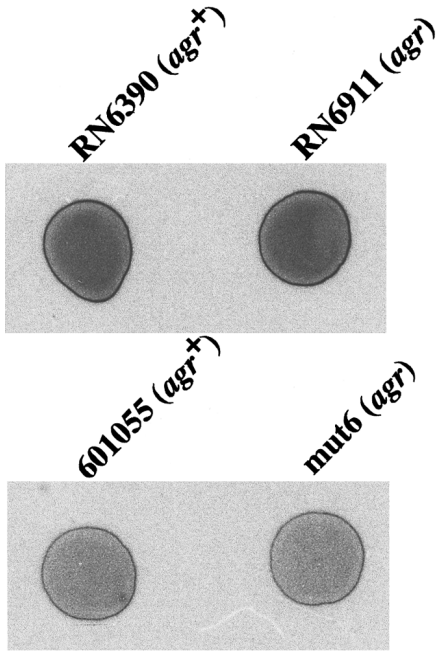

Figure 3. Detection of polysaccharide intercellular adhesin (PIA) in *Staphylococus aureus* wild-type and *agr* strains by immuno–dot-blot analysis. PIA was detected as described in Materials and Methods. agr^+, *agr*-positive; mut6, mutant 6.

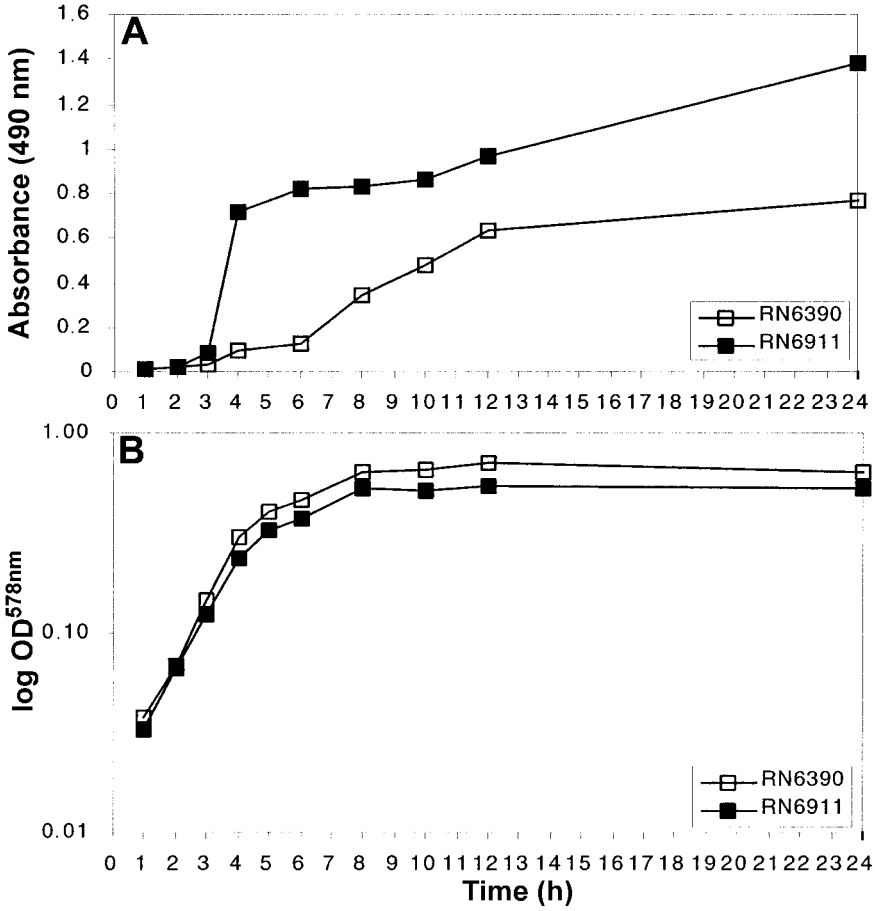

Figure 4. Biofilm formation of *Staphylococcus aureus* wild-type and *agr* strains during growth in microtiter plates. *S. aureus* RN6390 (*agr*-positive [agr^+]) and *S. aureus* RN6911 *(agr)* were grown for 12 h in tryptic soy broth supplemented with 0.5% glucose and were diluted 1:100 in freshly prepared medium. Biofilm assay was done as described in Materials and Methods. *A,* Biofilm formation after 1, 2, 3, 4, 6, 8, 10, 12, and 24 h of incubation; graphic representation of values obtained by photometric determination of absorbance at 490 nm. Data are mean of 16 microtiter plate wells. *B,* Bacterial growth monitored by optical density (OD) at 578 nm; logarithmic representation.

observed increase in biofilm formation of *agr* mutants was not found to be due to altered production of Atl or PIA, other *agr*-regulated factors probably contribute significantly to the attachment of *S. aureus* to polymer surfaces. We performed experiments that suggest an involvement of δ-toxin, which has surfactant-like properties [21], in the absence of adhesion of agr^+ strains to polystyrene. It remains obscure which advantage the strong production of δ-toxin offers to the bacteria. The erythrocytolytic and immunoinflammatory properties of δ-toxin, for example, are known [25], and, only recently, Mehlin et al. [26] identified δ-toxin as part of an inflammatory polypeptide complex, which may contribute to sepsis. We propose that δ-toxin might also serve as a surfactant in vivo that prevents adherence of staphylococcal cells to surfaces. Surfactants may also decrease the adherence of staphylococci to biomaterials. This was recently shown for designed synthetic surfactant polymers [27]. δ-toxin might exert similar effects during staphylococcal growth on indwelling devices. The bacteria could thus avoid unfavorable sticky situations, but further experiments on the role of δ-toxin in adherence are needed.

It appears that *S. aureus* has developed various pathways of pathogenesis to survive in a hostile environment. In an acute infection, the expression of host tissue–attacking toxins, as present in agr^+ strains, seems to be important for bacterial virulence, whereas, in chronic infections, an agr^- phenotype contributes to increased persistence. Our results suggest that the enhanced ability of *S. aureus agr* strains to adhere to polymers must be considered an advantage in catheter-associated infections. The high frequency of naturally occurring *agr* mutants (26%) supports the thesis that *agr* mutants have adapted to an ecologic niche in which adhesive properties are important.

Attenuation of acute infection in mice caused by *S. aureus* agr^+ cells was demonstrated by Mayville et al. [12] by inhibiting the agr system. Therefore, they proposed agr quorum-sensing blockers (on the basis of the thiolactone-containing *agr* pheromones) as potential antistaphylococcal therapeutic compounds.

By inhibiting the agr system of *S. aureus* RN6390 with the synthetic *S. epidermidis* pheromone and derivatives thereof in our study, we observed an enhanced adhesion to polystyrene. These results suggest that the application of quorum-sensing blockers in antistaphylococcal therapy should be limited to acute infections. The treatment of persistent staphylococcal infections by such agents, especially in patients with indwelling medical devices, is obviously counterproductive. In addition, the use of these substances might also transform an acute staphylococcal infection into a chronic infection.

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