Transcription of *Staphylococcus aureus* Fibronectin Binding Protein Genes Is Negatively Regulated by *agr* and an *agr*-Independent Mechanism

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The production of cell surface proteins in *Staphylococcus aureus* is generally down-regulated in the postexponential growth phase by the global regulator *agr*. The effector of this regulation is the RNAIII molecule, which is encoded within the *agr* locus. RNAIII seems to regulate most target genes at the level of transcription, but it also has an effect on the translation of some genes. To study the role of *agr* on the expression of fibronectin binding proteins (FnBPs), we investigated the transcription and translation of *fnb* genes in *agr* mutant strain WA250 and its parent strain, 8325-4. The results show that *fnb* genes are negatively regulated by *agr* and also by an *agr*-independent mechanism that restricts *fnb* mRNA synthesis to the early exponential phase of growth. Transcription and Western blot analysis of cell-associated FnBPs demonstrated that synthesis of both FnBPA and FnBPB in the wild-type and *agr* mutant strains took place preferentially during the first hour of growth and rapidly decreased after the second hour. We also confirmed previous results showing that the *agr* mutant strain has an increased capacity to bind fibronectin compared to its parent *agr*+ strain. However, while the concentrations of *fnb* mRNAs and proteins differed by a factor of 16 between the strains, the difference in fibronectin binding was only twofold, indicating that the binding of fibronectin to the bacteria is not proportional to the amount of FnBPs on their surface.

The initial step in an infectious disease is often adhesion to and colonization of host tissue surfaces. *Staphylococcus aureus*, which is a major human and animal pathogenic organism, has been shown to bind to several host matrix proteins and plasma proteins, such as fibronectin, fibrinogen, collagen (11), elastin (35), laminin (16, 41), prothrombin (4), thrombospondin (15), bone sialoprotein (37), and vitronectin (24, 25). For each of these binding functions, a corresponding surface-associated protein has been identified. The existence of an *S. aureus* extracellular matrix binding protein with broad specificity that is capable of binding several extracellular glycoproteins has also been reported (27). The role of some of these proteins in the pathogenesis of staphylococcal infections has been shown in animal models (10, 11, 28, 34).

Most *S. aureus* strains bind to fibronectin and two highly homologous fibronectin binding proteins (FnBPs), and their corresponding genes (*fnbA* and *fnbB*) have been identified (9, 12, 21). Mutants defective in either of the two genes adhered equally well to fibronectin-coated surfaces in vitro, while a double mutant was completely unable to adhere, indicating that both genes are expressed and contribute to fibronectin binding (13).

The ability of *S. aureus* cells to bind fibronectin is affected by two global regulatory systems, *agr* and *sar* (1, 3, 6, 7). Of these, *agr* is the best characterized and has been shown to act at the levels of both transcription and translation to regulate the production of many secreted toxins, enzymes, and cell surface proteins (29, 30, 36). Toxins and enzymes are generally positively controlled, while the production of cell surface proteins is negatively controlled (1, 18, 33). The effector of this positive and negative regulation is an RNA molecule, RNAIII (17, 30, 33), which is synthesized in response to the environmental concentration of an autocrine octapeptide synthesized by *agrB* and *agrD* (20). The octapeptide concentration is sensed by the *agrC* product, which resembles the signal sensor of classical two-component systems (20). AgrC is supposed to phosphorylate AgrA, which then activates transcription of the RNAIII-encoding gene. The *sar* locus, which appears to modulate the activity of *agr* (8, 14, 31), also seems to have a direct effect on fibronectin binding, which was decreased in *sar* mutants (3, 6, 7).

In several reports, *agr* mutants have been shown to bind more fibronectin than the corresponding wild-type strains (1, 6, 7). Considering the high sensitivity of FnBP to proteolytic degradation (13, 21), the increased fibronectin binding in the *agr* mutants could be due to decreased production of proteases rather than to increased synthesis of FnBPs.

To study the role of *agr* on the expression of FnBPs, we investigated the transcription and translation of *fnb* genes in an *agr* mutant and the corresponding wild-type strain. Our results show that the *fnb* genes are negatively regulated by *agr* and by an *agr*-independent mechanism that restricts FnBP synthesis to the early exponential phase of growth.

**MATERIALS AND METHODS**

**Bacterial strains.** *S. aureus* 8325-4 is our standard *agr*+ strain, which is a derivative of NTCC8325 cured of prophages (32). WA250, derived from 8325-4, has a Tn551 insertion in the *agrA* gene (29). RN6911 is a derivative of RN6390B in which the *agr* locus has been replaced with tetM (33). DU5883 is a derivative of 8325-4 in which the *fnbA* and *fnbB* genes have been inactivated by insertion mutagenesis (13). *Escherichia coli* MC1061 (5) was used as the host for plasmid constructions.

**Growth conditions.** *S. aureus* strains were routinely precultured in tryptic soy broth (TSB; Difco) for 16 to 18 h. Cells from 2 ml of preculture were transferred to 100 ml of brain heart infusion (BHI; Difco) in a 1-liter baffled flask and grown with shaking at 37°C until the cultures reached the late exponential phase of growth. Bacteria were harvested by centrifugation at 3000 × g for 1 min at 4°C. Cell pellets were washed once in PBS (0.1 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4) and resuspended to the desired concentration in PBS. Cells were sonicated in an ice-cold water bath for 30 s to disrupt the cell wall.

**RNA isolation and Northern blot analysis.** Cells were harvested by centrifugation at 3000 × g for 1 min at 4°C and resuspended in TRIzol reagent (Invitrogen;rvine, CA). Total RNA was isolated according to the manufacturer’s instructions. RNA was treated with RNA-free DNase (200 unit/ml; Promega; Madison, WI) to eliminate contaminating genomic DNA. After purification on a 1% agarose/TBE gel, RNA was transferred to a positively charged nylon membrane (Roche; Mannheim, Germany) using a vacuum blot apparatus (Bio-Rad; Hercules, CA). The membranes were then exposed to a UV cross-linker (Stratalinker; Stratagene; La Jolla, CA). The RNA was hybridized to a 32P-labeled *fnbA* mouse fetal liver cDNA probe, which was synthesized using the Superscript cDNA synthesis kit (Invitrogen). The probe was annealed to the membrane by incubating with ribonuclease-free water and 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 1 h. After hybridization, the membranes were washed three times in 2× SSC and 0.1% SDS, followed by 0.1% SDS. Finally, the membranes were exposed to X-ray film (SAR; Dupont; Wilmington, DE) for 1 to 2 days.
incubated on a rotary shaker at 37°C. Cultivation for fibronectin binding assays was done with TSB (Difco) supplemented with 0.6% yeast extract (Difco). Bacterial growth was monitored by measuring optical density at 600 nm (OD600). Wherever needed, antibiotics were added as follows: erythromycin, 10 µg ml⁻¹; tetracycline, 5 µg ml⁻¹.

Construction of plasmids and transformation. Competent E. coli cells were prepared and transformed as described by Sambrook et al. (38). Transformants were selected on Luria-Bertani plates containing 50 µg of ampicillin ml⁻¹. Chromosomal S. aureus DNA was prepared as described by Lotfalih et al. (26). Plasmid DNA was extracted by the alkaline lysis method (38) and further purified by using JetSorb (Genomed). pEX107 contains an internal fragment of the RNAII-encoding gene (nucleotides [nt] 1187 to 1565 in the published sequence [18]) in the antisense orientation behind the T3 promoter of pEX100 (30). For pWA1, a 534-bp DNA fragment (nt 185 to 719 in the published sequence [39]) containing part of the fnhA gene was synthesized by PCR using the primers 5'-CAATGATCGTTGTTGGGATGGGAC-3' and 5'-ACTTTACTTGTTACA-3. The PCR product was cloned into pGEM-T (Promega). The orientation of the insert, assessed by restriction enzyme cleavage, was such that an fnbA antisense RNA was produced from the P6 promoter of the plasmid.

In vitro synthesis of digoxigenin-labeled antisense RNA probes. pEX107 was used as the template for synthesis of an antisense RNAIII probe. The plasmid was linearized with EcoRI and transcribed by using T3 RNA polymerase as described by the supplier (DIG RNA Labeling Mix; Boehringer Mannheim). An antisense fnbA probe was synthesized from pWA1. The plasmid was digested with PstI, and the promoterless 3′ termini generated were removed by using the Klenow fragment of E. coli DNA polymerase I. The antisense transcript was generated by Sp6 polymerase as described above.

Northern blotting and primer extension analysis. Total S. aureus RNA was prepared as previously described (19). Northern blot analysis was performed as described by Sambrook et al. (38) by using positively charged nylon membranes (Boehringer Mannheim). Hybridization was carried out as described by the manufacturer (Boehringer Mannheim). Subsequently, the membranes were analyzed by chemiluminescence detection (DIG Luminescent Detection Kit; Boehringer Mannheim). Primer extension experiments were performed as previously described (31), by using 1 pmol of primer 5′-GTCTTCATATGTTGCGGTTGTGCAGCA-3′ (nt 428 to 453 [39]) for fnhA or 1 pmol of primer 5′-CGCGAAGGTTTCATTTTTGGTTGC-3′ (nt 820 to 842 [21]) for fnbB labeled with [γ-³²P]ATP (Amersham). Twenty micrograms of S. aureus total RNA was used as the template. Radioactivity was detected by a radioisotope imaging system (PhosphorImager 445SI; Molecular Dynamics). Northern blot and primer extension images were processed by using ImageQuant software.

Analysis of FnBPs by Western blotting. Cell-associated FnBPs were released by lysostaphin treatment of equal number of cells (OD₆₀₀ of 1.2) as previously described (19). Culture supernatants were dialyzed against water and concentrated 100-fold by hophilization. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% acrylamide resolving gels as previously described (22). After being transferred to nitrocellulose membranes (Schleicher & Schuell), FnBPs were detected with goat anti-serum against the fibronectin binding D domains of FnBPA from S. aureus (gift from L. K. Rantamäki, University of Helsinki, Helsinki, Finland). For development, rabbit anti-goat peroxidase-conjugated antibody (DAKO) was used as recommended by the supplier.

Assay for fibronectin binding. Binding of fibronectin to S. aureus cells was measured as described earlier (9). Washed S. aureus cells (10⁶ CFU) harvested at different times during growth were incubated with the thirty²⁻labeled (90,000 cpm) 24-KDa amino-terminal fragment of bovine fibronectin (21) in phosphate-buffered saline (PBS) containing 0.1% Tween 20 in a total volume of 0.3 ml.

RESULTS

Effect of agr on fnb transcription. Transcription of the fnb genes in strain 8325-4 and the agr mutant WA250 was analyzed by Northern blotting. Samples for RNA analysis were taken at intervals during the exponential and postexponential phases of growth. In numerous experiments using different culture media (BHI, TSB, and casein hydrolysate-yeast extract with and without glucose), fnb mRNA was detected only in the agr mutant (Fig. 1), suggesting that transcription of the fnb genes is negatively regulated by agr. Analysis of twofold serial dilutions of total RNA from both strains revealed that the agr mutant produced at least 16 times as much fnb mRNA as the wild-type strain (data not shown). Unlike that of other negatively regulated genes (i.e., spa and coa) which are constitutively expressed in agr mutants (23, 40), transcription of fnb was restricted to the first hour of growth (lag phase and first cell division).

As expected, no RNAIII was detected in the agr mutant (WA250) throughout the growth experiment (data not shown), indicating that the down-regulation of fnb expression at the beginning of the exponential growth phase was due to an agr-independent mechanism. This was confirmed by the observation of the same pattern of fnb expression in another agr mutant, RN6911, lacking the entire agr locus, including the RNAIII gene. In wild-type cells, large amounts of RNAIII were present at zero time; these decreased dramatically during the first 30 min of growth and then increased again after 2 to 3 h (Fig. 1). The presence of RNAIII during the lag phase and the early exponential phase of growth thus seemed to repress the transcription of fnb genes completely.

When wild-type bacteria were precultured in BHI instead of TSB, much less RNAIII was present at zero time and the level decreased below the detection limit within the first hour of growth in fresh BHI medium. Under these conditions, significant amounts of fnb mRNAs were found in the 1-h sample (see lane 0 in Fig. 2). When these cells, without any detectable RNAIII, were used to inoculate fresh BHI medium, there was no further increase in fnb mRNAs, in spite of the fact that RNAIII remained below the detection limit until 2 h (Fig. 2). Essentially the same result was obtained with agr mutant strain WA250 (data not shown). Together, these results show that transcription of the fnb genes is restricted to the first hour of growth (1 to 2 cell divisions) immediately after the bacteria have been transferred from a stationary-phase culture to fresh
medium and that this temporal regulation is independent of \textit{agr}.

Primer extension experiments were carried out to verify the Northern blot results and to check that both \textit{fnb} genes were expressed. Based on the differences between the nucleotide sequences in the 5' end of the \textit{fnb} genes (21), two nucleotide primers were designed to anneal specifically to the \textit{fnbA} and \textit{fnbB} mRNAs, respectively. Product lengths were calculated according to the reported locations of the transcription start points (13) as 402 nt for \textit{fnbA} and 389 nt for \textit{fnbB}. As shown in Fig. 3, products of the expected sizes were obtained when total RNA from the \textit{agr} mutant was used as the template while no products were obtained with equal amounts of wild-type RNA. As for the Northern blot analysis, \textit{fnb} transcripts were detected only during the first hour in the mutant strain. This confirmed that both genes were expressed and that they were negatively regulated by \textit{agr}.

**Analysis of cell-associated FnBPs in \textit{agr}+ and \textit{agr} mutant strains.** Production of cell-associated FnBPs during the exponential and postexponential phases of growth was assessed by Western (immunoblotting) analysis of lysostaphin extracts from wild-type and \textit{agr} mutant cells. The antibodies used for detection were directed against the fibronectin binding domains (D1 to D3), which are 95% identical in FnBPA and FnBPB. Two major proteins with apparent molecular masses of around 200 kDa were seen in extracts from both the wild type and the \textit{agr} mutant (Fig. 4), which is in agreement with the reported values for the native forms of FnBPA and FnBPB (12, 13, 21). Several smaller bands of variable sizes which reacted with the antibodies probably represent degradation products of the FnBPs, as they were not seen in extracts from a mutant lacking both FnBPA and FnBPB (DU5883) (Fig. 4). Analysis of twofold dilution series of extracts from the \textit{agr} mutant revealed that the amount of FnBPs was more than 16 times higher than that in the wild-type strain, which is in agreement with the difference in \textit{fnb} mRNA concentration between the strains.

As seen in Fig. 4, de novo synthesis of FnBPs took place preferentially during the first hour of growth, which is consistent with the transcription analysis (Fig. 1 and 2). Full-length FnBPs decreased dramatically between 2 and 3 h and were not seen in extracts from bacteria taken after 16 h of growth (Fig. 4, lane zero samples represent preculture cells grown for 16 h). The nature of the smaller bands seen in time zero samples is not known.

As only trace amounts of FnBPs, or their degradation products, were found in culture supernatants from the \textit{agr} mutant during the exponential phase of growth (data not shown), we conclude that the majority of FnBPs were associated with the bacterial cells. No proteins were detected by the anti-FnBP serum in supernatants from the \textit{wild-type} strain during the exponential phase of growth.

**Binding of fibronectin to \textit{agr}+ and \textit{agr} mutant strains.** Previous studies have shown that stationary-phase cells of \textit{agr} mutants bind 0.5 to 4 times as much fibronectin as do those of the corresponding wild-type strain (1, 6, 7). To correlate fibronectin binding with our present data on the expression of the \textit{fnb} genes, wild-type and \textit{agr} mutant cells, harvested at hourly intervals during the early phase of growth, were incubated with radiolabeled fibronectin as described in Materials and Methods. As shown in Fig. 5, fibronectin binding increased significantly during the first hour of growth in both strains, the mutant reaching twice the level of the wild-type strain. This was consistent with the simultaneous increase in the expression of the \textit{fnb} genes and suggests that the fibronectin binding was mediated by the de novo-synthesized FnBPs. However, while the concentrations of \textit{fnb} mRNAs and proteins differed between the strains by a factor of 16 the difference in fibronectin binding was only twofold, indicating that the in vitro binding of
fibronectin to the bacteria is not proportional to the amount of FnBPs on their surface.

**DISCUSSION**

In the present study, we have shown that the transcription of fnb genes in *S. aureus* is negatively controlled by agr and also by another, agr-independent, mechanism. Under the conditions used, fnbA and fnbB seem to be coordinately regulated, which is consistent with previous studies using fnb promoter fusions (13). It has been shown earlier that transcription of the genes coding for protein A (spa) and coagulase (coa) are also negatively regulated by agr and that the effector of this regulation is RNAIII (23, 40). In agr mutants, lacking RNAIII, spa and coa are therefore transcribed throughout the exponential and post-exponential phases of growth (23, 40). In contrast, synthesis of fnb mRNA in two different agr mutants (WA250 and RN6911) was restricted to the first hour of growth, corresponding to the lag phase and the first cell division. Since RN6911 lacks the whole agr locus, including the RNAIII gene, it can be con-

cluded that this temporal expression of the fnb genes must be due to agr-independent regulation.

According to the general model for the agr system, genes that are negatively regulated by agr should be expressed in wild-type strains during the early exponential phase of growth, before RNAIII starts to accumulate (17, 29, 36). This has also been demonstrated for spa and coa (23, 40). However, since fnb expression was restricted to the first hour of growth and high concentrations of RNA III were present in the wild-type bacteria at the beginning of this period of time, only trace amounts of fnb mRNA could be detected. Although the concentration of RNAIII decreased rapidly during the first 30 min of growth, trace amounts were still present after 1 h (Fig. 1). This is consistent with an RNAIII half-life of 15 min (2). Our results also indicate that transcription of the fnb genes is much more sensitive to RNAIII repression than is that of coa, which was expressed even at relatively high RNAIII concentrations (23).

In wild-type cultures with less initial RNAIII (precultured in BHI), the concentration of RNAIII decreased below the detection limit within the first hour of growth and allowed some transcription of fnb to occur. However, transcription again seemed to be restricted to the first hour of growth although RNAIII was not detected. After 1 h of growth, fnb transcription did not continue even when the bacteria were shifted into fresh medium (Fig. 2; data not shown for the agr mutant). This suggests that it is not some factor in the fresh culture medium that stimulates transcription of the fnb genes during the early exponential phase of growth, but rather it is the metabolic status of the bacterial cells that determines whether transcription takes place or not. It thus seems that stationary-phase bacteria which are transferred to fresh medium become competent for fnb transcription while cells that have been growing exponentially for 1 h do not.

The rapid increase in cell-associated FnBPs and fibronectin binding during the first hour of growth was consistent with the results of the mRNA analysis in both wild-type and agr mutant cells. However, while the fnb mRNA or FnBP levels differed by a factor of 16 between the strains the difference in fibronectin binding was only twofold. A possible explanation for this discrepancy is that other cell surface components that are up-regulated in the agr mutants interfere with fibronectin binding or that binding is simply not proportional to the number of FnBPs on the bacterial cells because of sterical reasons.

In summary, the present results show that expression of the fnb genes in *S. aureus* is negatively regulated by agr, and by an agr-independent mechanism, in such a way that synthesis of FnBPs is restricted to a short period of time in the early exponential phase of growth. This means that the ability of *S. aureus* cells to adhere to fibronectin in vivo probably changes dramatically during the course of infection. To fully understand the role of fibronectin binding in the infection process, the expression of agr and the fnb genes needs to be studied in vivo.

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