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Influence of *agr* on Fibrinogen Binding in *Staphylococcus aureus* Newman

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The ability of *Staphylococcus aureus* to bind fibrinogen is believed to be important in promoting bacterial adherence to both intravascular catheters and host tissues during infection. We investigated the influence of the global regulator *agr* on the fibrinogen binding capacity and its relationship to the expression of coagulase (encoded by *coa*) and clumping factor (encoded by *clfA*) in strain Newman. Strains were obtained by transducing site-specific mutations of *clfA*, *coa*, and *agr* into strain Newman to obtain single, double, and triple mutants of the respective genes. As expected, the *clfA* mutant bound less soluble ^{125}I-labeled fibrinogen than the corresponding *coa* mutant in *agr*$^+$ strains; however, with *agr* mutant strains, the upregulation in fibrinogen binding capacity correlated mostly with the increased expression and transcription of coagulase as shown by Western (immunoblot) and Northern (RNA) blot analysis. In particular, the *coa agr* double mutant resulted in a significant reduction in fibrinogen binding compared with that of the *agr* mutant. The contribution of *clfA* to fibrinogen binding in *agr*-negative strains was less than that of *coa* (32,740 ± 1,189 versus 18,141 ± 334 and 38,919 ± 1,021 cpm for *clfA agr*, *coa agr*, and the single *agr* mutant, respectively). Thus, coagulase is a major binding protein for soluble fibrinogen in the *agr*-negative background. In in vitro microtiter and catheter adherence assays with solid-phase fibrinogen, clumping factor, but not coagulase, plays a major role in binding to immobilized fibrinogen. *coa* transcription was negatively modulated by *agr* and occurred mainly during the exponential growth phase. In contrast, *clfA* transcription was *agr* independent and was strongest during the postexponential phase. Although an *agr coa clfA* triple mutant bound less soluble fibrinogen than the *agr coa* double mutant (8,504 ± 831 versus 18,141 ± 334 cpm), significant residual fibrinogen binding capacity remained in the triple mutant, thus suggesting an additional fibrinogen binding component. By using direct ligand affinity blotting with ^{125}I-fibrinogen, we could identify coagulase and an additional unidentified 52-kDa protein as a fibrinogen binding component in cell extracts. This band was absent in the extract of the *coa clfA* double mutant.

Staphylococcus aureus is an important human pathogen that causes a variety of infections. Plasma and matrix proteins such as fibrinogen or fibronectin have been thought to act as bridging molecules to facilitate the attachment of the bacteria to various surface substrata (e.g., catheters or endothelium cells) (7, 9, 30). Recent ex vivo data by Vaudaux and coworkers (29) have highlighted the important role of fibrinogen in mediating *S. aureus* adherence to intravascular catheters. In that regard, genes for several *S. aureus* surface proteins with specific binding activities to fibrinogen have been cloned and sequenced. Among these, the coagulase (*coa*) (21, 25) and clumping factor (*clfA*) (20) genes are the best described. Coagulase is responsible for the ability of *S. aureus* to clot plasma, while clumping factor mediates cell clumping in the presence of fibrinogen. The genes for clumping factor (*clfA*) and coagulase (*coa*) reveal no sequence similarity and are not physically linked. Recombinant clumping factor (20) and a fusion protein containing the C terminus of recombinant coagulase (25) were shown to bind fibrinogen. With a Tn*551*-mediated *clfA* mutant, it was further demonstrated that clumping factor mediates bacterial binding to solid substrata (e.g., catheters) coated with plasma proteins (29). In contrast, a *coa* mutant did not (21).

Two additional fibrinogen binding proteins, FibA (3, 4) and FbpA (10), with various degrees of sequence homology to coagulase have subsequently been described. In addition, an *S. aureus* surface protein that exhibits broad-spectrum binding to several mammalian matrix proteins, including fibrinogen (Map [12]), was characterized. Conceivably, this protein may also contribute to the overall fibrinogen binding of *S. aureus*. Because neither site-specific mutants nor antibodies against FibA, FbpA, and Map are available, the exact role of these proteins in fibrinogen binding by staphylococcal cells remains to be determined.

An evaluation of the binding properties of *S. aureus* to host proteins will likely contribute to understanding of the infectious processes. In addition to examining the specific adhesion-receptor interaction on the biochemical level, it seems reasonable to explore the regulation and expression of these adhesion molecules. The regulation of fibrinogen binding proteins in *S. aureus* is only partially understood. Analysis of mutants defective in global regulatory loci such as *agr* (1, 13) and *sar* (2, 8) indicated that at least some of the fibrinogen binding proteins are regulated by one or both of these regulators. For instance, the *sar* locus seems to be involved in the upregulation of clumping activity (8). In contrast, the *agr* locus has been thought to be involved in repressing the expression of coagulase (1, 13).

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TABLE 1. Fibrinogen binding, clumping activity, and coagulase activity in *S. aureus* Newman and derivatives

Strain	Genotype	^{125}I-fibrinogen binding (cpm/10^8 cells ± SE)	Clumping activity (titer)[a]	Cell-associated coagulase activity (titer)[b]
Newman	Wild type	13,268 ± 1,971	128	16
DU5852	*clfA*::Tn*917*	6,359 ± 284	ND[c]	16
DU5872	Δ*coa*::*tetK*	10,499 ± 729	128	ND
ALC620	*clfA*::Tn*917* Δ*coa*::*tetK*	4,304 ± 322	ND	ND
ALC355	Δ*agr*::*tetM*	38,919 ± 1,021	128	256
ALC473	Δ*agr*::*tetM clfA*::Tn*917*	32,740 ± 1,189	ND	256
ALC619	Δ*agr*::*tetM* Δ*coa*::*tetK*	18,141 ± 334	128	ND
ALC621	Δ*agr*::*tetM clfA*::Tn*917* Δ*coa*::*tetK*	8,504 ± 831	ND	ND

[a] Reciprocal of the highest dilution of fibrinogen (starting with 10 μg per well) showing cell clumping (10^8 cells per well).
[b] Reciprocal of the highest dilution of lysed protoplasts from postexponential cells, which shows clotting after incubation with rabbit plasma.
[c] ND, not detectable.

In this study, the influence of *agr* on fibrinogen binding was investigated. Site-specific mutants of *coa* and *clfA* in *agr*-positive and *agr*-negative backgrounds were constructed and analyzed. Our data provided evidence that in an *agr*-positive background, the clumping factor is the major contributor of soluble fibrinogen binding activity, while in an *agr*-negative background, the coagulase is a major binding component. However, only clumping factor mediates *S. aureus* binding to immobilized fibrinogen. Additional expression studies indicated that the *coa* gene is transcribed primarily during the exponential growth phase and is upregulated in *agr*-negative strains. In contrast, the *clfA* gene is strongly expressed during the postexponential phase in an *agr*-independent manner.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Strains were grown from stock culture on tryptic soy agar with 4% sheep blood and checked for hemolysin production. Cells were precultured overnight in CYPG (23) in appropriate antibiotics, diluted 1:100 in 10 ml of CYPG, and grown with aeration at 37°C. The antibiotics were used at the following concentrations: erythromycin, 10 μg/ml; tetracycline, 5 μg/ml; and minocycline, 2 μg/ml.

Construction of mutant strains. Bacteriophage φ11 was used to transduce the *agr* mutation from strain RN6911 (Δ*agr*::*tetM*) (24) into strain Newman, DU5852 (*clfA*::Tn*917* [20]), and DU5872 (Δ*coa*::*tetK* [21]). The method for the preparation of phage lysates and transduction was previously described (8). To construct the *coa agr* double mutant, we transduced the Δ*agr*::*tetM* mutation into DU5872 and selected for minocycline resistance. This is feasible because the *tetM* gene confers broad-spectrum resistance against tetracycline derivatives, including minocycline, whereas the *tetK* marker in the *coa* mutant DU5872 is narrow spectrum and therefore confers only tetracycline resistance but not minocycline resistance. The *clfA* mutation was transduced from DU5852 into the *coa* mutant DU5872 and into the *agr coa* double mutant ALC619 with selection for erythromycin resistance.

The following primers were used for PCRs to generate probes for Southern blotting: *clfA* upper primer, TAAAAAGAGGGAATAAAATGA; *clfA* lower primer, CACTTGTATTAACCGCTTGAT (nucleotides 285 to 929 [20]); and *agr* upper primer, CAACATTGGCATTCATTACT; *agr* lower primer, TTACACC ACTCTCCTCACTG (nucleotides 555 to 1754 [13]). Chromosomal DNA was prepared and digested with *Eco*RI and *Pst*I, and then the fragments were hybridized with ^{32}P-labeled PCR fragments (8). All transductants revealed the anticipated changes in the hybridization patterns (data not shown). In addition, all strains demonstrated the expected phenotypes in clumping and coagulase activities and in hemolysin production on blood agar plates.

Measurement of cell clumping and coagulase activity. Cell clumping was tested in microtiter plates by mixing 10 μl of washed bacterial cells (10^8 CFU) with 50 μl of fibrinogen, serially diluted twofold from a starting concentration of 1 μg/ml in phosphate-buffered saline (PBS). The reciprocal of the highest dilution of fibrinogen which induced clumping after 10 min was recorded. Coagulase activity was measured by addition of 100 μl of serial twofold dilutions of sample solutions to 200 μl of rabbit plasma (Difco Laboratories, Detroit, Mich.). The titer was the reciprocal of the highest dilution of samples that caused clotting after a 4-h incubation period at 37°C.

Binding of soluble ^{125}I-fibrinogen to bacterial cells. Equivalent numbers of washed cells (5×10^8 CFU) harvested from the postexponential phase were incubated with ^{125}I-labeled fibrinogen (~100,000 cpm) for 2 h at 37°C, and the bound radioactivity was measured (8). Strains were tested in quadruplicate in at least three independent experiments with the same relative results. Results from one typical experiment are shown in Table 1.

Bacterial adherence to solid-phase fibrinogen. Two methods were used to evaluate the attachment of bacteria to solid-phase fibrinogen. In the first method, microtiter plates (Immunolon 2; Dynatech Laboratories, Chantilly, Va.) were coated with serial dilutions of fibrinogen containing 2 mg of bovine serum albumin (BSA) per ml in PBS. After incubation at 37°C for 1 h, the plates were washed four times with PBS, and 50 μl of washed bacterial cells (5×10^8 CFU) was applied to individual wells. Plates were agitated at room temperature for 2 h. The wells were washed four times with PBS for 10 min each. Bound cells were fixed with 25% formaldehyde and stained with crystal violet. The microtiter plate was read in an enzyme-linked immunosorbent assay (ELISA) reader at 560 nm. The background values from BSA-coated wells ($1{,}000 \times A_{560} = 72 \pm 9.7$) were subtracted from sample values.

For the second method, cell binding to fibrinogen-coated catheters was assayed as previously described (7). Briefly, sections of urethane catheters (Cook, Inc., Bloomington, Ind.) were coated with a solution of fibrinogen (2 mg/ml) and BSA (2 mg/ml) in PBS, washed extensively, and incubated with [*methyl*-^{3}H]thymidine-labeled *S. aureus* cells (10^9 CFU) for 2 h. Catheters were washed four times, and the bound radioactivity was measured. Cells were labeled by inoculating an overnight culture (1:200 dilution) into 10 ml of CYPG containing 200 μCi of [*methyl*-^{3}H]thymidine. After growing (50 rpm at 37°C) for 18 h, cells were harvested and adjusted to the same optical density. All strains showed similar specific activities.

Protein isolation, SDS-PAGE, and Western blotting (immunoblotting). Cell-associated proteins were prepared from equivalent numbers of cells (2.5×10^{10} CFU) after digestion with lysostaphin in the presence of a hypertonic buffer (30% raffinose) and proteinase inhibitors (6). The digestion mixture was centrifuged ($5{,}000 \times g$ for 10 min). The supernatant contained the cell wall extract (600 μl), while the pellet was lysed in 400 μl of double-distilled H_2O to obtain protoplast lysates. Extracellular proteins were precipitated from culture supernatants with 12% trichloroacetic acid and dissolved in sodium dodecyl sulfate (SDS)-sample buffer to gain a 20-fold concentration. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on SDS–10% polyacrylamide gels (15) and transferred to nitrocellulose filters by electroblotting. To detect clumping factor and coagulase, filters were incubated with 2% BSA and 0.1% human serum to minimize the binding of antibodies to protein A. The blots were incubated with rabbit antibodies (dilution, 1:2,000) against coagulase (a gift from H. Igarashi, Tokyo Metropolitan Research Laboratory of Public Health) or against recombinant clumping factor (19) followed by incubation with the F(ab′)$_2$ fragment of sheep anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (1:5,000 dilution, Jackson ImmunoResearch, West Grove, Pa.) and the developing substrate. Protein A was detected with chicken anti-protein A antibodies (1:3,000 dilution) and the F(ab′)$_2$ fragment of rabbit anti-chicken IgG conjugated to alkaline phosphatase (10). Fibrinogen binding proteins were detected by incubation of the blot with [^{125}I]fibrinogen (10^6 cpm) for 2 h at room temperature. The blots were washed three times in PBS with 0.1% Nonidet P-40 and subjected to autoradiography. As an alternative method to detect fibrinogen binding proteins, blots were incubated with IgG-depleted fibrinogen followed by goat anti-fibrinogen antibodies conjugated to alkaline phosphatase as previously described (10).

Northern (RNA) blot hybridization. Cells obtained from various growth phases were disrupted with 0.1-mm-diameter glass beads in a high-speed homogenizer, and RNA was isolated as described previously (5). Five micrograms each of RNA was applied to a 1% agarose–0.66 M formaldehyde gel and transferred onto a nylon membrane. Blots were hybridized with ^{32}P-labeled probes (8). An 872-bp *Eco*RI-*Hin*dIII gel-purified fragment from a plasmid containing the *coa* gene (pNCOA2) (21) was used as a probe for the *coa* transcript. The *clfA* transcript was detected with an identical PCR fragment used for Southern blotting (described above).

RESULTS

Fibrinogen binding to strain Newman and derivatives. In prior studies, we and others have observed that inactivation of the *agr* locus has led to an increase in fibrinogen binding activity (8, 13). Because site-specific mutants defective in coagulase and clumping factor expression were readily available, we wanted to explore the net effect of these mutations on fibrinogen binding capacity in both *agr*-positive and *agr*-negative strains. As expected from earlier studies (20), mutation in *clfA* resulted in a significant reduction in fibrinogen binding in the *agr*-positive strain compared with that of the parental strain Newman (Table 1). Although the fibrinogen binding capacity of the *coa*-negative mutant was lower than that of the parent, the magnitude of the decrease was less than that of the *clfA* mutant. Mutations in both *clfA* and *coa* genes revealed an additive effect in the reduction of fibrinogen binding, because the *coa clfA* double mutant bound less fibrinogen than the single mutants. However, residual but significant fibrinogen capacity remained in the *clfA coa* double mutant (see ALC620 in Table 1).

In an *agr*-negative background, the fibrinogen binding capacity of all strains was up-regulated compared with that of the respective *agr*-positive strains. In this genetic background, the *coa* mutation resulted in a significant reduction in fibrinogen binding (ALC619 in Table 1). In contrast, the contribution of clumping factor to fibrinogen binding in *agr*-negative strains was much more modest (32,740 ± 1,189 cpm for the *clfA agr* double mutant versus 38,919 ± 1,021 cpm for the single *agr* mutant). However, the role of clumping factor in fibrinogen binding was more clearly observed when the *agr coa clfA* triple mutant was compared with the *coa agr* double mutant (Table 1). The binding of ^{125}I-fibrinogen to cells was inhibited with unlabeled fibrinogen. In contrast, preincubation of cells with IgG or addition of purified protein A to labeled fibrinogen had no effect on binding (data not shown). Thus, the observed fibrinogen binding was specific and could not be attributed to contamination of fibrinogen with IgG. Collectively, these data suggested that coagulase may play a more prominent role in soluble fibrinogen binding in the *agr*-negative background. However, it should be stressed that the *agr coa clfA* triple mutant and the *coa clfA* double mutant were still able to bind fibrinogen above the background level.

Expression of coagulase and clumping factor in *agr*-positive and *agr*-negative strains. To get more insight into the molecular basis of the observed differences in fibrinogen binding between the *agr*-positive and *agr*-negative strains, the expression and activities of coagulase and clumping factor were examined. The clumping factor titer did not differ between strain Newman and its isogenic *agr* mutant, ALC355 (Table 1). Likewise, the amounts of clumping factor in cell wall extracts of both strains appeared to be equivalent as detected by immunoblotting, with the extract from the *clfA coa* mutant serving as a negative control (see cell wall extracts in Fig. 1A). These data implied that the expression of clumping factor is *agr* independent. The multiple-banding pattern for clumping factor on the immunoblot was consistent with those described previously (19). In both *clfA*-negative strains (*clfA coa* [ALC620] and *clfA coa agr* [ALC621]), reactive bands were detected in the lysates derived from the protoplast preparations (Fig. 1A). These bands may be due to the expression of the truncated N-terminal part of the clumping factor as a result of the Tn*551* insertion near the C terminus of the *clfA* gene in the *clfA* mutant (20, 21).

As implied by previous studies (1, 13) as well as ours (Table 1), it is likely that the expression of coagulase is negatively regulated by *agr*. A relative increase in coagulase expression was observed in the protoplast lysates of the *agr* mutant ALC355 compared with those of the wild type (Fig. 1B). However, little cell-associated coagulase could be detected in the cell wall extracts (Fig. 1B) in which protein A and clumping factor were mostly found (Fig. 1A). In addition to protein A, two protein bands were found to react with rabbit anti-coagulase antibody (Fig. 1B). Because both bands are absent in the *coa*-negative strains, they probably correspond to coagulase antigen.

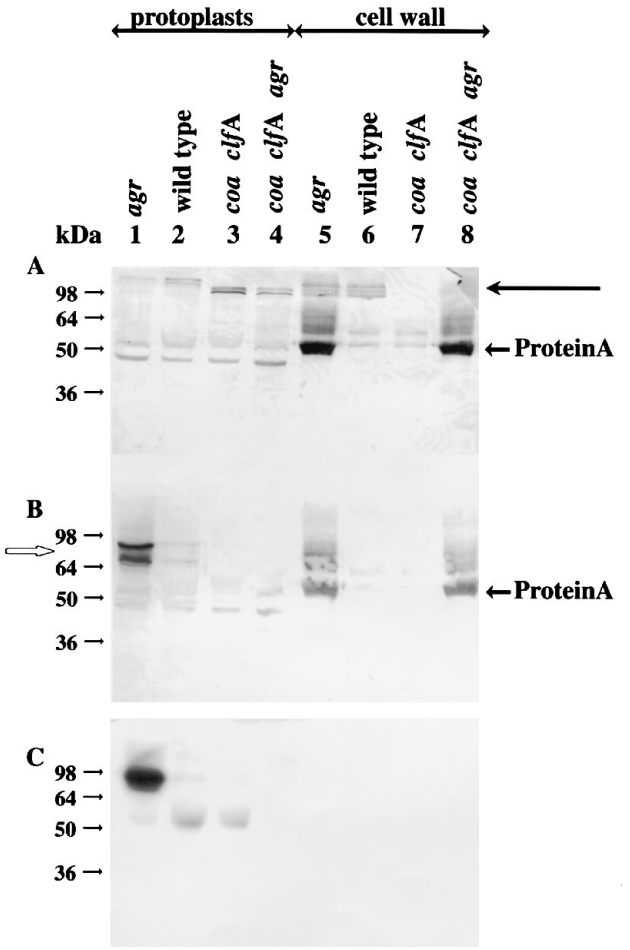

FIG. 1. Western blots of cell-associated proteins prepared from postexponential cells separated on 10% polyacrylamide–SDS gels. Cell wall extracts were derived from the soluble portion of lysostaphin digests of equivalent numbers of cells in a hypertonic buffer. Proteins from the remaining protoplasts were solubilized in SDS-PAGE sample buffer. For detection of clumping factor (A) and coagulase (B), the blots were incubated with rabbit anti-clumping factor or anti-coagulase antibodies (1:2,000) followed by goat anti-rabbit antibodies [F(ab′)$_2$ fragment] conjugated with alkaline phosphatase and developing substrate. For ^{125}I-fibrinogen binding (C), the blot was incubated with ^{125}I-fibrinogen (10^6 cpm). Molecular mass markers used were Seeblue prestained standards (Novex, San Diego, Calif.). Lanes 1 and 5, strain ALC355 (*agr*); lanes 2 and 6, strain Newman (wild type); lanes 3 and 7, strain ALC620 (*coa clfA*); lanes 4 and 8, strain ALC621 (*coa clfA agr*). Protein A was identified in blots identical to those shown here but developed with protein A-specific antibodies (data not shown). The long arrow (A) points to the clumping factor-specific bands, while the open arrow (B) points to the coagulase-specific bands; the ~90-kDa coagulase-specific band corresponds to the fibrinogen binding band in panel C (lane 1).

Fibrinogen binding to gel-separated proteins. We also examined the fibrinogen binding of protoplast lysates by ligand affinity blotting (Fig. 1C). In the *agr*-negative strain ALC355,

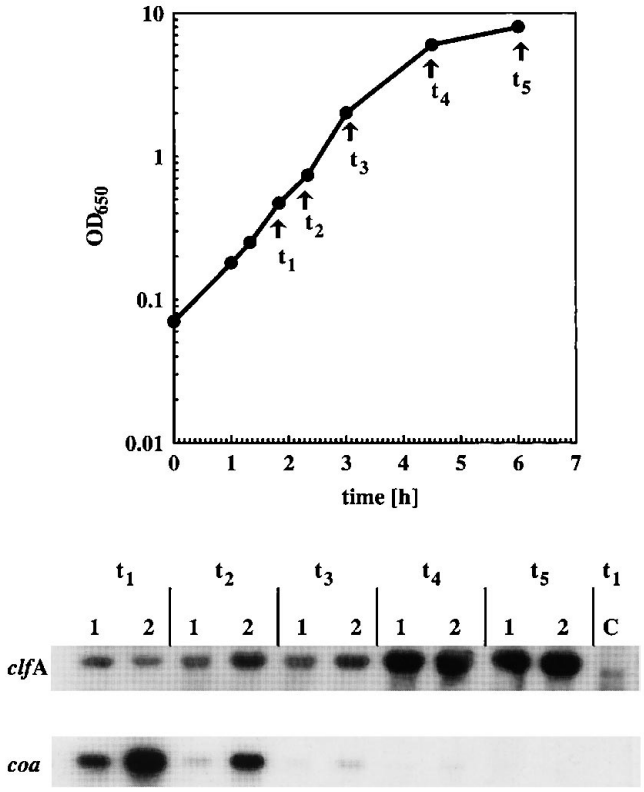

FIG. 2. Detection of *coa* and *clfA* transcripts by Northern blot analysis. RNA was purified from cells obtained at five time points (t_1 to t_5) during the growth cycle, and 5 μg was applied to each lane. An 871-bp *Eco*RI-*Hin*dIII fragment from the cloned *coa* gene (pNCOA2) was used as the *coa*-specific probe. Specific oligonucleotides were used in PCR to generate the *clfA*-specific probe (nucleotides 285 to 929 [20]). Lanes 1, strain Newman; lanes 2, strain ALC355 (*agr*). As a negative control, RNA obtained from the *agr clfA coa* triple mutant ALC621 was applied to lane C. OD_{650}, optical density at 650 nm.

one band that reacted with anti-coagulase antibodies also appeared to bind ^{125}I-fibrinogen strongly. By overlaying the autoradiograph with an identical blot probed with anti-coagulase antibody, it can be shown that this band probably corresponds to coagulase (Fig. 1B). This notion was also corroborated with the finding that this band was absent in the *coa agr* double mutant (Fig. 1B). Notably, the binding of fibrinogen to the clumping factor was not detectable in this ligand affinity blot even when cell wall extracts were assayed (data not shown).

One major additional band with an apparent molecular mass of 52 kDa also bound fibrinogen in the ligand binding assay (Fig. 1C). This band is upregulated in *agr*$^+$ strains and could not originate from the degradation of coagulase or clumping factor, because it was present in the *clfA coa* double mutant. The same protein band was detected after incubation with fibrinogen and anti-fibrinogen antibody conjugated with alkaline phosphatase but not with anti-protein A antibodies (data not shown). Thus, in addition to coagulase and clumping factor, our data imply the existence of an additional fibrinogen binding component in *S. aureus.*

Transcription of *coa* and *clfA*. We also investigated the effect of *agr* on the transcription of *clfA* and *coa* throughout the growth cycle (Fig. 2). The transcript level of *clfA* was highest in cells obtained during the postexponential phase. No difference in *clfA* transcription was observed between the *agr*-negative strain and the parental strain. In contrast to *clfA*, the *coa* transcript was detected mainly in cells from the exponential phase. In this instance, the *coa* transcription was enhanced in the *agr* mutant compared with that of strain Newman. Thus, the transcription of *coa* and *clfA* seemed to be regulated differentially, with *coa* being transcribed early in the growth cycle and *clfA* being transcribed mostly during the late growth cycle.

Binding of *S. aureus* to solid-phase fibrinogen. The role of clumping factor and coagulase in promoting *S. aureus* adherence to solid-phase fibrinogen was investigated with microtiter plates and catheters coated with fibrinogen. With both assays, the *clfA* mutant strains (DU5852, ALC620, ALC473, and ALC621) bound poorly to immobilized fibrinogen in comparison with the *clfA*-positive strains (Table 2). In contrast, the *coa* mutants (DU5872 and ALC619) attached to fibrinogen-coated microtiter plates or catheters as well as the corresponding *coa*-positive strains (Newman and ALC355) did, irrespective of the *agr* background. In the microtiter plate assay, the *agr* mutant ALC355 as well as the *agr coa* double mutant (ALC619) showed reduced binding compared with the respective *agr*-positive strains.

DISCUSSION

In this study, we provided evidence that both clumping factor and coagulase contribute to the fibrinogen binding capacity of *S. aureus*. However, major differences between these two proteins in terms of binding properties, expression, and gene

TABLE 2. Binding of *S. aureus* Newman and derivatives to solid-phase fibrinogen

Strain	Genotype	Binding to microtiter plates (1,000 × A_{560} [±SE]) coated with[a]:		Binding to fibrinogen-coated catheters (cpm ± SE)[b]
		100 μg of fibrinogen	25 μg of fibrinogen	
Newman	Wild type	314 ± 30	157 ± 42	697 ± 79
DU5852	*clfA*::Tn*917*	47 ± 14	4 ± 1	111 ± 50
DU5872	Δ*coa*::*tetK*	360 ± 58	141 ± 34	860 ± 161
ALC620	*clfA*::Tn*917* Δ*coa*::*tetK*	4 ± 3	0	91 ± 14
ALC355	Δ*agr*::*tetM*	84 ± 23	67 ± 16	556 ± 232
ALC473	Δ*agr*::*tetM clfA*::Tn*917*	5 ± 1	11 ± 4	74 ± 13
ALC619	Δ*agr*::*tetM* Δ*coa*::*tetK*	65 ± 9	78 ± 24	657 ± 69
ALC621	Δ*agr*::*tetM clfA*::Tn*917* Δ*coa*::*tetK*	27 ± 12	8 ± 1	113 ± 14

[a] Microtiter plates were coated with fibrinogen containing 2 mg of BSA per ml in PBS followed by incubation with washed bacterial cells (5×10^8 CFU). Bound cells were stained with crystal violet. A_{560} was measured in an ELISA reader. The background values from BSA-coated wells (1,000 × A_{560} = 72 ± 9.7) were subtracted from the sample values.

[b] Urethane catheters were coated with 2 mg of fibrinogen per ml and 2 mg of BSA per ml in PBS and incubated with [*methyl*-^{3}H]thymidine-labeled *S. aureus* cells (10^9 CFU), and the radioactivity of bound cells was measured. All strains showed similar specific activity (29,300 ± 4,020 cpm/10^8 CFU).

regulation were observed. Our fibrinogen binding assays confirmed earlier results (20, 29) that clumping factor is a major fibrinogen binding protein in strain Newman. However, in an *agr*-negative background, the capacity to bind soluble fibrinogen was markedly enhanced irrespective of the functioning status of the *clfA* gene. This was supported by the finding that the single *agr* mutant ALC355 as well as the *agr clfA* double mutant ALC473 showed significantly higher ^{125}I-fibrinogen binding than the corresponding *agr*-positive strain Newman and the *clfA* mutant DU5852. This extra fibrinogen binding capacity in the *agr*-negative background may be largely attributed to fibrinogen binding by coagulase. This notion is supported by the following observations. (i) The enhanced fibrinogen binding by the *agr*-negative strain ALC355 was associated with the upregulation of coagulase as shown by Western blotting (Fig. 1B) and Northern analysis (Fig. 2). (ii) Coagulase was detectable in cell lysates, and direct fibrinogen binding by coagulase could be demonstrated by ligand affinity blotting (Fig. 1C). (iii) The *coa agr* double mutant bound significantly less fibrinogen than the single *agr* mutant (Table 1).

In contrast to the findings of the ^{125}I-fibrinogen binding assay, binding to solid-phase fibrinogen in both *agr*-negative and *agr*-positive backgrounds could be attributed to clumping factor but not to coagulase. This is consistent with previous results (20, 29) that *clfA* is an important fibrinogen binding protein mediating *S. aureus* adherence to solid-phase fibrinogen. Although the molecular basis for the differential interaction of clumping factor and coagulase with soluble versus solid-phase fibrinogen remains unclear, several other microorganisms have been found to interact divergently with soluble and solid-phase fibrinogen (14, 17, 28). These findings are generally explained by conformational changes of fibronectin upon surface binding (27). Accordingly, the coagulase binding domain on the fibrinogen binding molecule may be masked or altered upon binding to a solid substratum. In this regard, Salzman et al. (26) have shown different modes of platelet interaction with soluble versus surface-bound fibrinogen. It is also plausible that the binding affinity of coagulase to fibrinogen may be too low to retain the bacteria on the surface. This would be consistent with the results of Dickinson et al. (11), who found evidence that coagulase may play a role in the attachment process of *S. aureus* to fibrinogen-coated surfaces in a flow chamber model, but only clumping factor was responsible for minimizing bacterial detachment. An alternative explanation may be that the C terminus of the coagulase molecule containing the fibrinogen binding domain is not exposed at the bacterial surface and thus can be reached only by soluble fibrinogen.

The expression of coagulase but not clumping factor was shown to be repressed in an *agr*-positive background as shown by Western blotting (Fig. 1). Both proteins could be identified in washed bacterial cells. However, most of the clumping factor was released from cells by lysostaphin digestion, whereas coagulase remained associated with the protoplasts after lysostaphin digestion. This may be explained by the missing cell wall anchoring motif of coagulase. Both molecules were also detectable in the culture supernatant (data not shown); however, the relative amounts of cell-bound versus secreted forms for these two proteins remain to be determined.

Northern blot analysis of the *clfA* and *coa* transcripts clearly demonstrated the differential regulation of these two genes throughout the growth cycle. The *clfA* gene is transcribed throughout the growth cycle and is highly expressed during the postexponential phase. Remarkably, the levels of *clfA* transcription of the wild-type and the *agr*-negative strains are similar. Thus, our finding implies an *agr*-independent signal for temporary regulation, especially during the late growth cycle. However, the *coa* transcript is produced mainly in the early exponential phase. In contrast to that of *clfA*, *coa* transcription is negatively regulated by *agr*, a regulatory system thought to be involved in repressing the transcription of some cell wall-associated protein genes such as protein A, fibronectin binding protein, and coagulase (1, 13). Thus, our data for coagulase are in concordance with the general understanding that inhibition of cell wall protein synthesis by the *agr* locus occurs mainly during the late growth cycle when RNA III, the *agr* regulatory molecule (24), is activated. In discordance with the finding of Lebeau et al. (16), we found no evidence of a positive modulation of *coa* transcription by *agr*. As they characterized strain RN6390 and its isogenic *agr* mutant RN6911, it is plausible that strain differences may account for the discrepancy observed. We also found that the *coa* transcription continued to be temporarily regulated in the *agr*-negative strain, with the *coa* transcript being at its strongest during the exponential phase. Thus, this observation implies that factors other than *agr* are involved in differential gene expression during the early part of the growth cycle in strain Newman.

Interestingly, coagulase activity and immunoreactive coagulase-specific bands were detected throughout the growth cycle, whereas *coa*-specific transcript was only detectable in the exponential growth phase. This discrepancy between transcription and protein expression implies that coagulase is stably expressed and may suffer little degradation within the cell. It is also plausible that coagulase may not be secreted well into the media in an *agr*-negative background during the postexponential phase, thereby resulting in accumulation of this protein within the cell. This notion is supported by our observation that the extracellular coagulase activity in the culture supernatants of the *agr*-negative strain obtained during the postexponential phase was less than that of the corresponding *agr*-positive strain. More quantitative assays and pulse-chase experiments will be required to clarify this issue in the future.

Using ligand-affinity blotting, we were not able to identify the clumping factor protein in the cellular extract. This may be due to loss of the fibrinogen binding property of clumping factor during protein preparation or denaturation during SDS-PAGE. This is in concordance with the results of Bodén and Flock (3), who failed to detect the clumping factor protein with similar methods. Interestingly, we were able to detect an unidentified fibrinogen binding protein (52 kDa), which was downregulated in the *agr*-negative strains but is distinct from coagulase, clumping factor, and protein A (Fig. 1C). Results from ^{125}I-fibrinogen binding studies also implicate an additional fibrinogen binding component, because the *clfA coa* double mutant was able to bind fibrinogen above the background level (Table 1). The fact that the *agr coa clfA* triple mutant still bound a residual but significant amount of soluble fibrinogen (Table 1) also hints at the existence of additional fibrinogen binding component(s) negatively regulated by *agr*. These yet unidentified fibrinogen binding components may correspond to Map, a previously identified protein with broad binding specificities to matrix proteins, including fibrinogen (12), and/or other novel binding proteins. These components cannot be FbpA, a 69-kDa fibrinogen binding protein described recently by us (10), because preliminary data indicated that strain Newman is negative for FbpA (data not shown). The size of the 52-kDa protein also differs from that of FibA, a recently described extracellular fibrinogen binding protein 19 kDa in size (4).

The fibrinogen binding property of the M protein has been shown to play a role in the resistance of group A streptococci to phagocytosis (31). Whether the binding of soluble fibrino-

gen to *S. aureus* cells via coagulase or other fibrinogen binding molecules contributes to the avoidance of the immune response is not clear. Recent virulence studies with specific *coa* mutants in animal models did not reveal a role for coagulase in virulence (22, 25). Interestingly, Mamo et al. (18) demonstrated that vaccination with a combination of two fibrinogen binding proteins, with one probably corresponding to purified coagulase, reduced the colonization of *S. aureus* in a mouse mastitis model. Since our data imply that the fibrinogen binding capacity is upregulated in an *agr*-negative background and little is known about the in vivo expression of the *agr* system during the course of an infection, it will be of clinical interest to reinvestigate the role of coagulase in *agr*-negative strains or in different states of infection.

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