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Factors Affecting the Collagen Binding Capacity of *Staphylococcus aureus*

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To determine whether the ability of *Staphylococcus aureus* to bind collagen involves an adhesin other than the collagen adhesin encoded by *cna*, we examined the collagen binding capacity (CBC) of 32 strains of *S. aureus*. With only two exceptions, a high CBC corresponded with the presence of *cna*. Both exceptions involved *cna*-positive strains with a low CBC. The first was a single strain (ACH5) that encoded but did not express *cna*. The second were the mucoid strains Smith diffuse and M, both of which encoded and expressed *cna* but bound only minimal amounts of collagen. Analysis of capsule mutants suggests that the reduced CBC observed in the mucoid strains was due to masking of the collagen adhesin on the cell surface and that this masking effect is restricted to heavily encapsulated strains. Differences in the CBC of the remaining *cna*-positive strains were correlated to variations in the level of *cna* transcription and were independent of the number of B domain repeats in the *cna* gene. In all *cna*-positive strains other than ACH5, *cna* transcription was temporally regulated, with *cna* mRNA levels being highest in cells taken from exponentially growing cultures and falling to almost undetectable levels as cultures entered the post-exponential growth phase. The CBC was also highest with cells taken from exponentially growing cultures. Mutation of *agr* resulted in a slight increase in *cna* transcription and a corresponding increase in CBC during the exponential growth phase but did not affect the temporal pattern of *cna* transcription. Mutation of *sar* resulted in a more dramatic increase in CBC and a delay in the post-exponential-phase repression of *cna* transcription. Mutation of both *sar* and *agr* had an additive effect on both CBC and *cna* transcription. We conclude that (i) *cna* encodes the primary collagen-binding adhesin in *S. aureus*, (ii) *sar* is the primary regulatory element controlling expression of *cna*, and (iii) the regulatory effects of *sar* and *agr* on *cna* transcription are independent of the interaction between *sar* and *agr*.

Staphylococcus aureus binds a number of host proteins, including fibronectin, laminin, vitronectin, fibrinogen, collagen, thrombospondin, elastin, osteopontin and bone sialoprotein (26). The *S. aureus* adhesins responsible for this binding have been termed MSCRAMMs to denote their role as microbial surface components recognizing adhesive matrix molecules (26). Genes encoding MSCRAMMs that bind fibronectin (*fnbA* and *fnbB*), fibrinogen (*fib*, *clfA*, and *fbpA*), elastin (*ebpS*), and collagen (*cna*) have been identified (26). Each of these genes encodes an adhesin with apparent specificity for a single host protein. In contrast, *map* appears to encode a broad-specificity MSCRAMM that mediates the low-level binding of several host proteins, including bone sialoprotein, fibronectin, fibrinogen, vitronectin, thrombospondin, collagen and osteopontin (15, 21). We recently demonstrated that *map* is encoded by most and possibly all *S. aureus* strains (33). *fib*, *clfA*, *ebpS*, and at least one of the two *fnb* genes are also highly conserved. In contrast, the collagen adhesin gene (*cna*) is relatively rare, being present in only 10 of the 25 strains that we examined (33).

Because *cna* is the only recognized *S. aureus* gene that encodes an adhesin that specifically binds collagen (30), the observation that most strains do not encode *cna* (33) suggests that most strains do not bind collagen. That inference is consistent with earlier surveys of *S. aureus* isolates (36) and is supported by the observation that mutation of *cna* in *S. aureus* Phillips eliminated the ability to bind collagen (28). However, *cna* mutagenesis has not been done in any strain other than Phillips, and it remains possible that at least some *cna*-negative *S. aureus* strains bind collagen either by virtue of the enhanced expression of a broad-specificity adhesin like Map or the presence of a collagen-binding MSCRAMM other than *cna*. Indeed, Nilsson et al. (22) reported that mutation of the staphylococcal accessory regulator (*sar*) in *S. aureus* DB resulted in a 16-fold enhancement in the capacity to bind type II collagen despite the fact that DB does not encode *cna*.

Identification and characterization of the *S. aureus* adhesins that contribute to collagen binding is important in light of the fact that *S. aureus* strains that cause musculoskeletal infections (e.g., septic arthritis and osteomyelitis) almost invariably bind collagen (2, 13, 26). This correlation suggests that therapeutic strategies aimed at the inhibition of collagen binding might be useful for the prevention and treatment of musculoskeletal infection. However, the development of such strategies will require a clear understanding of the bacterial factors that contribute to collagen binding. To that end, we characterized 32 strains of *S. aureus* with respect to their collagen binding capacity (CBC) and the presence and expression of *cna*. Our results indicate that (i) *cna* encodes the only *S. aureus* MSCRAMM that mediates the high-level binding of collagen, (ii) variations in CBC among *cna*-positive strains are due to capsule production and/or variations in the level of *cna* tran-

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TABLE 1. Strains used

Strain	*cna* (no. of B domains)	Reference	Description or comments
UAMS-1	Yes (1)	7	Clinical isolate (osteomyelitis)
U-6911	Yes (1)	8	*agr*-null mutant derived from UAMS-1
Phillips	Yes (2)	28	Clinical isolate (osteomyelitis)
FDA574	Yes (3)	30	Source of *cna* for cloning experiments
UAMS-639	Yes (4)	3	Clinical isolate (tracheal culture)
Becker	Yes (1)	16	Microencapsulated (type 8)
UAMS-604	Yes (3)	34	Clinical isolate (blood)
ACH2	Yes (2)	This study	Clinical isolate (septic arthritis)
ACH4	Yes (3)	This study	Clinical isolate (septic arthritis)
ACH5	Yes (3)	This study	Clinical isolate (septic arthritis)
SD	Yes (1)	6	Heavily encapsulated (type 2)
SC	Yes (1)	6	Capsule-deficient derivative of SD
M	Yes (1)	6	Heavily encapsulated (type 1)
MV	Yes (1)	18	Capsule-deficient derivative of M
CYL5556	Yes (1)	24	*cap1* mutant of M
CYL6194	Yes (1)	This study	*cap8* mutant of UAMS-1
CYL5972	Yes (1)	This study	*cap8* mutant of Becker
UAMS-2	No	This study	Clinical isolate (draining sinus tract)
UAMS-603	No	34	Clinical isolate (wound)
S6C	No	9	Hyperproducer of staphylococcal enterotoxin B
DB	No	4	Clinical isolate (blood)
11D2	No	4	*sarA* mutant derived from DB
ISP479C	No	7	Wild-type 8325-4 strain
ISP546	No	7	*agrA* mutant derived from ISP479C
RN6390	No	23	Wild-type 8325-4 strain
SarR	No	5	*sarA* mutant derived from RN6390
RN6911	No	23	*agr*-null mutant derived from RN6390
UAMS-173	No	This study	*sar agr* double mutant derived from RN6390
UAMS-174	Yes (3)	This study	RN6390 (*geh*::*cna*)
UAMS-175	Yes (3)	This study	SarR (*geh*::*cna*)
UAMS-171	Yes (3)	This study	*agr*-null mutant derived from UAMS-174
UAMS-172	Yes (3)	This study	*agr*-null mutant derived from UAMS-175
UAMS-176	Yes (3)	This study	RN6390 (pLI50::*cna*)
UAMS-177	Yes (3)	This study	SarR (pLI50::*cna*)
UAMS-88	No	This study	*S. epidermidis* control strain

scription and are independent of B-domain variations within the collagen adhesin itself, (iii) *cna* transcription is temporally regulated in a manner that is at least partially dependent on the *sar* regulatory locus, and (iv) the regulatory effects of *sar* on *cna* transcription are independent of the interaction between *sar* and *agr*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. aureus* strains used in this study are summarized in Table 1. Because the prototypical 8325-4 strain of *S. aureus* does not encode *cna* (33), we introduced *cna* into a representative 8325-4 strain (RN6390) and a *sar* mutant derived from RN6390 (SarR) (5). The *cna* gene was introduced both by transformation with a plasmid containing the FDA574 *cna* gene cloned into pLI50 (pLI50::*cna*) and by Φ11-mediated transduction from CYL574 (28). The latter results in the introduction of *cna* into the chromosomal lipase (*geh*) gene (*geh*::*cna*). Transformants were selected by plating on tryptic soy agar (TSA) containing 10 μg of chloramphenicol per ml and confirmed by plasmid profile analysis (data not shown). Transductants were selected on TSA containing 5 μg of tetracycline per ml and confirmed by Southern blot using *cna* and *geh* probes (data not shown). The RN6390 (*geh*::*cna*) and SarR (*geh*::*cna*) derivatives were designated UAMS-174 and UAMS-175, respectively (Table 1). We also generated *agr*-null mutants of UAMS-174 and UAMS-175 by Φ11-mediated transduction using RN6911 (23) as a donor strain. Transductants were selected on TSA containing 2 μg of minocycline per ml and confirmed by Southern blot using *cna*, *agr*, and *sar* probes (data not shown). The *agr*-null mutants of UAMS-174 and UAMS-175 were designated UAMS-171 and UAMS-172, respectively (Table 1).

Capsule-deficient mutants of the *cna*-positive, serotype 8 strains Becker and UAMS-1 were generated by allele replacement mutagenesis. The DNA region encoding the *cap8* gene cluster required for capsule biosynthesis has been cloned and sequenced (31). A derivative of Becker (CYL5972) with most of the *cap8* gene cluster deleted was constructed by using the temperature-sensitive delivery vector pCL52.1 (19). Briefly, a 3.8-kb *Sal*I fragment containing *cap8A*, *cap8B′*, and approximately 2.6 kb of the DNA upstream of the *cap8A* gene was ligated to a 4.4-kb *Sal*I fragment containing *cap8O*, *cap8P*, and approximately 2.0 kb downstream of the *cap8P* gene. The ligated fragment was cloned into the multiple cloning site of pCL52.1 to form pCL7842. The two fragments were shown by restriction enzyme digestion to be oriented in the same manner as found in the wild-type chromosome (data not shown). pCL7842 was used to transform *S. aureus* RN4220 and was then transduced from RN4220 into Becker by using phage 52A (19). RN4220 transformants and Becker transductants were isolated at the permissive temperature of 30°C. After a shift to the nonpermissive temperature (43°C) and selection for episomal integrants, a mutant (CYL5972) in which the plasmid had excised from the chromosome, leaving the deleted version of the *cap8* gene cluster, was isolated and verified as previously described (19).

To construct the UAMS-1 *cap8* deletion mutant (CYL6194), plasmid pCL7960 was constructed by cloning three DNA fragments in tandem into the multiple cloning site of pCL52.1. The three fragments consisted of (i) a 3.2-kb *Eco*RI-*Sal*I fragment containing *cap8B*, *cap8C*, *cap8D*, and *cap8E′*, (ii) a 1.7-kb *Sau*3A fragment containing the chloramphenicol resistance gene (*cat*) from pC194 (14), and (iii) a 4.4-kb *Sal*I fragment containing *cap8O*, *cap8P*, and approximately 2.0 kb of DNA downstream from the *cap8P* gene. The three fragments were ligated in such a way that the *cat* gene was flanked by the two *cap8* gene fragments in the same orientation found in the chromosome. The resulting plasmid (pCL7960) was transformed into RN4220 and then transduced into UAMS-1 as described above. After a shift to the nonpermissive temperature, derivatives of UAMS-1 containing *cat* were identified by plating on medium containing chloramphenicol. The fact that the intact *cap8* gene cluster in one of these (CYL6194) was replaced by the *cap8-cat* construct was confirmed by Southern blotting (data not shown). The capsule-deficient phenotypes of CYL5972 and CYL6194 were confirmed by rocket immunoelectrophoresis using a type-specific, rabbit anti-type 8 antiserum (31).

For the isolation of RNA for Northern blot analysis and for the collagen binding assays (see below), all strains were grown without antibiotic selection. Growth rate was not affected by any of the mutations under these conditions. In all cases, the stability of each mutation in the absence of selection was confirmed by plating on both selective and nonselective media (data not shown). The only

exceptions were the RN6390 and SarR strains carrying the pLI50::*cna* construct, both of which were grown in tryptic soy broth containing 5 μg of chloramphenicol per ml.

Northern blot analysis. Total cellular RNA was isolated as previously described (8). Because all mutations are defined by stable, chromosomal insertions, and because antibiotic selection affects growth rate, all strains were grown in tryptic soy broth without antibiotic selection. The similarity in optical density of each culture at the time points when RNA samples were collected was confirmed by measuring the optical density (A_{560}). Additionally, the stability of each mutation was confirmed by plating on selective and nonselective media at the conclusion of each experiment. RNA samples were denatured by using glyoxal and analyzed either by conventional Northern blotting (9) or by slot blotting. All gels were stained with ethidium bromide prior to transfer in order to confirm equal loads of RNA in each well. For slot blot analysis, RNA samples were vacuum drawn onto positively charged nylon membranes (Boehringer Mannheim, Indianapolis, Ind.), using a Bio-Dot SF slot blot apparatus (Bio-Rad Laboratories, Hercules, Calif.). After rinsing of each slot with Tris-EDTA buffer (0.1 M Tris-HCl [pH 8.0], 1 mM EDTA), the membrane was removed from the apparatus and the RNA was fixed to the membrane by using UV light (UV Crosslinker; Fisher Scientific, St. Louis, Mo.). The membrane was then processed according to our standard Northern blot protocol (8). Unless otherwise noted, 10 μg of total cellular RNA (based on A_{260}) was loaded in each lane or slot. Northern blotting was done with a digoxigenin-dUTP-labeled *cna* probe as previously described (8).

Collagen binding assays. Collagen binding assays were done with ^{125}I-labeled collagen (types I and II; Sigma Chemical Co., St. Louis, Mo.) as previously described (8). Briefly, cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended to an optical density (A_{560}) of 1.0 in PBS containing 0.1% bovine serum albumin and 0.1% Tween 20 (binding buffer). A 1.0-ml aliquot of the standardized cell suspension was placed in a 1.5-ml microcentrifuge tube that had been precoated overnight at 4°C with PBS containing 2% bovine serum. After addition of 10^5 dpm of ^{125}I-labeled collagen and incubation at room temperature with constant end-over-end mixing for 1 h, cells were pelleted and the bulk of the supernatant was removed. The tube was then centrifuged a second time, and the remaining supernatant was removed. The radioactivity retained in the pellet was measured in a gamma counter (8). Binding assays were done in duplicate, with the results reported as the average ± standard deviation.

Competition assays were done as described above except that unlabeled collagen, fibronectin, or fibrinogen was mixed with ^{125}I-labeled type I collagen immediately before addition to the standardized cell suspension. The amount of unlabeled protein was calculated to be in 10-fold excess with respect to labeled collagen, based on the assumption that 100% of the input collagen was labeled and recovered in the appropriate column fractions.

RESULTS

CBC as a function of *cna*. Collagen binding assays using 32 strains of *S. aureus* confirmed that strains that encode *cna* have a high CBC by comparison to *cna*-negative strains, all of which bound collagen at levels comparable to that of an *S. epidermidis* control (Fig. 1). In all cases, the results of our collagen binding assays were consistent with both type I (Fig. 1) and type II (Fig. 2) collagen. There were two exceptions to the correlation between *cna* and CBC, both of which involved *cna*-positive strains with a low CBC. The first was a single strain (ACH5) that had a CBC comparable to that of *cna*-negative strains (Fig. 1). Based on Northern blot analysis revealing the complete absence of *cna* mRNA (data not shown), the failure of ACH5 to bind collagen appears to arise from an as yet undefined transcriptional defect. The second exception involved the highly encapsulated strains Smith diffuse (SD) and M, both of which grow as mucoid colonies in vitro. As discussed below, the low CBC of SD and M appears to be due to masking of the collagen adhesin on the cell surface.

The CBC of *cna*-positive strains was competitively inhibited in the presence of unlabeled collagen but not in the presence of unlabeled fibrinogen or fibronectin (Fig. 3). In contrast, the CBC observed with *cna*-negative strains was not affected by the presence of any of the unlabeled proteins that we tested, including collagen (Fig. 3). These results indicate that the radioactivity observed in the pellet of *cna*-negative strains represents the background associated with our assay rather than the low-level binding of collagen by an adhesin present in strains that do not encode *cna*.

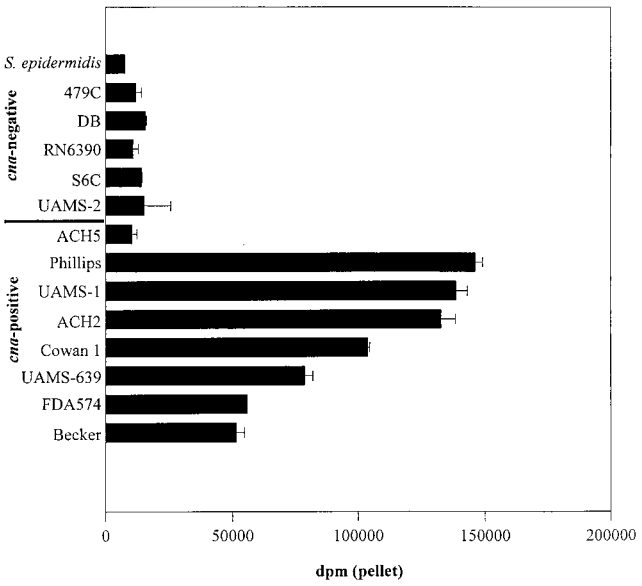


FIG. 1. Type I collagen binding as a function of *cna*. The CBC of *cna*-negative and *cna*-positive strains (Table 1) was determined by using type I collagen. *S. epidermidis* was included as a negative control. CBC was determined in cells from cultures in mid-exponential growth (4 h). Results are reported as averages of two assays; error bars indicate standard deviations.

CBC as a function of capsule production. The CBC of the SD and M strains was lower than that of other *cna*-positive strains (Fig. 4A) despite the fact that both strains encoded and expressed *cna* (Fig. 4B). To determine whether the low CBC was associated with the fact that these strains are heavily encapsulated, we compared the CBC of SD and M with that of capsule-deficient derivatives of each strain (Smith compact

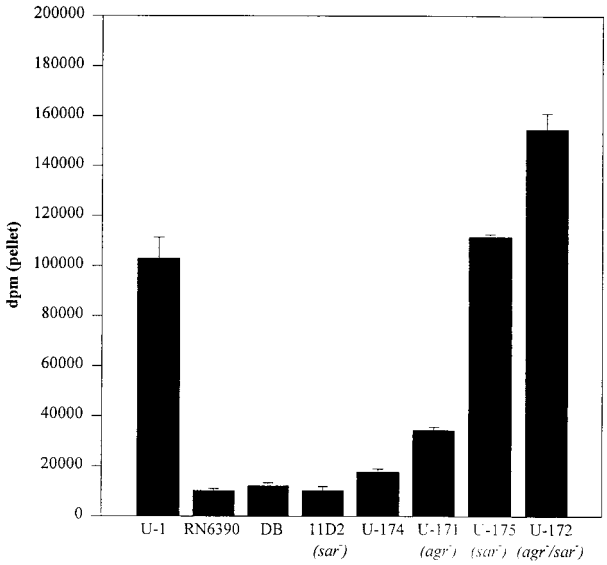


FIG. 2. Type II collagen binding as a function of *cna*. CBC was determined by using type II collagen. Assays were done with cells from cultures in mid-exponential growth (4 h). Results are reported as averages of two assays; error bars indicate standard deviations. *S. aureus* strains and their relevant genotypes are indicated below the chart.

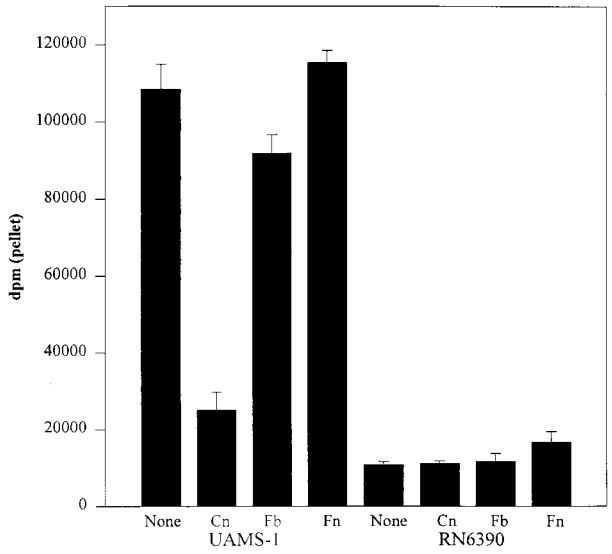


FIG. 3. Competition assays. Collagen binding assays were done with type I collagen and cells from exponential-phase (4-h) cultures of UAMS-1 (*cna* positive) and RN6390 (*cna* negative). Assays were done with equal amounts of ^{125}I-labeled collagen and an excess of unlabeled competitor protein. Results are reported as averages of two assays; error bars indicate standard deviations. None, no competitor protein; Cn, unlabeled collagen as competitor; Fb, unlabeled fibrinogen as competitor; Fn, unlabeled fibronectin as competitor.

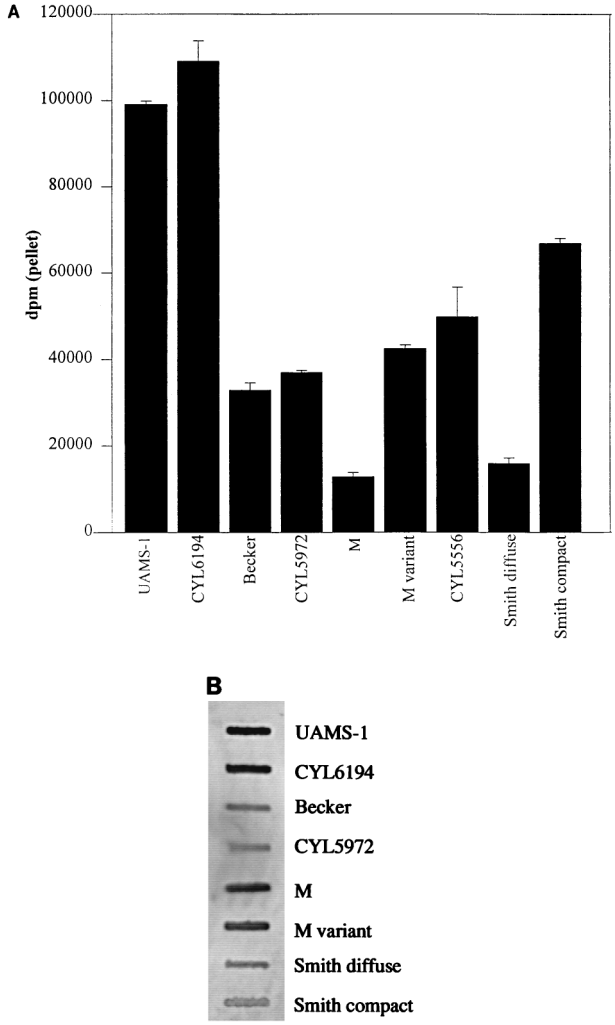


FIG. 4. CBC as a function of capsule production. (A) CBC of two microencapsulated strains (UAMS-1 and Becker), two mucoid strains (SD and M), and their respective capsule-deficient mutants. CBC was determined by using type I collagen. (B) Northern slot blotting with a *cna* probe and total cellular RNA from exponentially growing cultures of the indicated strains.

[SC] and M variant [MV], respectively). In both cases, the capsule-deficient strains had a CBC significantly higher than that of their parent strains (Fig. 4A). Although both SC and MV are spontaneous mutants that have not been characterized at the genetic level, the fact that the parent and mutant strains expressed similar levels of *cna* mRNA (Fig. 4B) indicates that the mutation responsible for the capsule-deficient phenotype of SC and MV did not affect *cna* transcription. Moreover, a subsequent experiment comparing the CBC of strain M and a derivative of M carrying a specific *cap* gene deletion (CYL5556) (24) confirmed that elimination of the capsule restored the ability to bind collagen (Fig. 4A). We conclude that the reduced CBC of SD and M is due to the presence of an extensive capsule that conceals the collagen adhesin and prevents its interaction with collagen. However, when we compared the CBC of UAMS-1 and Becker with that of *cap* mutants of each strain (CYL6194 and CYL5972, respectively), we observed only a slight increase in CBC with the capsule mutants (Fig. 4A). The relative amount of collagen binding in UAMS-1 and Becker and their capsule mutants did not change as a function of growth phase (i.e., the capsule mutants bound slightly more collagen when both exponential- and post-exponential-phase samples were examined) (data not shown). Because UAMS-1 and Becker are microencapsulated strains, these results suggest that any masking effect associated with the *S. aureus* capsule is limited to those strains that produce an abundance of capsular exopolysaccharide.

CBC variation in *cna*-positive strains. With the exceptions of ACH5, SD and M, all *cna*-positive strains had a CBC at least fivefold higher than that of any *cna*-negative strain (Fig. 1). However, we also observed a threefold difference in the CBC of *cna*-positive strains (Fig. 1). These differences were not correlated to structural variations in the *cna* gene itself. Specifically, our analysis included strains encoding *cna* variants with one (UAMS-1 and Becker), two (ACH2 and Phillips), three (Cowan, FDA574, ACH4, and UAMS-604), and four (UAMS-639) copies of the 561-bp B domain (26), and there was no correlation between the number of B domains and CBC (Fig. 1). Rather, the differences that we observed among *cna*-positive strains were correlated to differences in the level of *cna* transcription during exponential growth. For example, *S. aureus* Phillips had the highest CBC (Fig. 5A) and also appeared to have the highest level of *cna* mRNA (Fig. 5B). Conversely, Becker had the lowest CBC (Fig. 5A) and the lowest amount of *cna* mRNA (Fig. 5B). Strains that had a CBC between these extremes also appeared to express intermediate amounts of *cna* mRNA. These results suggest that with the exception of highly encapsulated strains of *S. aureus*, variations in CBC are a function of variations in *cna* transcription rather than structural differences within the collagen adhesin itself.

Temporal expression of *cna*. We recently demonstrated that *cna* transcription in *S. aureus* was temporally regulated, with expression being highest in exponentially growing cultures and falling to almost undetectable levels as cultures entered the post-exponential growth phase (8). Although two of the three *agr* mutants examined in the previous study appeared to express slightly elevated levels of *cna* mRNA during the expo-

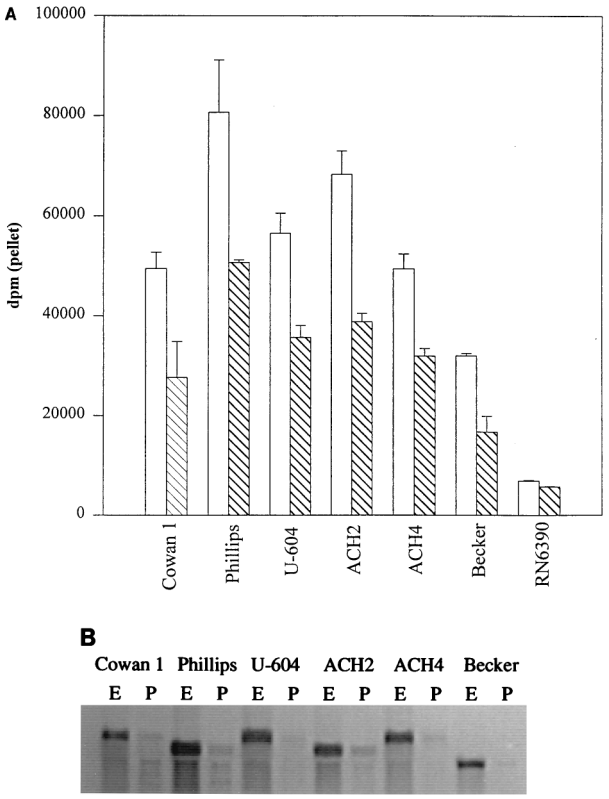


FIG. 5. CBC as a function of *cna* transcription. (A) CBC was determined by using type I collagen and cells taken from cultures in exponential (open bars) and post-exponential (hatched bars) growth. RN6390 was included as a *cna*-negative control. Error bars indicate standard deviations based on two assays. (B) Northern blotting done with a *cna* probe and total cellular RNA from early-exponential (E)- and post-exponential (P)-phase cultures of the six *cna*-positive strains shown in panel A.

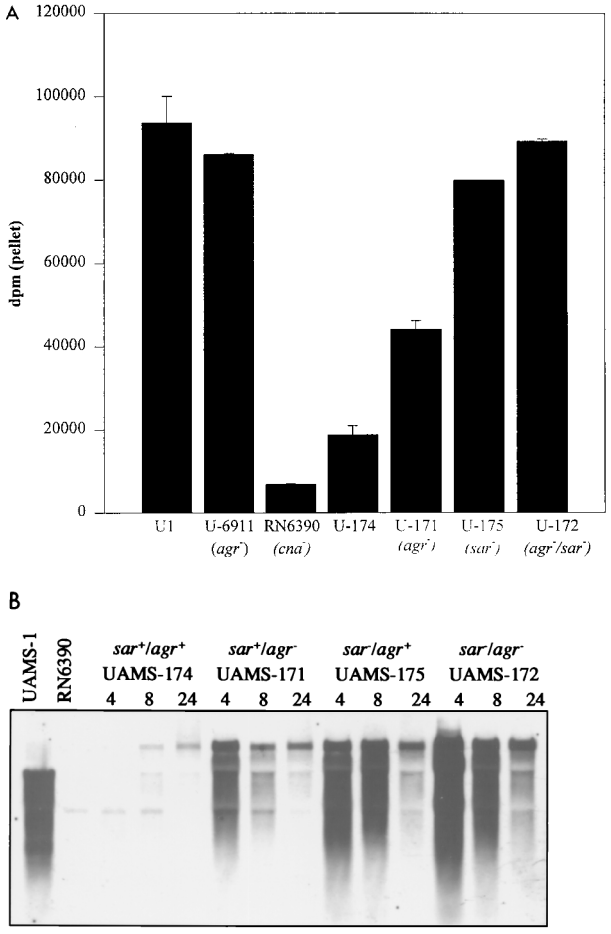
nential growth phase, mutation of *agr* did not alter the temporal pattern of *cna* transcription (8).

The temporal regulation of *cna* transcription was confirmed in this study. Specifically, Northern blot analysis of RNA samples taken from each of six *cna*-positive strains revealed that the level of *cna* mRNA was highest during the exponential growth phase and then fell to almost undetectable levels (Fig. 5B). The CBC of all six *cna*-positive strains was also highest when cells taken from exponential-phase cultures were assayed (Fig. 5A). However, despite the drastic drop in the level of *cna* mRNA present in stationary-phase samples, the CBC of all *cna*-positive strains remained higher than that of *cna*-negative strains (Fig. 5A). These results are consistent with our previous conclusion that the *S. aureus* collagen adhesin is relatively stable (8). The observation that the CBC of *cna*-negative strains did not change as a function of growth phase (data not shown) provides further support for our conclusion that the radioactivity detected in the pellet of *cna*-negative strains represents the background associated with our assay rather than collagen binding associated with an alternative collagen-binding adhesin.

Regulation of *cna* transcription. The previous suggestion that mutation of *agr* resulted in a slight increase in the CBC of some, but not all, *S. aureus* strains (8) was also confirmed during the course of this study. For instance, the CBC observed with a clinical osteomyelitis isolate (UAMS-1) was not significantly different from the CBC observed with an isogenic *agr*-null mutant (UAMS-6911) (Fig. 6A). However, we did observe an increase in the CBC of an *agr*-null mutant (UAMS-171) derived from UAMS-174 (Fig. 6A). Northern blot analysis confirmed that the increased CBC observed in UAMS-171 was a function of increased *cna* transcription (Fig. 6B). These results suggest that *agr* negatively regulates *cna* transcription during the exponential growth phase but that the regulatory influence of *agr* is strain dependent. Additionally, mutation of *agr* did not affect the temporal pattern of *cna* transcription (Fig. 6B).

To determine whether mutation of the staphylococcal accessory regulator (*sar*) had any effect on the temporal pattern of *cna* transcription, we introduced *cna* into RN6390 and an isogenic *sar* mutant (SarR) both as part of a multicopy plasmid (pLI50::*cna*) and as a single-copy chromosomal insertion (*geh*::*cna*). In both the plasmid-borne and chromosomal variants, the *cna*-positive derivatives of SarR (UAMS-177 and UAMS-175, respectively) had an elevated CBC by comparison to the corresponding *cna*-positive derivatives of RN6390 (UAMS-176 and UAMS-174, respectively) (Fig. 7A). Importantly, the increased CBC observed in the UAMS-175 *sar* mutant (Fig. 7A) exceeded the increase observed in the UAMS-171 *agr* mutant (Fig. 6A). Northern blot analysis confirmed that the increased CBC observed in the *sar* mutants was due to an increase in the level of *cna* transcription (Fig. 7B). To quantitate this increase, we repeated the Northern blot analysis using twofold dilutions of total cellular RNA taken from exponential-phase cultures of the *cna*-positive derivatives of RN6390 and SarR (Fig. 8). By comparison to UAMS-176 (RN6390, pLI50::*cna*), UAMS-177 (SarR, pLI50::*cna*) appeared to express at least eightfold more *cna* mRNA (Fig. 8). The increase was even more apparent when UAMS-174 (RN6390, *geh*::*cna*) and UAMS-175 (SarR, *geh*::*cna*) were compared. Specifically, UAMS-175 appeared to express *cna* at a level at least 32-fold higher than that for UAMS-174 (Fig. 8). Although less apparent, the increase in *cna* transcription seen in UAMS-175 was also reflected in the CBC. Specifically, UAMS-177 had a CBC approximately twice as high as that of UAMS-176, while UAMS-175 had a CBC approximately fourfold higher than that of UAMS-174 (Fig. 7A).

By comparison to UAMS-174, *cna* transcription in UAMS-175 not only was elevated during the exponential growth phase but also persisted at a relatively high level as cultures entered the post-exponential growth phase (Fig. 6B). These results suggest that the *sar* regulatory locus also contributes either directly or indirectly to the temporal regulation of *cna* transcription. The same temporal pattern of *cna* transcription was observed when we examined a *sar agr* double mutant (Fig. 6B). Additionally, the effect of the double mutation on *cna* transcription appeared to be additive, with the amount of *cna* mRNA in the *sar agr* double mutant being slightly higher than the amount observed in either the *sar* or the *agr* mutant strains (Fig. 6B). These results were also confirmed by our collagen binding assays. Specifically, the CBC observed during the exponential growth phase with the *sar agr* double mutant consistently exceeded that observed in the *sar* mutant in assays using both type I (Fig. 6A) and type II (Fig. 2) collagen. Interestingly, although the level of *cna* mRNA was increased during the post-exponential growth phase in both the *sar* mutant and the *sar agr* double mutant, the temporal pattern of collagen binding was unchanged compared to the wild-type strain. Specifically, the CBC of the *sar* mutants still peaked during the exponential growth phase and fell as cultures entered the post-exponential growth phase (Fig. 6A).



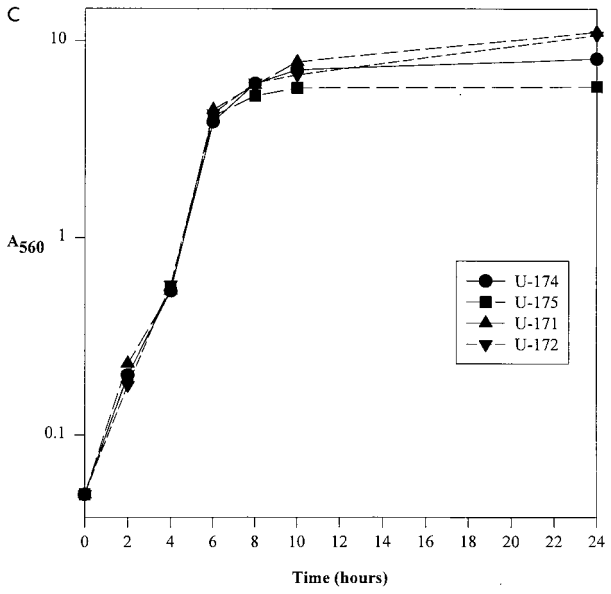


FIG. 6. Effect of *sar* and *agr* mutation on *cna* transcription and CBC. (A) CBC of *sar* and *agr* mutants was determined by using type I collagen and cells taken from exponential-phase cultures. *S. aureus* strains and their relevant genotypes are indicated below the chart. RN6390 was included as a negative control. (B) Northern blotting done with a *cna* probe and 5 μg of total cellular RNA taken from cultures during exponential (4 h) and post-exponential (8 and 24 h) growth phases. *S. aureus* strains and their relevant genotypes are indicated above the appropriate lanes. Exponential-phase (4-h) samples from UAMS-1 and RN6390 were included as positive and negative controls, respectively. It should be noted that UAMS-1 encodes a *cna* gene with a single B domain, while the *cna* variant introduced into the RN6390 strains contains three B domains. (C) Growth curve for RN6390 *agr* and *sar* mutants in panels A and B.

DISCUSSION

There is mounting evidence to suggest that *S. aureus* MSCRAMMs (adhesins) are important in the pathogenesis of various forms of staphylococcal disease. For example, the ability to bind fibronectin has been associated with the colonization of heart valves and endocarditis (17), while the production of a 19-kDa extracellular fibrinogen-binding protein has been associated with the colonization of wounds (25). The ability to bind fibronectin, fibrinogen, laminin, or thrombospondin has also been shown to promote the adherence of *S. aureus* to various biopolymers, including those used in the construction of indwelling medical devices (10, 11). Similarly, inactivation of the collagen adhesin gene (*cna*) has been correlated to reduced virulence in animal models of endocarditis and septic arthritis (12, 28). The latter is consistent with the observation that *S. aureus* isolates obtained from patients with septic arthritis and osteomyelitis generally exhibit an enhanced capacity to bind collagen (26). Moreover, we recently demonstrated that the collagen adhesin encoded by *cna* is present on the surface of *S. aureus* cells growing in bone (8). Taken together, these observations clearly suggest an important role for *S. aureus* adhesins in various forms of staphylococcal disease.

As part of our efforts to assess the role of adhesins in the pathobiology of *S. aureus*, we recently mapped each of the recognized adhesin genes within the *S. aureus* chromosome and assessed the prevalence of each gene among clinical isolates (33). The genes encoding the elastin-binding protein (*ebpS*), two fibrinogen-binding proteins (*fib* and *clfA*), and at least one of the two fibronectin-binding proteins (*fnbA* and/or *fnbB*) were present in every strain that we examined ($n = 25$). The *map* gene, which has been reported to encode an adhesin capable of mediating the low-level binding of a number of host proteins, was also present in all strains. In contrast, the *cna* collagen adhesin gene was present in only 10 of 25 strains (33).

The observation that *cna* is not present in most *S. aureus* strains is important because (i) *cna* is known to encode a high-affinity, collagen-specific adhesin (29, 30) and (ii) *S. aureus* strains isolated from patients with septic arthritis or osteomyelitis typically exhibit an enhanced capacity to bind collagen (2, 13). The fact that a relatively rare characteristic of the population is conserved among isolates that cause a specific form of staphylococcal disease suggests that the phenotype is associated with the pathogenesis of the disease. However, to date there has not been a comprehensive analysis establishing a direct correlation between the collagen adhesin encoded by *cna* and the ability to bind collagen. It therefore remains possible that *cna*-negative strains have some capacity to bind collagen as a function of either a broad-specificity adhesin (e.g., Map) or some as yet unrecognized adhesin. Indeed, Nilsson et al. (22) reported that mutation of *sar* in the *cna*-negative strain DB resulted in an enhanced capacity to bind type II collagen. Additionally, no binding was observed with type I collagen (22). Because DB does not encode *cna*, and because the collagen adhesin encoded by *cna* is known to bind both type I and type II collagen (26), these results suggest the existence of a second collagen adhesin that exhibits specificity for certain types of collagen.

We believe that the results presented here rule out the possibility that *S. aureus* encodes a second adhesin that makes a significant contribution to collagen binding by comparison to

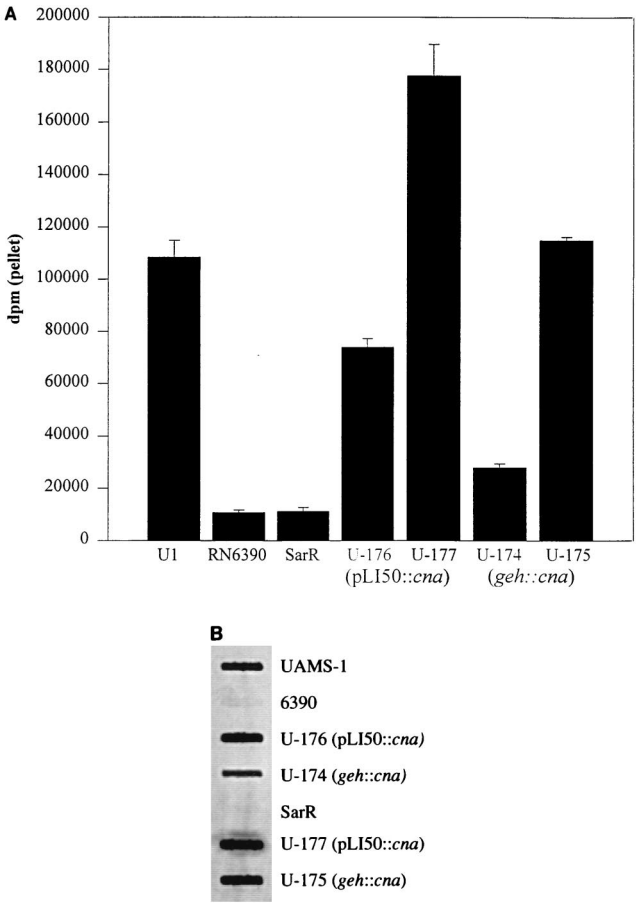


FIG. 7. Role of *sar* in *cna* transcription and CBC. (A) CBC of *cna*-positive variants of RN6390 and SarR. Plasmid (pLI50::*cna*) and chromosomal *cna* variants (*geh*::*cna*) are indicated below the chart. (B) Northern slot blotting using a *cna* probe and total cellular RNA from exponential-phase (4-h) cultures of the strains in panel A.

the adhesin encoded by *cna*. Specifically, our results for a number of *cna*-positive and *cna*-negative strains, using both type I and type II collagen, indicate that the high level of collagen binding in *S. aureus* is a direct function of the presence and expression of *cna*. We base that conclusion on the observations that (i) with only two exceptions, every *cna*-positive strain examined had a CBC at least fivefold higher than that of any *cna*-negative strain, (ii) the only exceptions to the correlation between *cna* and CBC can be explained either by the failure to transcribe *cna* or by the presence of an extensive capsule that masks the collagen adhesin itself (see below), (iii) in strains that were not heavily encapsulated and expressed *cna*, variations in CBC corresponded to the level of *cna* transcription, (iv) the alterations observed in the CBC of our *sar* and *agr* regulatory mutants can be explained by the effect of each mutation on *cna* transcription, and (v) none of the *cna*-negative strains examined bound collagen above the background level of our assay. The last is based on the observation that the amounts of radioactivity observed in pellets from all *cna*-negative strains were essentially identical, did not change as a function of growth phase, and were not reduced in the presence of other host proteins, including unlabeled collagen. In contrast, the binding of ^{125}I-labeled collagen in *cna*-positive strains was specifically inhibited by the presence of unlabeled collagen. It should be emphasized that our studies included the DB and 11D2 strains examined by Nilsson et al. (22) and that we did not observe collagen binding above background levels in either strain, using either type I or type II collagen. Indeed, type I and type II collagen worked equally well in our binding assays, and we did not observe any strain-dependent distinctions in the ability to bind different collagen types. Although we cannot explain the difference between our results and those reported by Nilsson et al. (22), we believe that our results demonstrate that the high-level binding of collagen by *S. aureus* is not restricted to certain types of collagen. However, we cannot rule out the possibility that *S. aureus* encodes a second adhesin that mediates collagen binding in a type-specific manner at a level below the sensitivity of our assay.

The observation that the highly encapsulated strains SD and M had a CBC lower than that of their corresponding capsule-deficient derivatives suggests that the presence of an extensive capsule may mask the adhesin on the surface of the bacterial cell. A similar masking effect helps the bacterial cell avoid phagocytosis by preventing recognition of C3b deposited on the cell surface (3). It is possible that masking of the collagen adhesin also helps the bacterial cell avoid recognition by host immune system. However, the *S. aureus* collagen adhesin appears to contribute to the colonization of musculoskeletal tissues (28), which suggests that the inhibition of collagen binding associated with capsule production might inhibit the colonization of at least some tissues. Although not conclusive, that observation is consistent with our demonstration that *S. aureus* SD is not virulent in a rabbit osteomyelitis model despite the fact that it encodes and expresses *cna* and is highly virulent in a murine peritonitis model of staphylococcal disease (35). However, given the fact that capsule-deficient mutants of UAMS-1 and Becker did not have a CBC significantly higher than that their microencapsulated parent strains, the inhibitory effect of the capsule is apparently restricted to those strains that produce an abundance of capsular exopolysaccharide.

Our results also demonstrate that variations in CBC among *cna*-positive strains is associated with the level *cna* transcription and is independent of any functional difference associated with structural variations in the collagen adhesin. Specifically, our studies included strains that encode each of four *cna* variants that differ in the number (one, two, three, or four) of B domains (30). The B domain is a 561-bp region that is directly repeated in strains that have multiple domains. Other than the fact that it is not responsible for binding collagen (27), the function of the B domain is unknown. One intriguing possibil-

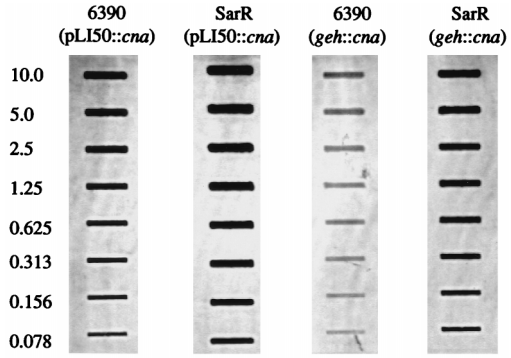


FIG. 8. Quantitative analysis of *cna* transcription in *sar* mutants, using two-fold serial dilutions of total cellular RNA from exponential-phase (4-h) cultures of *cna*-positive derivatives of RN6390 and SarR. RNA was analyzed by slot blotting using a probe specific for *cna*. Approximate amounts of RNA are indicated in micrograms at the left.

ity is that multiple B domains extend the ligand-binding A domain outward such that it is more exposed even in encapsulated strains. It should be noted that all of our capsule-deficient mutants were generated in strains that encode a *cna* gene with a single B domain. We are currently investigating the possibility that *cna* variants that have multiple B domains are less subject to the inhibitory effect of the capsule.

Finally, our results establish that the temporal pattern of *cna* transcription observed in our earlier experiments (8) is a consistent feature of all *cna*-positive strains. The temporal pattern observed with *cna* is characteristic of *S. aureus* surface proteins, most of which are regulated by the accessory gene regulator (*agr*) and/or the staphylococcal accessory regulator (*sar*). In a previous report, we demonstrated that mutation of *agr* had no effect on the temporal pattern of *cna* transcription (8). That finding was confirmed in this study. Our earlier study also suggested that mutation of *agr* resulted in an increase in *cna* transcription during the exponential growth phase in some, but not all, strains (8). The strain-dependent increase in the level of *cna* transcription during the exponential growth phase was also confirmed during the course of this study. Taken together, our results suggest that *agr* encodes or induces some factor that represses *cna* transcription during exponential growth but that the more complete repression observed as cultures enter the post-exponential growth phase requires an additional, *agr*-independent regulatory signal.

One possibility is the temporal signal described by Vandenesch et al. (37), who demonstrated that the induction of *hla* transcription requires induction of the *agr*-encoded RNA III effector molecule and an additional *agr*-independent regulatory signal that is induced during the transition between exponential and post-exponential growth. At present, the nature of this additional regulatory signal and its contribution to the regulation of *S. aureus* virulence factors other than *hla* are unknown. A second possibility is the staphylococcal accessory regulator (*sar*). To examine that possibility, we introduced *cna* into RN6390 and its otherwise isogenic SarR mutant. Our results indicate that mutation of *sar* results in a significant increase in the exponential-phase expression of *cna* and a delay in the post-exponential-phase repression of *cna* transcription. These results suggest that *sar* is the primary regulatory element controlling expression of *cna*. At present, we do not know whether the *sar*-mediated regulation of *cna* transcription is a function of a direct interaction between SarA and *cis* elements upstream of *cna* or involves an additional regulatory element that is not encoded within the *sar* locus.

Interestingly, mutation of both *sar* and *agr* had an additive effect on CBC by comparison to the corresponding *sar* and *agr* single mutants. One intriguing possibility that would explain these results is that the sensory component of the *agr* system somehow modulates a response regulator that is either encoded within *sar* or induced in response to some *sar*-encoded regulatory signal. Because mutation of the sensory component of two-component regulatory systems typically has a more moderate effect on phenotype than mutation of the response regulator (20), such as scenario would explain the moderate increase in CBC observed with an *agr* mutant and the more drastic increase in CBC observed with the *sar* mutant. However, mutation of *agr* has no effect on the production of any *sar* transcript (1). Moreover, the additive effect observed with *sar agr* double mutants would not be expected based on the scenario described above. In fact, an additive effect would not be expected in any scenario in which the *sar* and *agr* regulatory signals were interdependent. For that reason, we conclude that the regulatory effects exerted by the *sar* and *agr* loci on *cna* transcription are independent of each other.

Finally, although mutation of *sar* resulted in a delay in the post-exponential-phase repression of *cna* transcription, the delay did not have a significant impact on the post-exponential-phase CBC. Specifically, while collagen binding and *cna* transcription were highest during the exponential growth phase, the post-exponential-phase decline in CBC was apparent even in *sar* mutants. These results suggest that *sar* may have an impact on collagen binding at some posttranscriptional level. One possibility for the decrease in CBC despite the continued production of *cna* mRNA is that mutation of *sar* results in overproduction of proteases that degrade the collagen-binding adhesin. Indeed, mutation of *sar* is known to result in enhanced proteolytic activity (5). A second possibility is that the overproduction of the *cna*-encoded adhesin or some other surface protein affects the surface architecture of *S. aureus* in such a way that the adhesin cannot be appropriately localized to the cell surface. Indeed, the observation that the *cna*-encoded adhesin is anchored in the cell envelope in a manner that is distinct by comparison to other *S. aureus* surface proteins (32) suggests that the collagen adhesin may be differentially affected in strains carrying mutations that alter expression of multiple target genes. Although we cannot yet explain the discrepancy between *cna* transcription and CBC in post-exponential-phase cultures of *sar* mutants, it is important to note that the discrepancy involves the failure to bind collagen despite expression of *cna* rather than the ability to bind collagen in the absence of *cna* transcription. These results therefore do not detract from our conclusion that the collagen adhesin encoded by *cna* is the primary adhesin responsible for the ability of *S. aureus* to bind collagen.

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