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Transcriptional Regulation of the *Staphylococcus aureus* Collagen Adhesin Gene, *cna*

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We demonstrate that transcription of the *Staphylococcus aureus* collagen adhesin gene (*cna*) is temporally regulated, with expression being highest in exponentially growing cultures and falling to almost undetectable levels as cultures enter the post-exponential-growth phase. The temporal regulation of *cna* transcription was not affected by mutation of *agr*. We also demonstrate that the collagen adhesin is expressed by both *agr*$^+$ and *agr*-negative *S. aureus* cells growing in bone.

Staphylococcus aureus is a pathogenically diverse organism capable of producing a wide array of virulence factors. These virulence factors tend to be globally regulated, with expression of many surface proteins being repressed in favor of exoprotein synthesis as cultures enter the post-exponential-growth phase (13). Although several regulatory elements contribute to this global shift (5, 10, 19, 25, 27), the *agr* locus appears to play a central role in regulating the production of staphylococcal virulence factors (5, 11, 15). The importance of *agr* in the pathogenesis of staphylococcal disease is emphasized by studies demonstrating the reduced virulence of *agr* mutants in animal models of peritonitis (26), endophthalmitis (2), endocarditis (4), septic arthritis (1), and osteomyelitis (8).

Among the surface proteins produced by *S. aureus* are a diverse group of adhesins capable of binding host proteins present in the extracellular matrix. Although *S. aureus* can bind a variety of host proteins (16), isolates associated with musculoskeletal disease generally exhibit an enhanced propensity to bind collagen and/or bone sialoprotein (3, 12, 20–22). This observation is particularly relevant in light of the fact that most strains do not bind either of these matrix molecules (9, 20, 21). Taken together, these results suggest that the ability to bind collagen and/or bone sialoprotein may be directly involved in the colonization of bone and cartilage. Indeed, mutation of the collagen adhesin gene, *cna*, was recently shown to result in a reduced capacity to cause septic arthritis (17).

We have utilized a rabbit osteomyelitis model to evaluate the role of specific *S. aureus* virulence determinants in the pathogenesis of musculoskeletal disease (8). These experiments were undertaken with an *S. aureus* isolate (UAMS-1) obtained from the bone of a patient suffering from osteomyelitis. In our initial experiments, we generated an *agr*-negative derivative of UAMS-1 (UAMS-4) and established that mutation of *agr* resulted in a reduced capacity to cause osteomyelitis but did not eliminate the ability to colonize bone (8). Based on the hypothesis that the ability to bind collagen and/or bone sialoprotein may be important in the colonization of bone and the possibility that expression of these surface proteins is not repressed in an *agr* mutant, we carried out experiments aimed at defining the relative abilities of UAMS-1 and UAMS-4 to bind these matrix proteins. Because UAMS-1 did not bind appreciable amounts of bone sialoprotein but did exhibit a high binding capacity for collagen (8), we focused our efforts on the *S. aureus* collagen adhesin gene (*cna*).

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UAMS-4 was generated by φ11-mediated transduction using ISP546 (an 8325-4 strain) as a donor strain (8). In this study, we also used φ11-mediated transduction to move the *agrA*::Tn*551* insertion from ISP546 into the *cna*-positive strain Smith diffuse (SD). In both cases, transductants were selected for erythromycin resistance at 10 μg per ml and confirmed by Southern blot analysis with a DNA probe for *agr* and by Northern blot analysis with a probe specific for RNAIII (data not shown). UAMS-4 and the *agrA*::Tn*551* derivative of SD (SDA) were maintained on medium containing 20 μg of erythromycin per ml; however, because the insertion was stable in the absence of selection (data not shown), cells used for assessing the capacity to bind collagen and for the isolation of RNA for Northern blot analysis were taken from cultures grown in the absence of antibiotics. Mutation of *agr* did not affect the growth of either strain (data not shown).

To determine whether mutation of *agr* had an effect on *cna* transcription, we isolated total cellular RNA from UAMS-1, UAMS-4, SD, and SDA at 2-h intervals and did Northern blots (11) using a DNA probe corresponding to the UAMS-1 *cna* gene (9). In both UAMS-1 and SD, *cna* mRNA was detected during early exponential growth (2 h) and reached a maximum during the mid-exponential-growth phase (4 h) (Fig. 1). Little or no *cna* mRNA was observed during the post-exponential-growth phase. In comparison to SD, there appeared to be a slight increase in the exponential-phase amount of *cna* mRNA in SDA (Fig. 1). However, this increase was not apparent in UAMS-4. Moreover, the same temporal pattern of *cna* expression observed in UAMS-1 and SD was also observed in the *agr* mutants UAMS-4 and SDA (Fig. 1).

The results of our Northern blot analysis were consistent with the observation that UAMS-1 and UAMS-4 bound comparable amounts of collagen at all time points (Fig. 2). Similar results were obtained with strains SD and SDA (data not shown). It is interesting, however, that the reduction in *cna* transcription observed in post-exponential-phase samples from UAMS-1 and UAMS-4 (Fig. 1) did not coincide with a comparable reduction in the ability to bind collagen (Fig. 2). Specifically, while the collagen binding capacity (CBC) was highest

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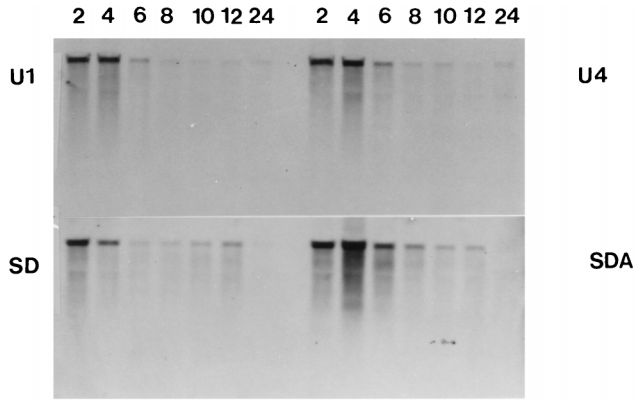

FIG. 1. Temporal regulation of *cna* transcription. Lane designations indicate the time points (h) at which samples for RNA analysis were taken. The 6-h time point corresponds to the transition between the exponential- and post-exponential-growth phases (data not shown). In all cases, 5 μg of total cellular RNA (as determined on the basis of A_{260} values) was hybridized with a probe corresponding to *cna*. All strains encode a form of *cna* with a single B domain. U1, UAMS-1; U4, UAMS-4; SD, Smith diffuse; SDA, Smith diffuse *agrA*::Tn*551*.

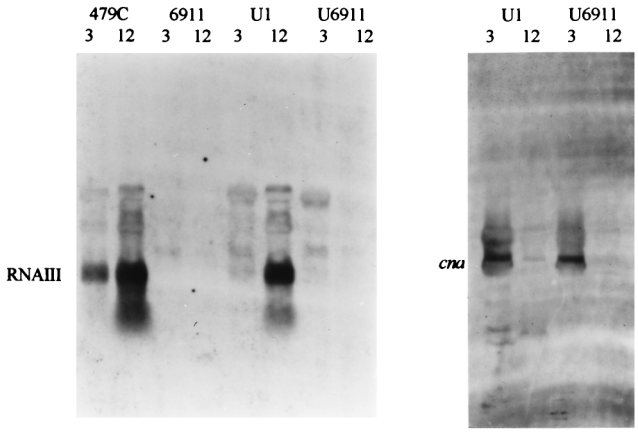

FIG. 3. Expression of *cna* in an *agr*-null mutant. Total cellular RNA was harvested at the indicated time points (3 and 12 h). ISP479C (479C) is an 8325-4 strain included as a positive control for RNAIII transcription. RN6911 (6911) is an *agr*-null mutant in which the region encoding RNAIII was replaced by *tetM* (13). U1, UAMS-1; U6911, UAMS-6911. Northern blots were done with 5 μg of total cellular RNA (as determined on the basis of A_{260} values) and DNA probes corresponding to RNAIII (left) or *cna* (right).

in cells taken from mid-exponential-phase cultures (4 h), it remained relatively high even in cells taken from overnight (24-h) cultures (Fig. 2). These results suggest that the collagen adhesin of *S. aureus* is relatively stable. That is an important observation, particularly in light of the fact that previous studies detailing the *S. aureus* CBC were done with cells taken from overnight cultures (3, 6, 12, 21). Indeed, Clark et al. (6) demonstrated that growth at 42°C resulted in a reduced CBC compared with growth at 37°C. Although it was unclear whether this effect was mediated at the transcriptional or posttranscriptional level, the observation that the comparison was done with cells taken from overnight cultures (6), together with our demonstration that *cna* transcription is reduced to minimal levels during the post-exponential-growth phase, suggests that the reduced CBC observed at the elevated temperature probably reflects instability of the collagen adhesin itself rather than an effect on the transcription of *cna.*

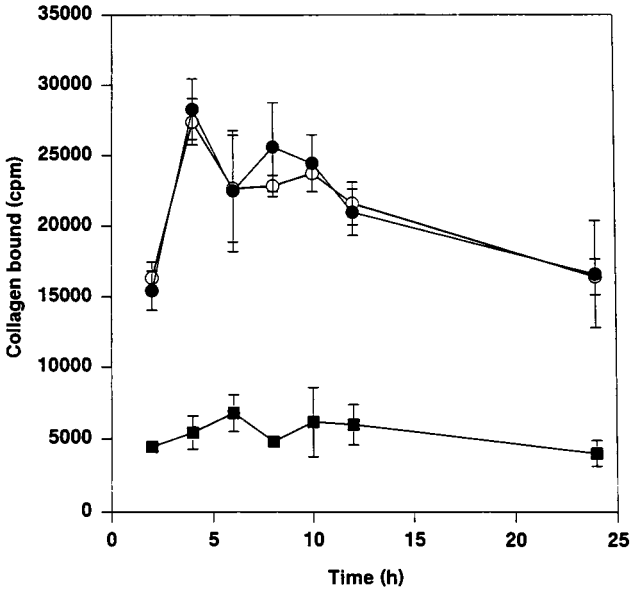

FIG. 2. Collagen binding capacities of UAMS-1 and UAMS-4. The collagen binding capacities of UAMS-1 (●), UAMS-4 (○), and ISP479C (■) were determined at the indicated time points. ISP479C was included as a negative control because it does not contain *cna* (data not shown). Results are reported as counts per minute of ^{125}I-collagen retained in the pellet after two rounds of centrifugation and aspiration of the supernatant. Results shown are the averages of two independent experiments. Error bars indicate the standard deviation at each time point.

The results discussed above suggest that the temporal regulation of *cna* transcription is not mediated by *agr*. However, when we examined transcription of the *agr* target genes *hla* and *spa* in UAMS-4, we found that, in comparison to UAMS-1, *hla* transcription was essentially unchanged while the transcription of *spa* was only slightly elevated (data not shown). Although these results were not particularly surprising given the complexity of the regulatory events associated with *agr* (13, 15, 27) and the recognized strain-dependent differences in *agr*-mediated regulation (7), they also left open the possibilities that (i) the *agrA*::Tn*551* insertion in UAMS-4 did not completely inactivate the *agr* locus (despite our inability to detect RNAIII in UAMS-4) and (ii) *agr* contributes to the regulation of *cna* transcription in *S. aureus* strains other than UAMS-1. To address the first of these possibilities, we generated an *agr*-null mutant of UAMS-1. This mutant was generated by ϕ11-mediated transduction using RN6911 (an *agr*-null mutant in which the *agr* locus has been replaced by the *tetM* tetracycline resistance determinant) as a donor strain (13). Transductants were selected for tetracycline resistance at 5 μg per ml and confirmed as described above (data not shown). As was the case with UAMS-4, the temporal pattern of *cna* transcription observed in UAMS-1 was also observed in the UAMS-1 *agr*-null mutant (UAMS-6911) (Fig. 3). Because the deletion in UAMS-6911 includes the regions encoding both RNAII and RNAIII (13), these results rule out the possibility that the temporal regulation of *cna* transcription in UAMS-4 is due to the residual production of RNAIII.

To address the possibility that *agr* contributes to the regulation of *cna* in other *S. aureus* strains, we introduced *cna* into ISP479C and ISP546 (13, 26). ISP479C is an 8325-4 strain that does not contain *cna* (data not shown). ISP546 is an *agrA*::Tn*551* derivative of ISP479C (13, 26). To generate *cna*-positive derivatives of each strain, *S. aureus* CYL574 (17) was used as a donor strain for the ϕ11-mediated transduction of

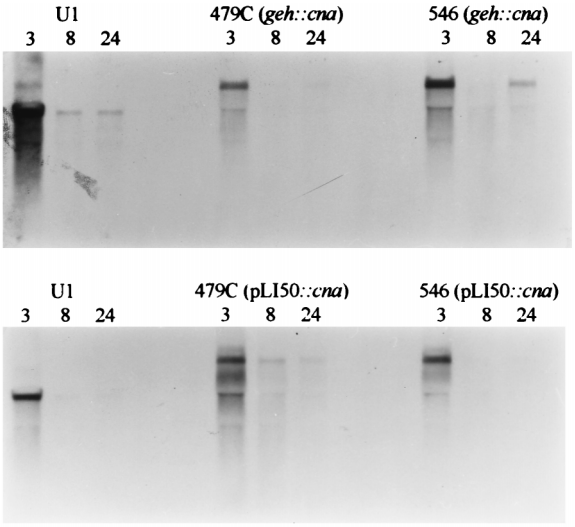

FIG. 4. Temporal regulation of *cna* transcription in ISP479C and ISP546. Total cellular RNA was harvested from derivatives of ISP479C (479C) and ISP546 (546) in which *cna* was introduced either by transduction (*geh*::*cna*) or by transformation (pLI50::*cna*). Samples collected during the exponential-growth phase (3 h) and during the post-exponential-growth phase (8 and 24 h) were hybridized with a probe corresponding to *cna*. RNA samples collected from UAMS-1 (U1) at the corresponding time points were included as a positive control. Importantly, the *cna* gene of UAMS-1 has a single B domain (11), while the FDA574 *cna* gene, used in the transduction and transformation experiments, has three B domains (16). The presence of the additional B domains is reflected in the increased size of the *cna* mRNA present in the ISP479C and ISP546 derivatives.

cna into the lipase structural gene (*geh*). Transductants were selected for tetracycline resistance at 5 μg per ml and confirmed by Southern blotting with *cna*, *geh*, and *agr* probes (data not shown). We also generated *cna*-positive derivatives of ISP479C and ISP546 by transformation, using a pLI50 (14) construct containing the entire *cna* structural gene from FDA574 and an additional 500 bp of the region upstream from the *cna* start codon (9). Transformants were selected for chloramphenicol resistance at 10 μg per ml. The same temporal pattern of *cna* transcription observed in UAMS-1 and SD was observed in all of the *cna*$^+$ derivatives of ISP479C and ISP546 (Fig. 4). These results demonstrate that the *agr*-independent temporal regulation of *cna* transcription is not strain dependent. Additionally, because *cna* is encoded within a discrete genetic element that is not present in all strains of *S. aureus* (9), it is of interest that the temporal regulation of *cna* was consistent both in strains that are naturally *cna* positive (UAMS-1 and SD) and in strains into which *cna* was introduced in the absence of any other DNA (ISP479C). These results demonstrate that the temporal regulation of *cna* transcription involves a regulatory element that is not part of the *cna* genetic element itself.

The observation that regulation of *cna* transcription is independent of *agr* is consistent with the recent demonstration that mutation of *agr* has no effect on expression of the fibrinogen binding protein gene *clfA* (28). However, because *clfA* was preferentially expressed during the post-exponential-growth phase (28), while *cna* transcription was highest during exponential growth, transcription of *cna* and *clfA* either is not coordinately regulated or is regulated in an inverse manner. In fact, because the other *S. aureus* genes that are preferentially expressed during the exponential-growth phase (e.g., *spa* and *coa*) are negatively regulated by *agr* (13), the results presented here demonstrate that *cna* transcription is controlled in a manner unlike that of any other *S. aureus* virulence factor examined to date.

There is evidence to suggest the existence of at least five regulatory loci in *S. aureus*. Two of these (*xpr* and *sae*) are

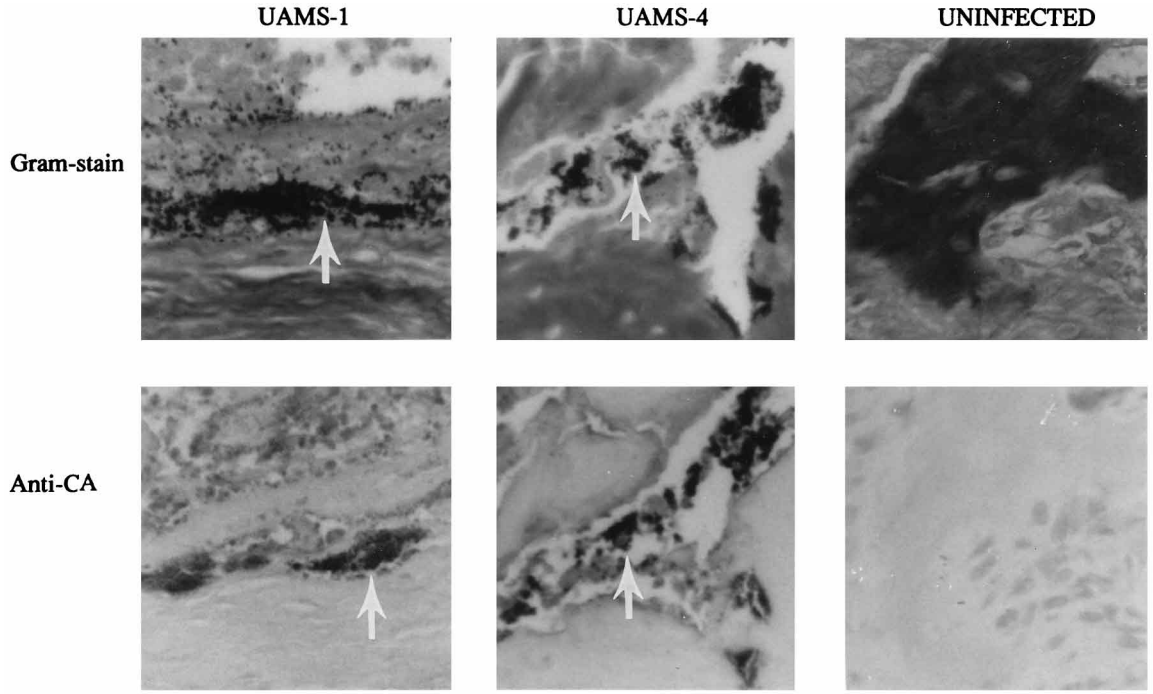

FIG. 5. Expression of the *S. aureus* collagen adhesin in bone. Serial histological sections from rabbits infected with UAMS-1 or UAMS-4 were examined by Gram stain (upper panels) or by IHC analysis with an anti-CA antibody (lower panels). Regions containing gram-positive cocci that were positive by IHC analysis are indicated by arrows. Sections from an uninfected rabbit were included as a negative control. The dark region in the Gram-stained section from the uninfected rabbit (upper right) is due to the nuclear fast red counterstain.

defined only by the presence of a Tn*551* insertion (10, 25), while two others (*sar* and *agr*) have been cloned and sequenced (5, 19). The fifth is an undefined temporal regulatory signal that is required for expression of at least some staphylococcal exoproteins (e.g., alpha-toxin) (27). At present, we have not determined whether any of these other regulatory elements contribute to the transcriptional regulation of *cna*. One of the difficulties in that regard revolves around the fact that none of the *S. aureus* strains in which the *xpr* and *sar* regulatory mutations have been introduced contain *cna* (data not shown). However, while we have not identified the regulatory signal responsible for the temporal regulation of *cna* transcription, we have addressed the issue of whether the collagen adhesin is expressed in vivo and whether mutation of *agr* has an effect on the in vivo expression of *cna* that is not apparent in vitro. Specifically, we used a polyclonal rabbit antiserum raised against the collagen adhesin (anti-CA) for the immunohistochemical (IHC) analysis of bone sections taken from rabbits infected with UAMS-1 or UAMS-4 (8). The anti-CA antibody was generated against a 17-kDa peptide spanning residues 151 to 297 (18). For IHC analysis, radii from rabbits infected with UAMS-1 or UAMS-4 were fixed in formalin and decalcified with formic acid prior to embedding in paraffin (8). Gram stains (24) were prepared from each block to confirm the presence of intraosseous bacteria. Sections were then treated with hot citrate buffer (Citra Solution; BioGenex, San Ramon, Calif.) as an antigen retrieval step (23). Endogenous rabbit immunoglobulin G (IgG) was blocked with AffiniPure Fab fragment goat anti-rabbit IgG heavy and light chain antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted to a final concentration of 0.8 μg per ml in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). After the blocking antibody was removed, 100 μl of the anti-CA antibody diluted 1:500 in PBS-BSA was added to each section. The anti-CA antibody was detected by using peroxidase-conjugated AffiniPure goat anti-rabbit IgG heavy and light chains diluted to a final concentration of 1.6 μg per ml in PBS-BSA. Peroxidase-labeled antibody was detected by using 3,3′-diaminobenzidine tetrachloride–nickel-cobalt according to the manufacturer's directions (Zymed Laboratories, Inc., San Francisco, Calif.). Each section was counterstained with nuclear fast red for 1 min before mounting.

Positive signals of apparently equal intensity were observed in the bones of rabbits infected with UAMS-1 and rabbits infected with UAMS-4 (Fig. 5). Moreover, the histologic areas that were positive by IHC corresponded precisely to areas in which bacteria were detected by Gram stain (Fig. 5). No signal was observed in the absence of the anti-CA antibody (data not shown) or in bones from uninfected control rabbits (Fig. 5). These results demonstrate that the collagen adhesin is exposed on the surface of *S. aureus* growing in bone. Because comparison of our collagen binding data and Northern blot data suggests that the collagen adhesin persists on the cell surface even after *cna* transcription is reduced to minimal levels, it could be argued that the presence of the adhesin on the cell surface does not reflect the active transcription of *cna*. However, it should be noted that the histologic sections used in these studies were taken from rabbits after a 4-week postinfection period (8). Given such an extended infection period, we believe the results of our IHC analysis strongly suggest that *cna* transcription occurs in vivo at the site of infection. Finally, our results demonstrate that mutation of *agr* has no affect on the in vivo expression of *cna*. Although not conclusive, these results suggest that the residual pathogenicity of UAMS-4 in our osteomyelitis model may be due, at least in part, to the continued ability to express *cna* in a regulated manner even in the absence of a functional *agr* locus.

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