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Functional Analysis of the *Staphylococcus aureus* Collagen Adhesin B Domain

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The *Staphylococcus aureus* collagen adhesin (CNA) occurs in at least four forms that differ in the number (one, two, three, or four) of B domains. The B domains contain 187 amino acids and are located between the domains that anchor CNA to the cell envelope and the ligand-binding A domain. To determine whether a B domain is required for functional expression of CNA, we cloned the 2B *cna* gene from *S. aureus* strain Phillips and then eliminated both B domains by overlapping PCR. The absence of a B domain did not affect processing of the collagen adhesin to the cell surface or the ability to bind collagen. Based on our recent demonstration that the capsule can mask CNA on the surface of *S. aureus* cells (A. F. Gillaspy et al., Infect. Immun. 66:3170–3178, 1998), we also investigated the possibility that multiple B domains can extend the ligand-binding A domain outward from the cell surface and thereby overcome the inhibitory effect of the capsule. Specifically, we cloned the naturally occurring 4B CNA variant from *S. aureus* UAMS-639 and, by successive elimination of B domains, generated 1, 2, and 3B variants that are isogenic with respect to the 4B clone. After introducing each variant into microencapsulated and heavily encapsulated strains of *S. aureus* and growing cells under conditions known to affect capsule production (e.g., growth on Columbia agar), we correlated capsule production with exposure of CNA on the cell surface and the ability to bind collagen. Under no circumstance was the masking effect of the capsule reduced by the presence of multiple B domains. These results indicate that the B domains do not extend the ligand-binding A domain outward in a fashion that can overcome the inhibition of collagen binding associated with capsule production.

Staphylococcus aureus can bind a variety of proteins present in the host extracellular matrix (ECM). The ability to bind ECM proteins is a function of ligand-specific adhesins collectively referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (24). The MSCRAMM adhesins share a common structural organization that includes (i) an N-terminal signal sequence, (ii) a nonrepetitive region that is often responsible for binding of the ECM protein, (iii) a repetitive region that exhibits strain-dependent variability with respect to length, and (iv) a C-terminus anchoring domain that includes an LPXTG anchoring motif, a hydrophobic membrane-spanning domain, and a carboxy-terminal tail rich in positively charged amino acids (24, 31).

Although the *S. aureus* collagen-binding adhesin (CNA) shares these architectural features, it is unique by comparison to other MSCRAMMs. For example, the gene (*cna*) encoding CNA is the only recognized MSCRAMM gene that is not present in all *S. aureus* strains (34). Additionally, although it contains the LPXTG anchoring motif, there is evidence to suggest that CNA may be anchored to the cell via its hydrophobic membrane-spanning domain rather than a covalent linkage to the cell wall peptidoglycan (32). The repetitive domain in CNA is remarkably large, consisting of between one and four copies of a 187-amino-acid region designated the B domain (8). Additionally, while the repetitive regions in other MSCRAMMs are essential for functional exposure of the ligand-binding domain (11) or are directly involved in binding the target protein (33, 37), the repetitive B domain(s) of CNA has not been associated with any function. Indeed, recent data suggest that the B domain is not required for collagen binding (27).

Comparisons between *cna*-positive and *cna*-negative strains of *S. aureus* clearly indicate that CNA is the primary determinant of the ability to bind collagen (7). However, *cna*-positive strains also exhibit significant differences with respect to collagen binding capacity (CBC). These differences appear to be related to the level of *cna* transcription rather than functional differences correlated to the number of B domains (7). However, it remains possible that the number of B domains is biologically relevant at least under some circumstances. For instance, our comparison of heavily encapsulated strains and their corresponding capsule mutants demonstrated that the capsule can mask CNA on the cell surface to an extent that effectively limits its ability to bind collagen (7). That is an interesting observation because it suggests that two phenotypes (collagen binding and capsule production) that are both thought to contribute to the pathogenesis of staphylococcal infection (20, 25) are not compatible with each other. However, the heavily encapsulated strains that we examined (M and Smith diffuse) are not representative of the microencapsulated serotype 5 and 8 strains most often associated with human infection (2, 3, 14, 15, 26, 35). Additionally, both strains encode a CNA variant with a single B domain (7). These observations led us to question whether capsule production inhibits collagen binding under biologically relevant conditions and, if so, whether multiple B domains act like a stalk to extend the ligand-binding A domain outward from the cell surface and

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)	Location[a]
B1	GGATCCACAGCTTCCGGTTTAATAGGTGTA	−475
B2	GGATCCAGGCCACTCTTAGTCTGCTTACAT	3659
B3	**TCGGTTTTTTGATTGGTTTT**TCAGTATTAG	1596
B4	**AAAACCAATCAAAAAAACCGA**ATAAACCAATC	3280
A1	GATCAGATTCAAGGTGGACAGC	625
A2	CTGCTCAAAAGGTTTGGGAAGG	1307
16S-1	CCTATAAGACTGGGATAACTTCGGG	119
16S-2	CTTTGAGTTTCAACCTTGCGGTCG	910

[a] Locations of *cna* primers are relative to the ATG start codon; locations of 16S rRNA gene primers are based on GenBank accession no. X68417. Orientations of the B2 and B3 primers are opposite those of the B1, B4, A1, and A1 primers. The underlined bases in the B2 primer define a *Bam*HI site that was added to facilitate cloning; the given location of 3659 corresponds to the adenine adjacent to this *Bam*HI site. Given their opposite orientations, the boldfaced bases in the B3 and B4 primers are complementary to each other. Double underlines indicate bases on the opposite end of the B domains. More directly, the first 10 bases in the B3 primer correspond to the 5′ end of the W domain, with position 1596 corresponding to the last base at the 3′ end of the A domain. Similarly, the first 10 bases in the B4 primer correspond to the 3′ end of the A domain, with position 3280 corresponding to the 5′ end of the W domain.

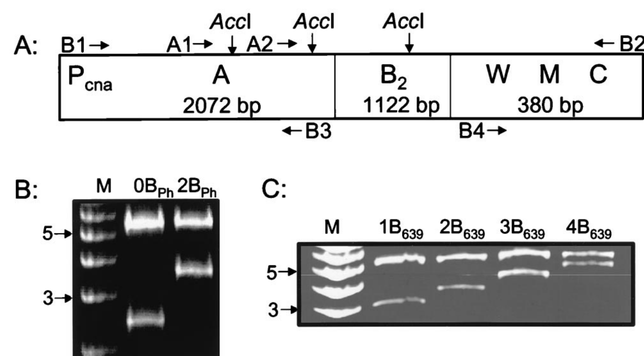

FIG. 1. Verification of *cna* clones. (A) Schematic representation of *cna*. Domains are indicated by uppercase letters within boxes. The location of each primer and its orientation is indicated by an arrow. The relative location of each *Acc*I site is also indicated. (B) Electrophoretic confirmation of isogenic 0B and 2B *cna* clones. (C) Electrophoretic confirmation of the isogenic clones derived from the UAMS-639 4B *cna* variant. In both panels, the upper band is the vector (pLI50), with the size variation in the lower band reflecting the number of B domains. Lanes M contain molecular size markers, with the approximate sizes of selected markers indicated to the left.

thereby overcome the inhibition associated with capsule production.

To address these issues, we constructed isogenic *cna* variants containing one, two, three, or four B domains. We also constructed a *cna* variant that does not include a B domain. Each variant was introduced into microencapsulated and heavily encapsulated strains of *S. aureus* and compared with respect to exposure of CNA on the cell surface and the ability to bind collagen.

MATERIALS AND METHODS

Bacterial strains. Phillips and UAMS-639 are *S. aureus* isolates that encode the 2B and 4B *cna* variants, respectively (8). UAMS-128, Newman, and Wright are *cna*-negative strains. UAMS-128 is representative of the prototypical phage group III strain 8325-4. Newman and Wright are microencapsulated strains representative of capsular serotypes 5 and 8, respectively (5, 18). A capsule-deficient mutant of Newman was generated by inactivating specific *cap* loci, using pCL7960 as previously described (7). Smith diffuse (SD) is a heavily encapsulated serotype 2 strain (7). Smith compact (SC) is a spontaneous capsule-deficient mutant of SD (7). For studies assessing the ability to bind fibronectin, *cna* clones were introduced into a derivative of 8325-4 (DU5883; kindly provided by Tim Foster, Trinity University, Dublin, Ireland) carrying mutations inactivating the *fnbA* and *fnbB* genes.

Growth conditions. Because *cna* is maximally expressed during exponential growth (7, 9), most assays were done with cells taken from exponentially growing tryptic soy broth (TSB) cultures maintained under aerobic conditions at 37°C. However, because capsule production is highest during the postexponential growth phase and when cells are grown on solid media (17), some experiments were done with bacteria harvested from postexponential-phase TSB cultures or from Columbia agar plates. Where required to ensure the maintenance of plasmids (see below), the medium was supplemented with 5 μg of chloramphenicol per ml.

Cloning of natural *cna* variants. To generate the 2B and 4B *cna* clones, we synthesized a pair of oligonucleotide primers (B1 and B2) that could be used to amplify each variant, using genomic DNA from Phillips (2B) and UAMS-639 (4B), respectively. The location of the B1 primer (Table 1) dictated that each amplification product included the upstream region containing the endogenous promoter for each variant (Fig. 1A). Nucleotide sequences of the oligonucleotide primers and their locations relative to the *cna* structural gene are given in Table 1.

PCR amplification of the 2B *cna* variant was done by using *Taq* polymerase (Qiagen, Inc., Valencia, Calif.) with cycling parameters of (i) 94°C for 2 min, (ii) 94°C for 1 min, (iii) 50°C for 1.5 min, (iv) 72°C for 1.5 min, (v) repetition of steps ii through iv for 30 cycles, and (vi) 10-min extension at 72°C. Amplification of the 4.5-kb 4B variant was done using an Extended Long PCR kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's recommendations. Amplification products were ligated into the pCR2.1 TOPO vector (Invitrogen, Inc., Carlsbad, Calif.) and introduced into *Escherichia coli* by electroporation. Transformants were selected on tryptic soy agar containing 100 μg of ampicillin per ml.

After verifying the fidelity of each clone by restriction digest analysis and by nucleotide sequencing (data not shown), we subcloned amplified fragments into the *E. coli*-*S. aureus* shuttle vector pLI50. Ligation products were introduced into *E. coli* Ultracomp TOP10F′ cells (Invitrogen) by electroporation. Plasmids isolated from the Ultracomp TOP10F′ transformants were verified by restriction enzyme analysis (Fig. 1B and C) and then introduced into *S. aureus* RN4220 by electroporation (4). Transformants were selected on tryptic soy agar containing 5 μg of chloramphenicol per ml. Plasmids were introduced into the experimental strains either by electroporation or by ϕ11-mediated transduction (7).

Generation of isogenic CNA variants. To generate a *cna* variant lacking a B domain, we synthesized oligonucleotide primers (B3 and B4) corresponding to the regions immediately upstream and downstream of the B domains. These oligonucleotides were synthesized with an orientation that primed DNA synthesis away from the B domains, with each primer having the reverse orientation with respect to one of the two primers (B1 or B2) used to amplify the full-length *cna* variants discussed above (Fig. 1A). The B1 and B3 primers were used to amplify a 2,070-bp fragment containing the *cna* promoter region and the ligand-binding A domain, while the B2 and B4 primers were used to amplify a 380-bp fragment containing the cell envelope-spanning domains and the cytoplasmic tail (Fig. 1A). Importantly, the 5′ ends of the B3 and B4 primers contained 20-bp regions that were directly complementary to each other (Table 1).

After amplification of the 2,070- and 380-bp fragments from the genome of *S. aureus* Phillips, the two fragments were gel purified and mixed in PCR buffer containing deoxynucleoside triphosphates and *Taq* polymerase at a large fragment-to-small fragment ratio of 20:1. The fragment mixture was denatured by heating and allowed to reanneal; then, without adding primers, a fill-in reaction was done during five amplification cycles. At that point, the B1 and B2 primers were added, and the amplification continued for an additional 35 cycles. The resulting 2,450-bp products were gel purified and cloned into the pCR2.1 vector described above. DNA sequencing confirmed that both of the B domains in the Phillips *cna* clone were eliminated without introducing frameshift mutations at the junction between the A domain and the wall-spanning (W) domain (data not shown).

To generate the isogenic 1B, 2B, and 3B variants, the natural 4B pCR2.1 clone from UAMS-639 was partially digested with *Acc*I, which has two restriction sites located upstream of the B-domain region and an additional restriction site within each B domain (Fig. 1A). The resulting fragments were examined by electrophoresis. Those products that had a size consistent with the presence of one, two, or three B domains were gel purified and recircularized by using T4 DNA ligase. Each plasmid variant was then introduced into *E. coli* Ultracomp TOP10F′ cells by electroporation. To confirm that neither of the *Acc*I sites upstream of the B domains had been cleaved, we designed a primer (A1) corresponding to the region upstream of both sites and a second primer (A2) corresponding to the region between the A-domain *Acc*I sites and in the same orientation as the A1 primer. Using these primers together with the B3 primer, we confirmed that we could amplify a fragment of the appropriate size (i.e., the size expected if the region containing both *Acc*I sites was retained) from all clones (data not shown). We then confirmed that we had eliminated one, two, or three B domains by restriction analysis of each plasmid clone (Fig. 1C), by PCR amplification using the B1 and B2 primers and by DNA sequencing (data not shown). Each *cna* variant was then subcloned into pLI50 and introduced first into *E. coli* and then into RN4220 as described above. Each clone was then introduced into the experimental strains by ϕ11-mediated transduction.

Northern blot analysis. Total cellular RNA was isolated from bacteria harvested from exponentially growing TSB cultures as previously described (9). Briefly, overnight cultures were diluted 1:100 into fresh TSB prewarmed to 37°C. After 4 h, the optical density (A_{560}) was determined to ensure that all cultures were in the exponential growth phase. A 10-ml sample was immediately mixed with ice-cold acetone-ethanol (1:1) and processed for RNA by using RNAzol (7). Northern slot blots were done with a *cna*-specific probe as previously described (7). To ensure that quantitative differences in the amount of *cna* mRNA were not due to unequal loading, we carried out parallel Northern blot analyses using a fragment of the *S. aureus* 16S rRNA gene (Genbank accession no. X68417) as a probe. Sequences of the primers used to amplify the 792-bp 16S rRNA probe are given in Table 1.

Western blot analysis. Bacterial cells from overnight TSB cultures were subcultured to 20 ml of fresh, prewarmed TSB to yield a starting optical density (A_{560}) of 0.05. After 4 h, cells were harvested by centrifugation, washed, and then resuspended in 1 ml of TEG buffer (25 mM EGTA, 25 mM Tris-HCl [pH 8.0]). After addition of 200 μg of lysostaphin and incubation at 37°C for 20 min, protoplasts were transferred to BioPulverizer tubes containing 0.1-mm-diameter silica beads (Bio 101, Vista, Calif.) and then lysed in an FP120 FastPrep (Savant Instruments, Inc., Holbrook, N.Y.) set at 6.5 m/s and run for 40 s. After lysis, tubes were cooled on ice and then centrifuged. The supernatant was removed, and the protein content of each sample determined by the Bradford protein assay (Bio-Rad Laboratories, Hercules, Calif.).

Proteins were resolved for Western blot analysis by electrophoresis under denaturing conditions using 10 to 20% gradient gels and a Bio-Rad Mini-PROTEAN II electrophoresis chamber. Duplicate gels were run so that one gel could be transferred and a second gel stained to ensure equal loads between lanes. To standardize the loads on both gels, a 60-μl mixture containing 50 μg of protein in sample buffer was boiled and then split into 25-μl fractions for loading onto each gel. After electrophoresis, one gel was stained with Coomassie blue while the other was transferred to a polyvinylidene difluoride membrane. Membranes were processed at room temperature by (i) blocking for 2 h with Tris-buffered saline containing 0.1% Tween 20 and 5% dry milk, (ii) blocking a second time with the same buffer containing 100 μg of human Fc fragment (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.), (iii) incubation for 1 h in blocking buffer containing 100 μg of human Fc fragment and 10 μl of primary antibody (see below), and (iv) incubation for 1 h in blocking buffer containing 10 μl of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) and 10 μl of horadish peroxidase-conjugated antibiotin antibody. The antibiotin antibody was added to allow simultaneous detection of the biotinylated size markers (New England Biolabs, Beverly, Mass.). Blots were developed by using the Lumiglo substrate (New England Biolabs) according to the manufacturer's recommendations.

FACS analysis. Surface expression of CNA was assessed by fluorescence-activated cell sorting (FACS). Cells for FACS analysis were grown in TSB at 37°C with appropriate antibiotic selection. After 4 h, cells were harvested by centrifugation, washed once with ice-cold phosphate-buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 0.1% sodium azide [pH 7.4]) and then resuspended in PBS to a density of 10^8 CFU per ml. To minimize nonspecific binding of IgG to protein A, 27 μg of purified human IgG Fc fragment (Jackson ImmunoResearch) was added and the cell suspension was incubated with end-over-end rocking for 30 min. The amount of Fc fragment required to eliminate background activity associated with protein A was empirically determined by carrying out parallel reactions using rabbit nonimmune serum (data not shown). After blocking protein A, the cell suspension was washed with ice-cold PBS and then resuspended in PBS containing primary antibody. The primary antibody was an IgG preparation purified from rabbit polyclonal antisera generated by immunizing rabbits with a 55-kDa peptide corresponding to the CNA ligand-binding A domain (9). After incubating at 37°C for 30 min, the suspension was washed with ice-cold PBS and then incubated for an additional 30 min in the presence of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG Fab_2 fragments (Jackson ImmunoResearch). Cells were then washed, fixed, and assayed by FACS analysis as previously described (11).

Binding assays. Binding assays were done with cells grown in TSB or harvested from Columbia agar plates. In the case of broth cultures, cells were harvested by centrifugation, washed with PBS, and then 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). When assays were done with cells grown on solid media, bacterial colonies were harvested after overnight growth, washed with PBS, and then resuspended in binding buffer to an optical density of 1.0. Once the cell suspensions were prepared, binding assays were done with ^{125}I-labeled type I collagen or fibronectin as previously described (7). All assays were done in quadruplicate, with the results reported as average ± standard error of the mean.

Shear force studies. Shear force studies were done as previously described (19), with minor modifications. Briefly, bacteria were harvested from TSB cultures and resuspended in PBS to a cell density of 10^7 CFU per ml. Assays were done with glass coverslips coated with type II collagen (Sigma Chemical Co., St. Louis, Mo.). The purity of the collagen was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Collagen-coated coverslips were prepared as previously described (28, 29). Briefly, collagen was dissolved in 3.0% glacial acetic acid at a final concentration of 2.44 mg per ml; 20 μl of the collagen solution was placed on a glass coverslip (no. 1, 24 by 50 mm; Corning Inc., Corning, N.Y.) in the region corresponding to the area of flow (approximately 60 mm^2). After incubation in a humid atmosphere for 1 h, coverslips were rinsed with PBS and then assembled into the flow chamber. The amount of immobilized collagen on the coverslip was estimated by assaying the protein content of the rinse, using a modified Lowry assay (Sigma). The collagen concentration on the coverslip was 9.8 ± 0.9 μg per cm^2 (data not shown). To verify that the entire area of flow was coated with collagen, preliminary studies were performed to confirm that the level of nonspecific adhesion was insignificant (data not shown).

A parallel-plate flow chamber was used (1) to mimic dynamic *in vivo* conditions. The flow chamber was cleaned and disinfected before each use. After assembly of the flow chamber with a collagen-coated coverslip, the chamber was filled with PBS. Because wall shear rate is a function of the flow chamber geometry (6), different wall shear rates could be attained by varying the flow rate through the chamber. Flow rate was controlled using a syringe pump (model 44; Harvard Apparatus, South Natick, Mass.). The flow chamber assembly was mounted onto a computer-driven stage (Ludl Mac 2000; Ludl Electronic Products, Hawthorne, N.Y.) of an Olympus IMT-2 phase-contrast microscope (Olympus Corp., Lake Success, N.Y.). The microscope stage was maintained at 37°C in an air-curtain incubator. A charge-coupled device camera (CCTV Corp., South Hackensack, N.J.) was used to obtain images at four equidistant points on the coverslip. The images from the camera were sent to a VCR (model HR-VP422U; JVC, Elmwood Park, N.J.), where they were recorded. The captured images were digitized by a frame grabber board (LG-3; Scion Corp., Frederick, Md.) on a computer (Quadra 950; Apple Computer, Inc.) at a rate of 10 frames per second. The public-domain program NIH Image (26a) was used to analyze captured images. The field of view used for counting adherent cells was 0.0206 mm^2. The cell counts obtained from all four images were averaged for each experiment. The adherent cell densities were not significantly different at randomly selected points on the coverslip. We assumed that adhering cells did not affect the downstream bulk cell concentration, since the number of adherent cells was negligible compared to the bulk concentration of the cell suspension (data not shown).

RESULTS

Requirement for a B domain. To determine whether processing or function of the CNA adhesin is dependent on the presence of at least one B domain, we assessed the presence of CNA on the cell surface and compared the CBC of the Phillips 2B clone and the isogenic derivative in which both B domains had been eliminated. The fact that the 0B and 2B variants were isogenic was confirmed by DNA sequencing and by Northern blot analysis demonstrating that both variants were expressed at equivalent levels (data not shown). Comparison of the 0B and 2B variants in each of three *S. aureus* strains confirmed that there was no significant difference in the exposure of CNA on the cell surface (Fig. 2A) or the ability to bind collagen (Fig. 2B). These results indicate that the B domain has little or no impact on transport of the CNA adhesin to the cell surface or on presentation of the ligand-binding A domain.

Comparison of isogenic *cna* variants. It is difficult to make definitive conclusions about the contribution of the B domain(s) to collagen binding because the number of strains that produce certain variants is limited (UAMS-639 is the only recognized strain that produces the 4B variant) and because comparisons are complicated by differences in the level of *cna* transcription (7). To address these issues, we generated isogenic variants of *cna* derived from the UAMS-639 4B clone. The isogenicity of the 1B, 2B, and 3B clones with respect to the 4B clone was confirmed by DNA sequencing (data not shown) and by Northern blot analysis demonstrating that all four variants were expressed at indistinguishable levels (Fig. 3A). Additionally, as determined by Western blotting, the amount of CNA in cell lysates prepared with each variant was the same (Fig. 3B), and we did not observe a significant difference between any of the clones in our FACS analysis (Fig. 4A). However, quantitative binding assays revealed a reduced CBC with the 4B CNA variant (Fig. 4B). Although this reduction was modest, the fact that the results obtained with the 1B, 2B, and 3B variants using four different assays (Northern blotting Western blotting, FACS analysis, and quantitative collagen

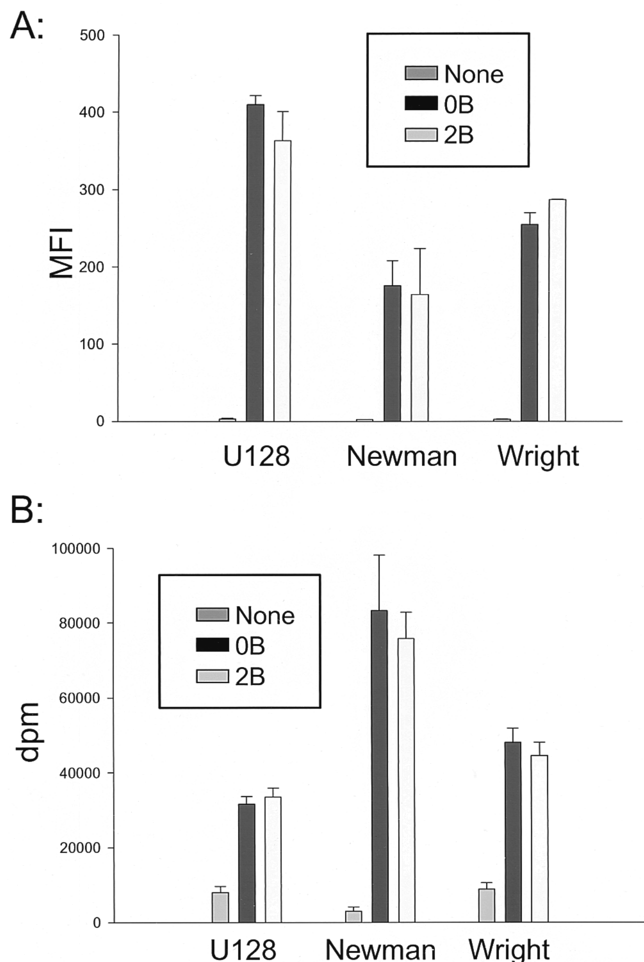

FIG. 2. Functional comparison of 0B and 2B variants. The 0B and 2B *cna* clones were introduced into the *cna*-negative strains UAMS-128 (U128), Newman, and Wright and then assayed by FACS (A) and quantitative collagen binding assay (B). Results of FACS are reported as mean fluorescence intensity (MFI); results of ^{125}I-collagen binding assays are reported as disintegrations per minute (dpm). "None" refers to the parent strains without a *cna* clone. The results shown represent the average of two assays, each of which was repeated twice. Error bars indicate standard error of the mean.

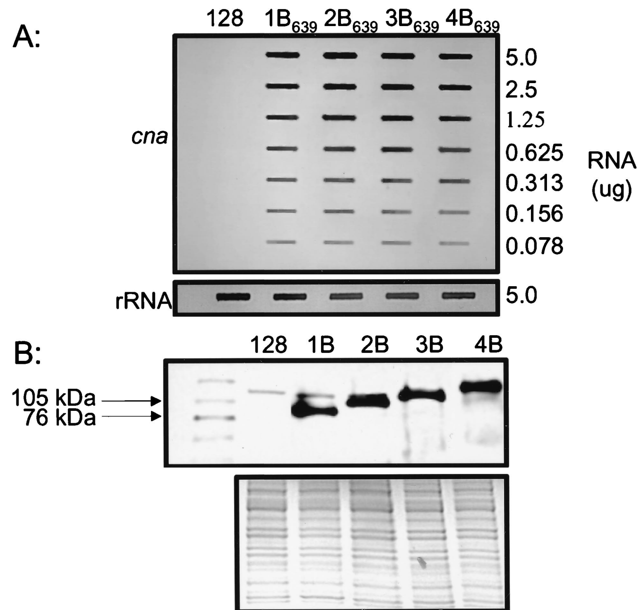

FIG. 3. Analysis of isogenic *cna* clones. The 1B, 2B, 3B, and 4B *cna* variants derived from UAMS-639 clone were introduced into the *cna*-negative strain UAMS-128 (U128) and examined by Northern (A) and Western (B) blotting. The lower portion of panel B is a stained gel identical to the gel used for the Western blotting. Probes used for each Northern blot are indicated to the left of panel A.

binding assays) were comparable, while the results of collagen binding assays with the 4B clone were low (Fig. 4B) by comparison to the level of *cna* transcription (Fig. 3A), and the presence of CNA on the cell surface (Fig. 4A) suggested that the 4B variant may not bind collagen as efficiently as CNA variants having fewer B domains. In fact, the combined results obtained when the *cna* variants were compared in seven different strains of *S. aureus*, each of which was assayed at least twice, revealed an almost linear decline that was directly correlated to an increase in the number of B domains (Fig. 4C). Statistical analysis confirmed a significant difference ($P < 0.0009$) between the 1B CNA variant and both the 3B and 4B variants (Fig. 4C).

The fact that the difference in CBC observed with the 1B CNA variant and the 3B and 4B variants was statistically significant does not mean that the difference is biologically relevant. In an attempt to address this issue, we did shear force studies using conditions that create shear forces approximating those associated with blood flow (29). These studies revealed that the 3B and 4B variants also adhered to a collagen-coated substrate less efficiently than an isogenic 1B variant (Fig. 4D). Again, while the effect was modest, it was both reproducible and statistically significant ($P < 0.05$). It should also be noted that the differences between the 1B variant and the 3B and 4B variants was apparent only at intermediate shear forces; presumably, no difference was observed at lower or higher shear forces because the former is insufficient to inhibit attachment while the latter is high enough to have the same relative effect on all CNA variants.

Fibronectin binding. Hienz et al. (12) reported that mutation of *cna* resulted in a reduced capacity to bind fibronectin. Additionally, a search of the GenBank database for proteins similar to a single B domain at the amino acid level revealed some similarity (~30%) with three different streptococcal fibronectin-binding proteins (S33851, S42798, and U31980). Based on that finding, we introduced each of our isogenic *cna* clones as well as the 0B variant derived from Phillips into an 8325-4 derivative unable to bind fibronectin (DU5883). In no case was the ability to bind fibronectin altered by comparison to the parent strain (data not shown).

Correlation between number of B domains, capsule production, and CBC. The results discussed above suggest that multiple B domains do not promote and may actually impair the ability to bind to collagen. However, it remains possible that the B domains serve some function that is correlated to collagen binding but only under certain conditions. As discussed above, one possibility is that multiple B domains can relieve the inhibition of collagen binding associated with capsule production by extending the ligand-binding A domain outward from the cell surface. To address this possibility, we introduced each of the isogenic *cna* clones into the microencapsulated strain Newman and its capsule mutant and into the heavily encapsulated strain SD and its capsule mutant (SC). Comparison of the CBC observed with Newman and its capsule mutant revealed no significant degree of inhibition, while comparison

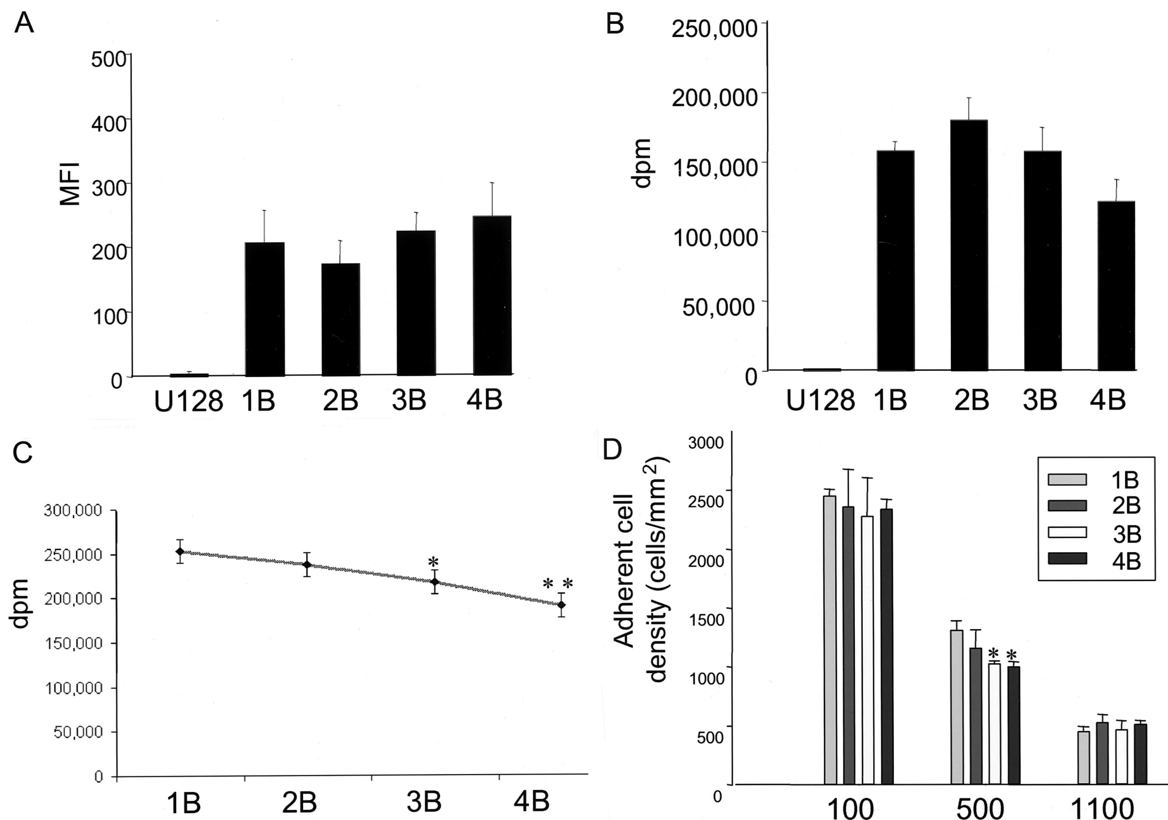

FIG. 4. Functional comparison of isogenic CNA variants. Each of the isogenic *cna* clones was introduced into the *cna*-negative strain UAMS-128 (U128) and assayed by FACS (A) and quantitative collagen binding assay (B). Results of FACS are reported as mean fluorescence intensity (MFI); results of ^{125}I-collagen binding assays are reported as disintegrations per minute (dpm). Results shown represent the average of two experiments with each experiment including duplicate assays. Results are shown as mean ± standard error of the mean. (C) Cumulative results of all collagen binding assays ($n = 30$). (D) Results of shear force studies. Units on the *x* axis are per second. Results shown represent the average of two assays, each of which was repeated twice. Error bars indicate standard error of the mean. The asterisks in panels C and D indicate statistical significance ($P < 0.05$).

of the CBC of SD and its capsule mutant (SC) confirmed that all four CNA variants were masked to approximately the same extent (data not shown). While these results confirm our earlier conclusion (7) that the capsule can mask CNA on the surface of *S. aureus* cells, the prominence of serotype 5 strains like Newman (3, 14, 15, 35) also suggests that this masking may not be biologically relevant with respect to human infection. At the very least, these results suggest that multiple B domains do not offer an advantage with respect to overcoming the inhibition associated with capsule production. However, these results must be interpreted with caution because it is possible that under the growth conditions used for these experiments (mid-exponential-phase TSB cultures), Newman simply does not make enough capsule to inhibit the ability of any CNA variant to bind collagen. To address this possibility, we carried out a final set of experiments in which we assessed the CBC of the Wright strain grown under conditions known to enhance capsule production. Wright was chosen for these experiments because its colony morphology suggested that it was more encapsulated than Newman. However, even when we assayed cells taken from different growth phases (Fig. 5A to C) or harvested from Columbia agar (Fig. 5D), we could not demonstrate an elevated CBC correlated with multiple B domains. Indeed, as was observed in all of our experiments, the 4B CNA variant consistently yielded the lowest CBC regardless of the strain or growth conditions used.

DISCUSSION

Our previous results demonstrating that the capsule can mask the CNA adhesin on the surface of *S. aureus* cells were limited to the heavily encapsulated serotype 1 and 2 strains M and SD (7). Such heavily encapsulated strains are not representative of the microencapsulated serotype 5 and 8 strains most commonly associated with human infection (20, 23, 35). Additionally, we examined two microencapsulated serotype 8 strains (UAMS-1 and Becker) in our previous study (7) and did not observe a significant reduction in collagen binding by comparison to their corresponding capsule mutants. These results suggest that the masking we observed with M and SD may not be biologically relevant. That may also account for the discrepancy between our results and previous studies (20) demonstrating that capsule production does not significantly influence the ability to bind fibronectin, bone sialoprotein, or collagen. There is also evidence to suggest that staphylococcal capsule does not limit the deposition of antibody or complement on the cell surface (16, 38). However, Ohtomo and Yoshida (22) demonstrated that the SD capsule can mask the MSCRAMM adhesin(s) responsible for fibrinogen binding even when the capsule is not maximally produced. Moreover, with respect to collagen binding, the earlier studies concluding that the capsule does not inhibit collagen binding must be interpreted with caution because the comparisons were done with a strain

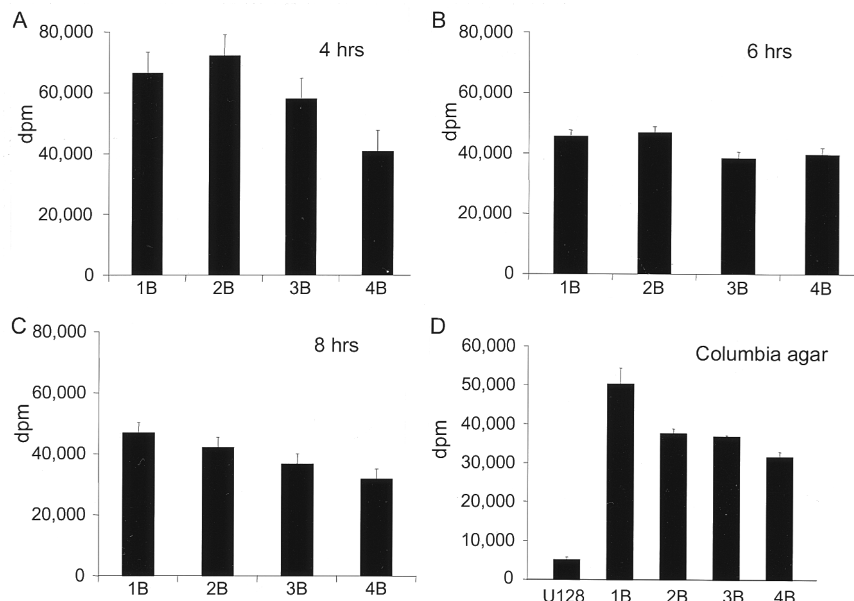

FIG. 5. Quantitative collagen binding as a function of growth conditions. *S. aureus* Wright was transformed with each isogenic *cna* clone and examined by quantitative collagen binding assay using cells taken from exponentially growing cultures (A) and from post-exponential-phase cultures (B and C). (D) Results obtained when the cells used in the collagen binding assay were harvested from Columbia agar. Results of ^{125}I-collagen binding assays are reported as disintegrations per minute (dpm). Results shown represent the average of two experiments, each including duplicate assays. Results are shown as mean ± standard error of the mean.

(Reynolds) that does not encode *cna* (20). Finally, because *cna* is maximally expressed during the exponential growth phase (9), for our earlier collagen binding assays we used cells taken from exponentially growing broth cultures (7). However, capsule production is highest in cells taken from post-exponential-phase cultures and higher still when cells are grown on solid medium (17). It is therefore possible that our earlier results with serotype 8 strains (7) did not accurately assess the potential for capsule inhibition.

A second consideration with respect to our earlier work (7) is that both M and SD encode the *cna* variant with a single B domain. Because some strains encode a *cna* variant with as many as four B domains (8), the overall size of the CNA adhesin can vary by as much as 561 amino acids. Although repetitive sequences are routinely found in bacterial genes, and many of these have no known function, the repeats are rarely of the magnitude of the CNA B-domain repeats. Moreover, the repetitive domains of other *S. aureus* adhesins are required either as the ligand-binding domain itself or for presentation of the ligand-binding domain (11, 33, 37). Our results confirm earlier reports (27) that a B domain is not required for the functional exposure of CNA. However, that does not preclude the possibility that multiple B domains have a bearing on the overall capacity to bind collagen, at least under some circumstances. Based on the preceding discussion, one possibility is that multiple B domains extend the ligand-binding A domain outward from the cell surface and thereby relieve the inhibition associated with capsule production.

We initially attempted to address this possibility by cloning each the naturally occurring CNA variants encoding one, two, three, or four B domains (8) and introducing each clone into strains producing various amounts of capsule. Although we could not demonstrate any circumstance in which the presence of multiple B domains was correlated with an elevated CBC, these studies were difficult to interpret because each of the natural *cna* clones, all of which included the promoter endogenous to their respective parent strains, were expressed at different levels even when introduced into the same genetic background (data not shown). To overcome that limitation, we generated isogenic *cna* variants with one, two, three, or four B domains and introduced each variant into different strains of *S. aureus*. The results confirmed our earlier report (7) suggesting that the capsule can mask the CNA adhesin; however, this masking effect was observed irrespective of the number of B domains. More directly, when we compared the CBC of SD and SC derivatives transformed with each isogenic clone, we found that the level of inhibition was not correlated to the number of B domains (data not shown).

We also examined the CBC of the serotype 5 and 8 strains Newman and Wright after transforming these strains with each *cna* variant and growing both strains under conditions known to enhance capsule production. Because capsule production is highest during the post-exponential growth phase (17), we first examined cells taken from post-exponential-phase broth cultures. However, we did not observe any effect other than a general decline in the overall CBC. Because *cna* is preferentially expressed during the exponential growth phase (7, 9), this decline in CBC could reflect reduced production of CNA. However, the CNA adhesin appears to be very stable (7), and it is also possible that the decline reflects increased capsule production rather than some effect directly related to CNA. For example, Ohtomo and Yoshida (22) demonstrated that a growth-phase-dependent decline in fibrinogen binding was due to increased capsule production rather than a decrease in production of the fibrinogen-binding protein(s). That would suggest that the decline we observed may have been at least partly due to the increased production of capsular polysaccharides, in which case the lack of a correlation between the number of B domains and an elevated CBC would provide further support for the hypothesis that the B domains do not act like a stalk. However, Lee et al. (17) established that as much as 98% of capsular polysaccharide could be recovered from the superna-

tant of cells grown in liquid medium regardless of whether cells are harvested in exponential or stationary phase and that, by comparison to broth cultures, growth of *S. aureus* on solid medium results in an ~300-fold increase in the production of capsular polysaccharide (17). Based on that finding, we also examined the CBC of cells harvested from Columbia agar. Again, we could not establish a correlation between reduced capsule inhibition of collagen binding and the number of B domains. We believe that these results rule out the possibility that the B domains can extend the ligand-binding A domain outward to an extent that has an impact on the correlation between collagen binding and capsule production.

Rich et al. (27) recently characterized the structural characterization of the *S. aureus* CNA. Although they did not include experiments assessing CNA function, they did present convincing evidence that CNA has a mosaic architecture in that (i) the presence of a B domain does not alter the conformation of the A domain and (ii) the presence of multiple B domains does not alter the conformation of any single B domain. It was also noted that CNA was functional even in the absence of a B domain. Our results are consistent with their suggestion that the B domain is not required for CNA function. However, that does not preclude the possibility that multiple B domains have an impact on the functional status of CNA. Indeed, Rich et al. (27) suggested that "these B domain repeats could affect the function or the stability of the A domain, they may serve as a 'stalk,' projecting the A domain away from the bacterial surface and positioning the A domain for binding to collagen, or they may possess binding sites for ligands other than collagen." We addressed all of these possibilities during the course of the studies reported here.

Specifically, while our results effectively rule out the possibility that the B domains function as a stalk as least as it pertains to extending the A domain beyond a capsule boundary, they also suggest that multiple B domains may have an impact on the "function or stability of the A domain" in that they appear to limit the interaction between CNA and collagen. While the reduction we observed with the 3B and 4B variants was modest (~25%), the combined analysis of multiple strains grown under various conditions confirmed an almost linear decline in CBC that was directly correlated to the increase in the number of B domains. Perhaps more importantly, our shear force studies confirmed that strains producing CNA variants with multiple B domains have a reduced capacity to adhere to a collagen-coated substrate. Because these studies were done by using conditions that cause shear forces comparable to those observed in blood, it is possible that the differences we observed have an impact on the ability of *S. aureus* to adhere to collagen within host tissues. However, as yet we have no data suggesting a mechanism for the reduced binding observed with an increasing number of B domains or demonstrating whether the reduction in collagen binding associated with multiple B domains is biologically relevant.

The results discussed above could be interpreted in two ways. The first is that the 4B *cna* variant represents the primordial form of *cna* and that subsequent deletions have reduced the need to maintain such a large, repetitive gene without reducing (and perhaps even increasing) the ability to bind collagen. In this case, the failure to identify a natural 0B variant could simply reflect the fact that a single B domain is a less efficient recombination substrate rather than functional pressure to maintain the B domain. On the other hand, the loss of B domains could offer an immunological advantage. For instance, Gravekamp et al. (10) recently demonstrated that multiple repeats of an 82-amino-acid domain in the alpha C protein of group B streptococci are associated with decreased virulence, apparently because the presence of multiple repeats makes the alpha C protein a more accessible target for antibody. Interestingly, the increased antibody accessibility observed by Gravekamp et al. (10) appeared to be related to extension of the protein beyond the group B capsular polysaccharide. While that does not appear to be the case with CNA, we are nevertheless investigating the possibility that a CNA-specific immune response is more effective against strains expressing *cna* variants with multiple B domains.

The second possibility is essentially the opposite hypothesis in that it suggests that the *cna* gene has acquired additional B domains via some recombinational process. This hypothesis presupposes that multiple B domains serve some useful biological function. Having ruled out the possibility that multiple B domains allow the bacterium to bind collagen without interference from the capsule, it is difficult to envision what this function might be. One possibility is that multiple B domains effectively limit the interaction between the cell and a collagen-containing substrate and thereby increase the bacterium's ability to escape a localized site of infection. At present, we have no evidence to support this hypothesis. Indeed, it is certainly possible that the B domains either are irrelevant with respect to collagen binding or serve some purpose unrelated to collagen binding. With regard to the latter, our results rule out the possibility that the CNA B domains have an impact on the ability to bind fibronectin.

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