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Genetic Evidence that Bound Coagulase of *Staphylococcus aureus* Is Not Clumping Factor

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Staphylococcus aureus **Newman cells carry a surface receptor for fibrinogen called clumping factor. The bacteria also express coagulase, an extracellular protein that binds to prothrombin to form a complex with thrombinlike activity which coverts fibrinogen to fibrin. We have confirmed a recent report (M. K. Bodén and J.-I. Flock, Infect. Immun. 57:2358–2363, 1989) that coagulase can bind to fibrinogen as well as to prothrombin and also that a fraction of coagulase is firmly attached to the cell. A mutant with a deletion in the chromosomal *coa* gene was isolated by allelic replacement. Allelic replacement either was directly selected by electrotransformation of *S. aureus* R3N4220 with a nonreplicating suicide plasmid, pCOA18, carrying the Δ*coa*::Tc^r mutation or occurred after transduction of the integrated pCOA18 plasmid. The *coa* mutant was completely devoid of coagulase activity but interacted both with soluble fibrinogen and with solid-phase fibrinogen with the same avidity as the parental strain. This strongly suggests that the bound form of coagulase is not clumping factor and is not responsible for the adherence of *S. aureus* Newman to solid-phase fibrinogen. The fibrinogen binding determinant of coagulase was located in the C terminus of the protein, by analyzing truncated fusion proteins, in contrast to the prothrombin-binding region which was located in the N terminus.**

Most strains of *Staphylococcus aureus* express coagulase, an extracellular protein that stimulates the conversion of fibrinogen to fibrin to cause the formation of clots in mammalian plasma (13). Coagulase does not have enzymatic activity—it binds to prothrombin with 1:1 stoichiometry, and the complex becomes proteolytically active and initiates polymerization of fibrin (11, 18). Thus, prothrombin is activated without being converted to thrombin.

S. aureus cells form clumps when they are mixed with plasma. Fibrinogen binds to a receptor (called clumping factor) which is located on the bacterial cell surface (10, 13, 36). This factor may also be at least partly responsible for promoting the adherence of bacteria to traumatized tissue and to indwelling medical devices such as catheters and prostheses, which become coated with fibrinogen and other plasma proteins after implantation (5, 40, 41).

The identity of clumping factor has remained elusive. It was originally thought that clumping factor was a cell-bound form of coagulase (bound coagulase) (13). However, in 1954, Duthie (7) suggested that coagulase and clumping factor were separate entities. Since this work was published, there have been several reports claiming to have identified, and in some cases to have purified, clumping factor (6, 22, 37, 39). Recently, Bodén and Flock (3, 4) showed that coagulase could bind to fibrinogen as well as to prothrombin and that a proportion of the normally extracellular protein was tightly associated with the cell surface. They suggested that this cell-bound form of coagulase is, in fact, clumping factor.

Eight serotypes of coagulase have been identified by neutralization tests (38). Recently, the primary structures of coagulases representing serotypes I, II, and III have been determined (15, 16, 28). The predicted molecular weights of the mature (extracellular) forms of the coagulases vary according to the number of tandem repeats of a 27-residue sequence at the carboxy terminus. Thus, the coagulases of strains 8325-4 and Newman (serotype III; predicted molecular mass, 68,904 Da) have five repeats (27), strain 213 coagulase (serotype II; predicted molecular mass, 71,749 Da) has six repeats (15) and strain BB coagulase (serotype I; predicted molecular mass, 77,377 Da) has eight repeats (16). By testing proteolytically generated peptides and deletion mutants (17, 18, 27), the region of coagulase that binds prothrombin was shown to be in the N terminus. These parts of the three coagulases contain sequences that have only 50% identical residues (see Fig. 1) and are probably responsible for the antigenic differences. Elsewhere, the proteins are more than 90% identical.

The purpose of this investigation was to determine if the suggestion that bound coagulase is clumping factor could withstand the scrutiny of genetic analysis. We have previously isolated a coagulase-deficient mutant of strain 8325-4 by allelic replacement (28). This *coa* mutation, which lacked 0.9 kb of DNA internal to the *coa* gene, and a deletion isolated here which lacked all but about 50 bp of the *coa* gene were introduced into *S. aureus* Newman, a strain which has been widely used for studies on clumping factor. The mutants were tested for their ability to clump in a solution of fibrinogen and for adherence to fibrinogen-coated plastic surfaces.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Tables 1 and 2, respectively.

Bacterial growth media and antibiotics. *Escherichia coli* strains harboring plasmids were routinely grown in L broth and L agar (24) incorporating antibiotics (ampicillin [Ap], 100 μg/ml; kanamycin [Ka], 50 μg/ml; chloramphenicol [Cm], 7 μg/ml) where appropriate. *S. aureus* strains were grown in Trypticase soy broth or agar incorporating antibiotics (chloramphenicol, 7 μg/ml; erythromycin [Em], 10 μg/ml; tetracycline [Tc], 2, 15, or 30 μg/ml) where appropriate.

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Purification of fibrinogen. Fibrinogen used for Western affinity blotting was purified as follows. A 200-mg amount of human fibrinogen (Sigma) was dissolved in 50 ml of phosphate-buffered saline (PBS) and passed through a gelatin-Sepharose column to remove contaminating fibronectin. Gelatin-Sepharose was prepared by coupling 10 mg of gelatin (BDH, Poole, United Kingdom) to 100 ml of CNBr-activated Sepharose 4B (Pharmacia) by using the procedure described by the manufacturer. The eluate was then passed through a column of protein A coupled to Sepharose (Pharmacia) to remove contaminating immunoglobulins. The eluate was concentrated by freeze-drying and tested by Western blotting as follows. The fibrinogen solution contained no detectable fibronectin when 5 μg was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 5% acrylamide) and probed with a monoclonal antibody to human fibronectin. No immunoglobulin was detectable when 5 μg of protein was fractionated by SDS-PAGE and probed with peroxidase-conjugated protein A.

A slightly different purification scheme was used to prepare fibrinogen for measuring the attachment of *S. aureus* to this protein immobilized on solid surfaces. Fibrinogen purchased from IMCO (Stockholm, Sweden) was fractionated by Sephacryl S-300 chromatography and then by affinity chromatography on gelatin-Sepharose. This purified fibrinogen was a gift from A. Haeberli, Thrombosis Research Laboratory, University Hospital, Berne, Switzerland.

Measurement of cell clumping. Cell clumping was tested by using a 2% (wt/vol) solution of human fibrinogen (Sigma) in PBS (0.018 M K_2HPO_4, 0.01 M KH_2PO_4, 0.145 M NaCl [pH 7.4]). Clumping was estimated qualitatively by adding 20 μl of fibrinogen solution to a saline suspension of bacterial cells made from freshly grown colonies by using a wire loop. Clumping was measured quantitatively in microtiter trays. Serial twofold dilutions of a 2% (wt/vol) fibrinogen solution were made in PBS, and 50 μl was incubated with 20 μl of a suspension of 2×10^9 washed bacterial cells. The reciprocal of the highest dilution of fibrinogen showing clumping after 3 min was recorded as the titer (37).

Measurement of attachment of bacteria to solid-phase fibrinogen. We have recently modified a previously described staphylococcal adherence assay (40–42) to measure the ability of *S. aureus* to adhere to low levels of surface-adsorbed fibrinogen. Two-sided coverslips (8 by 9 mm) made of polymethylmethacrylate (PMMA) were prepared as previously described (42). They were incubated for 60 min at 37°C with low concentrations (ranging from 0.125 to 1 μg/ml) of purified fibrinogen and were then rinsed once in PBS. It was ascertained that the PMMA surfaces were coated in a dose-dependent manner with fibrinogen amounts ranging from 16 to 145 ng per coverslip (11 to 101 ng/cm^2) when a ^{3}H-labelled preparation of fibrinogen was used (35). Fibrinogen-coated coverslips were then incubated with 4×10^6 CFU of [^{3}H]thymidine-labelled *S. aureus* per ml for 60 min at 37°C, as previously described (42). The adherence medium was PBS with 1 mM Ca^{2+} and 0.5 mM Mg^{2+}, supplemented with 5 mg of human serum albumin per ml which prevented nonspecific adherence of *S. aureus* (40–42). Coverslips were then rinsed, and the number of attached bacteria was estimated from radioactive counts (42). PMMA coverslips coated with albumin were used as controls (40–42).

Measurement of coagulase activity. Coagulase activity was measured by adding 0.5 ml of serial twofold dilutions of *S. aureus* culture supernatants made in PBS to 0.5 ml of a 1:3 dilution of rabbit plasma in PBS. The titer was the reciprocal of the highest dilution of the supernatant showing evidence of clotting after 24 h of incubation at 37°C.

SDS-PAGE and Western blotting. SDS-PAGE was performed by standard procedures (21) with 12.5% acrylamide gels. Proteins were extracted from *S. aureus* cells as follows. Whole cells from 1.5 ml of exponential- or 0.4 ml of stationary-phase cultures were washed twice and boiled in final sample buffer (21). Culture supernatants were concentrated by using 25% (vol/vol) trichloroacetic acid, and the precipitated protein was dissolved in final sample buffer. Proteins were released from *E. coli* cells by using a sonicator (MSE) (5 bursts of 0.5 min with 0.5-min interruptions for cooling). The cells from a stationary-phase broth culture were concentrated fivefold. Proteins were separated by SDS-PAGE and transferred from the gel to nitrocellulose membranes by use of a semidry blotter (Bio-Rad) and 48 mM Tris-HCl–39 mM glycine (pH 9.2) with 20% methanol. Filters were blocked with a 10% (wt/vol) solution of skim milk in PBS. Fibrinogen-binding proteins were detected by one of the following methods: (i) incubation of the filters with 50 μg of purified fibrinogen per ml (see above) and then with a monoclonal antibody recognizing an epitope in the midsection of the alpha-chain (American Biogenetic Sciences, Notre Dame, Ind.; 1:100 dilution) and finally with goat peroxidase-conjugated anti-mouse immunoglobulin G serum (Sigma; 1:500 dilution); or (ii) by incubation of the filters with purified fibrinogen and then with peroxidase-conjugated anti-human fibrinogen (Sigma; 1:500 dilution). Identical results were obtained by both procedures. Fibronectin was detected by using a monoclonal antibody to fibronectin (ICN Pharmaceuticals; 1:100 dilution) and then goat anti-mouse immunoglobulin G serum (Sigma; 1:500 dilution) and peroxidase-conjugated protein A (Sigma; 1:500 dilution). Rabbit anti-Newman coagulase serum (a gift from H. Igarashi, Tokyo Metropolitan Research Laboratory of Public Health) was used to detect coagulase in Western blots. The bound antibody was recognized by using a protein A peroxidase conjugate.

Manipulation of DNA. DNA-modifying enzymes were purchased from the Boehringer Corp. or from Promega and were used according to the manufacturers' instructions. DNA manipulations were performed by using standard procedures (2, 31). DNA hybridization was performed by the method of Southern (34). *S. aureus* genomic DNA was purified by a modification of the method of Mekalanos (23) as described by O'Reilly et al. (25). Probe DNA was nick translated (30) by using [α-^{32}P]dATP (New England Nuclear).

Transfer of DNA. Plasmid DNA was transformed into *E. coli* cells made competent by $CaCl_2$ treatment and into *S. aureus* by electroporation (26).

Transduction. Transduction in *S. aureus* was performed by the method of Asheshov (1) by using bacteriophage 85.

Construction of Δ*coa*::Tc^r. The *coa* gene of strain 8325-4 contains a single *Hin*dIII site and a single *Eco*RI site (Fig. 1). A deletion of DNA located between these sites was involved in the formation of the coagulase mutation Δ*coa*::Em^r (Fig. 1) (28). To construct a deletion lacking the entire coding sequence, the exonuclease *Bal* 31 was used to remove DNA located 5′ to the *Eco*RI site and 3′ to the *Hin*dIII site in a controlled reaction (Fig. 2). To facilitate exonuclease digestion, the strain 8325-4 *coa* gene was cloned from pCOA14 into a vector which lacked both of these sites. This vector was constructed here by cleaving plasmid pBluescript with both *Eco*RI and *Hin*dIII and then treating it with DNA polymerase I Klenow fragment to fill in the single-stranded

TABLE 1. Bacterial strains

Strain	Relevant genotype	Properties	Source or reference
S. aureus			
Newman	*coa*$^+$	High level of clumping factor	8
DU5809	Δ*coa*::Em^r	Mutant of strain 8325-4 defective in coagulase	28
DU5856	Δ*coa*::Em^r	Mutant of strain Newman defective in coagulase; transduction from DU5809	This study
RN4220		Mutant of 8325-4 capable of stably maintaining shuttle plasmids	20
DU5861	Δ*coa*::Tc^r	Mutant of RN4220 defective in coagulase	This study
DU5872	Δ*coa*::Tc^r	Mutant of strain Newman defective in coagulase; transduction from DU5861	This study
DU5864	*coa*::pCOA18	RN4220 with integrated pCOA18	This study
DU5854	Δ*coa*::Tc^r	Mutant of strain Newman defective in coagulase; transduction from DU5864	This study
E. coli TB1	*ara thi rpsL hspR* Δ*lac-pro*XIII φ80*lacIZ*ΔM15		44

ends prior to ligation and transformation into *E. coli* TB1. Plasmid DNA from Ap^r transformants was tested for loss of these two sites and for retention of the *Kpn*I site in the multiple cloning site. One such plasmid, pBluescript (E-H), was kept. The 5.2-kb *Kpn*I fragment from pCOA14 carrying the 8325-4 *coa* gene (27) was then cloned into the *Kpn*I site in pBluescript (E-H), forming pCOA17. This plasmid was then cleaved at the unique *Eco*RI and *Hin*dIII sites within the *coa* gene and treated with *Bal* 31 exonuclease to resect the remaining *coa* DNA, ca. 500 bp, in both the 5′ and 3′ directions (Fig. 2). The DNA was treated with Klenow polymerase and ligated with a *Hin*dIII fragment from the *S. aureus* plasmid pCW59 which carries a tetracycline resistance determinant. This fragment was also treated with Klenow polymerase. Ap^r transformants were tested for tetracycline resistance by replica plating onto L agar containing 2 μg of tetracycline per ml. One plasmid (pCOA18) had the expected structure, which was verified by detailed restriction mapping and by Southern hybridization. The *coa* mutation carried by pCOA18 was designated Δ*coa*::Tc^r.

Cloning the coagulase gene from strain Newman. Genomic DNA from strain Newman was cleaved with *Xba*I and ligated with pUC19 DNA cleaved with the same enzyme. Recombinants carrying the Newman *coa* gene were identified by colony hybridization (9) by using the 0.9-kb *Eco*RI-*Hin*dIII fragment from within the *coa* gene of strain 8325-4 as the probe. One recombinant pNCOA1 was analyzed and shown to carry a 7.7-kb *Xba*I fragment (Fig. 1). The *coa* gene was subcloned on a 4.5-kb *Kpn*I fragment into pUCKan18 cut with *Kpn*I. The 3′ *Xba*I site in the *coa* fragment was retained. One of the *Kpn*I sites is located within the 7.7-kb *Xba*I fragment, while the other is derived from the polycloning site of the vector. A shuttle plasmid (pNCOA5) was then constructed by ligating the chloramphenicol-resistant plasmid pSK265 into the unique *Bam*HI site in pNCOA2.

Construction of plasmids expressing truncated coagulases. A deletion that removed the coding sequences located 3′ to the *Hin*dIII site in the *coa* structural gene cloned from strain Newman was constructed by subcloning a *Kpn*I-*Hin*dIII fragment from pNCOA1 into pUCKan18 cut with the same enzymes. Ka^r transformants that were Lac$^-$ were grown in broth, lysed, and tested for plasma clotting activity. One

TABLE 2. Plasmids

Plasmid	Host	Marker(s)	Relevant properties	Source or reference
pCW59	*S. aureus*	Cm^r Tc^r	Cloning vector	43
pSK265	*S. aureus*	Cm^r	Cloning vector	14
pUC19	*E. coli*	Ap^r	Cloning vector	44
pUCKan19	*E. coli*	Ka^r	Cloning vector	29
pUCKan18	*E. coli*	Ka^r	Cloning vector; polylinker sites in opposite orientation to pUCKan19	29
pBluescript	*E. coli*	Ap^r	Cloning vector	32
pBluescript (E-H)	*E. coli*	Ap^r	pBluescript lacking *Eco*RI and *Hin*dIII sites	This study
pCOA5	*E. coli*	Ap^r	5.2-kb *Kpn*I fragment from 8325-4 carrying *coa* in pUC19	27
pCOA14	*E. coli*	Ap^r	5.2-kb *Kpn*I fragment from 8325-4 carrying *coa* in pUC19; opposite orientation by pCOA5	27
pCOA17	*E. coli*	Ap^r	5.2-kb *Kpn*I *coa* fragment of 8325-4 in pBluescript (E-H)	This study
pCOA18	*E. coli*	Ap^r Tc^r	pBluescript (E-H) with Δ*coa*::Tc^r mutation	This study
pNCOA1	*E. coli*	Ap^r	7.7-kb *Xba*I *coa* fragment from strain Newman in pUC19	This study
pNCOA2	*E. coli*	Ka^r	4.5-kb *Kpn*I strain Newman *coa* fragment from pNCOA1 in pUCKan18	This study
pNCOA3	*E. coli*	Ka^r	3.3-kb *Kpn*I-*Hin*dIII Newman *coa* region in pUCKan18	This study
pNCOA5	Shuttle	Ka^r Cm^r	pNCOA2 + pSK265; linked at *Bam*HI sites	This study
pNCOA6	Shuttle	Ka^r Cm^r	pNCOA3 + pSK265; linked at *Kpn*I sites	This study
pNCOA8	*E. coli*	Ka^r	1.3-kb *Hin*dIII-*Bam*HI *coa* fragment from pNCOA2 in pUCKan19	This study

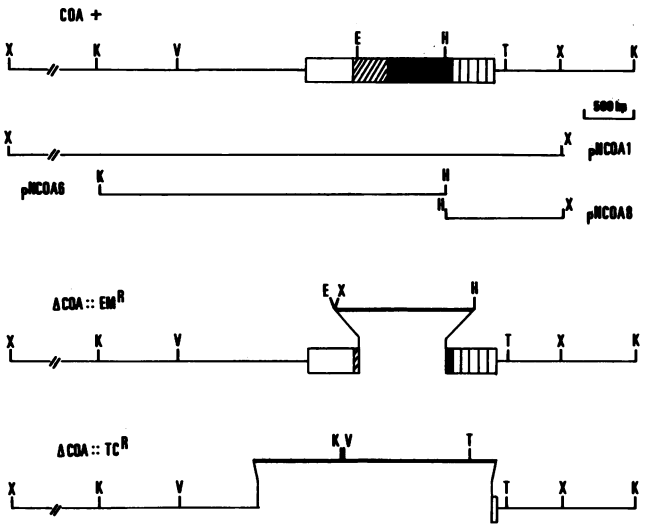

FIG. 1. Structure of the *coa* locus of *S. aureus* 8325-4 and *coa* mutants. The boxed region represents the *coa* gene and the coagulase protein, with the direction of transcription from left to right. The unshaded part of the boxed region represents the N-terminal amino acid residues that diverge between the coagulases of strains BB, 8325-4, and 213. The solid shaded part of the boxed region represents the residues that are conserved between the three sequences. In strains 213 and 8325-4, the conserved residues extend further towards the N terminus (cross-hatched region). The parallel vertical lines indicate the 27 residue repeats located in the C terminus. The single thin lines below the coa$^+$ locus in the upper part of the diagram indicate the DNA fragments cloned into pUC19 in the construction of pNCOA1, into pUCKan18 in the construction of pNCOA6, and into pUCKan19 in the construction of pNCOA8. The structures of Δ*coa*::Em^r and Δ*coa*::Tc^r are shown below. Restriction endonuclease cleavage sites are abbreviated as follows: X, *Xba*I; K, *Kpn*I; V, *Eco*RV, E, *Eco*RI; H, *Hin*dIII; T, *Taq*I.

derivative, pNCOA3, was kept. A shuttle plasmid was constructed from pNCOA3 by inserting pSK265 at the *Kpn*I site, forming pNCOA6. This plasmid was then transformed into *S. aureus* with selection for Cm^r.

A *lacZ-coa* in-frame fusion expressing only the C-terminal 177 residues of coagulase was constructed by cutting pNCOA2 with *Hin*dIII (in *coa*) and *Bam*HI (in the polycloning site adjacent to the *Kpn*I site used in constructing pNCOA2), treating it with Klenow polymerase, and ligating it into the *Hin*cII site of pUCKan19. Ka^r transformants that were white on X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside) were screened for derivatives with the expected insert. These were subsequently tested for expression of a fibrinogen-binding protein by Western blotting. The plasmid expressing the LacZ-Coa fusion protein in *E. coli* was called pNCOA8.

RESULTS

Transfer of the Δ*coa*::Em^r mutation to *S. aureus* Newman. It has been suggested that a cell-bound form of coagulase in *S. aureus* Newman is clumping factor (4). The objective of this research was to test this notion genetically with coagulase-deficient mutants of the clumping-factor-positive strain *S. aureus* Newman. We had previously isolated a Coa$^-$ mutant of *S. aureus* 8325-4 (28), a strain which expresses low levels of both coagulase and clumping factor (Table 3). The Δ*coa*::Em^r mutation had previously been constructed in vitro by replacing DNA between the *Eco*RI and *Hin*dIII sites in the *coa* gene of 8325-4 with a fragment expressing erythromycin resistance (28) (Fig. 1). The mutation was introduced into strain 8325-4 by allelic replacement, forming DU5809 (28). In this article, we report the transfer of the Δ*coa*::Em^r mutation into strain Newman by transduction. It should be noted that strains Newman and 8325-4 are closely related; there is no impediment to the transfer of chromosomal markers between the strains by transduction. They are of a similar phage type, their coagulases are both serotype III (11a), and their *coa* genes have indistinguishable restriction maps (unpublished data). Southern blotting verified that the transductant carried the expected changes in the chromosomal *coa* locus (data not shown). The Em^r Newman transductant (DU5856) was completely deficient in coagulase activity but retained clumping factor activity (Table 3), suggesting that coagulase and clumping factor are distinct.

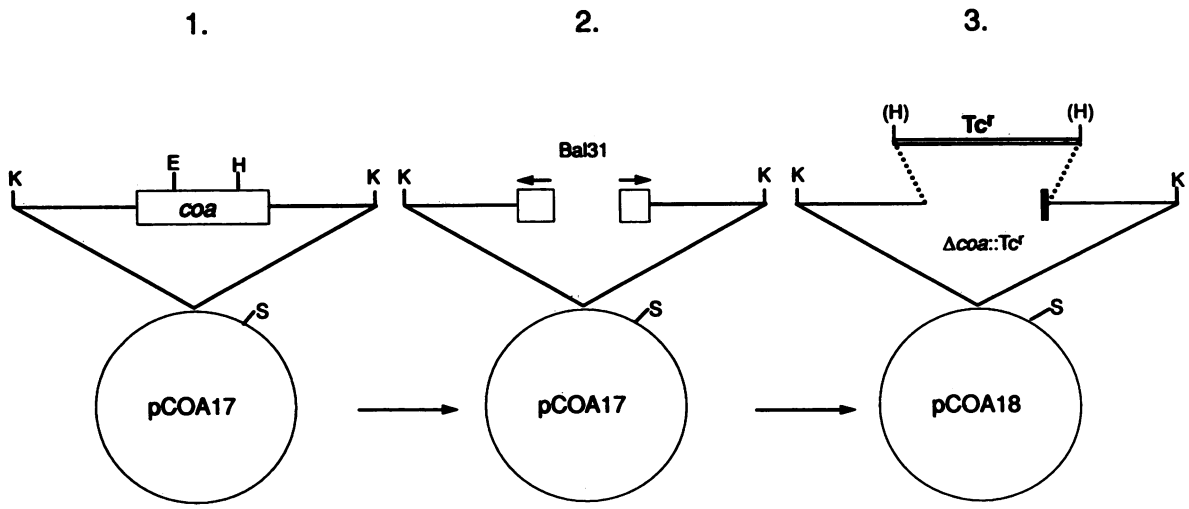

FIG. 2. Construction of Δ*coa*::Tc^r. The circles represent the plasmid vector pBluescript (E-H). The open box depicts the coagulase gene, and the parallel lines depict the Tc^r fragment from pCW59. Restriction endonuclease sites are abbreviated as follows: K, *Kpn*I; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I.

TABLE 3. Coagulase and clumping titers

Strain	Coagulase titer	Clumping titer
Newman	512	4,096
DU5856 Δ*coa*::Em^r	<2	4,096
DU5854 Δ*coa*::Tc^r	<2	4,096
8325-4	64	256
DU5809 Δ*coa*::Em^r	<2	256
DU5809(pNCOA5)	512	256

Newman Δ*coa*::Tc^r. To eliminate the possibility that the clumping phenotype of Newman Δ*coa*::Em^r was due to expression of remaining *coa* coding sequences, a larger deletion mutant (Δ*coa*::Tc^r) was constructed by removing DNA 5′ to the *Eco*RI site and 3′ to the *Hin*dIII site in *coa* (Fig. 2).

The plasmid carrying Δ*coa*::Tc^r, pCOA18, does not have a replication system that functions in *S. aureus*. For the Tc^r marker to be inherited and expressed in *S. aureus* after introduction of the plasmid into the gram-positive host by electroporation, recombination between the plasmid and the chromosome must occur. A single crossover will result in the plasmid becoming integrated into the chromosome with a concomitant duplication of the *coa* locus (Fig. 3A). A double crossover is required for allelic replacement (Fig. 3B). Plasmid pCOA18 was transformed into *S. aureus* RN4220 by electroporation, with selection for colonies that were resistant to 2 μg of tetracycline per ml. One colony of the 50 tested was Coa$^-$ and was presumed to have acquired the Δ*coa*::Tc^r mutation by allelic replacement. This strain was designated DU5861. The remaining transformants were Coa$^+$ and were thought to have a complete copy of the plasmid integrated into the *coa* locus. The Tc^r marker of this strain (DU5861) was subsequently transduced into strain Newman. All of the transductants tested were Coa$^-$, thus demonstrating linkage between the drug resistance marker and the coagulase mutation. DU5861 was presumed to have acquired the Δ*coa*::Tc^r mutation by allelic replacement. One transductant (DU5872, Newman Δ*coa*::Tc^r) was kept for further analysis.

The remaining Tc^r pCOA18 transformants of strain RN4220 expressed coagulase activity. They were presumed to have arisen by integration of plasmid pCOA18 into the *coa* locus. The transformants fell into two groups, those that expressed low-level tetracycline resistance (<15 μg/ml; 6 of 49) and those that expressed high-level tetracycline resistance (>30 μg/ml; 42 of 49). It was assumed that the low-level Tc^r Coa$^+$ transformants contained a single copy of pCOA18 inserted into the *coa* locus. One strain, DU5864, was analyzed further.

Transduction of *coa*::pCOA18. It has been reported that propagation of a transducing phage on a strain carrying an integrated copy of a plasmid such as pCOA18, which carries an in vitro-constructed mutation, and subsequently infecting a wild-type recipient and selecting for the drug marker associated with the mutation promotes plasmid excision and allelic replacement at a high frequency (33). Accordingly, phage 85 was propagated on the Coa$^+$ pCOA18 integrant DU5864 (RN4220 *coa*::pCOA18), and Tc^r transductants were selected in Newman Coa$^+$. In one experiment, 6 of 31 transductants were coagulase deficient and expressed low-level Tc^r, suggesting that plasmid excision had occurred, leaving the Δ*coa*::Tc^r mutation in the *coa* locus (Fig. 3). One such transductant, strain DU5854, was kept for further analysis.

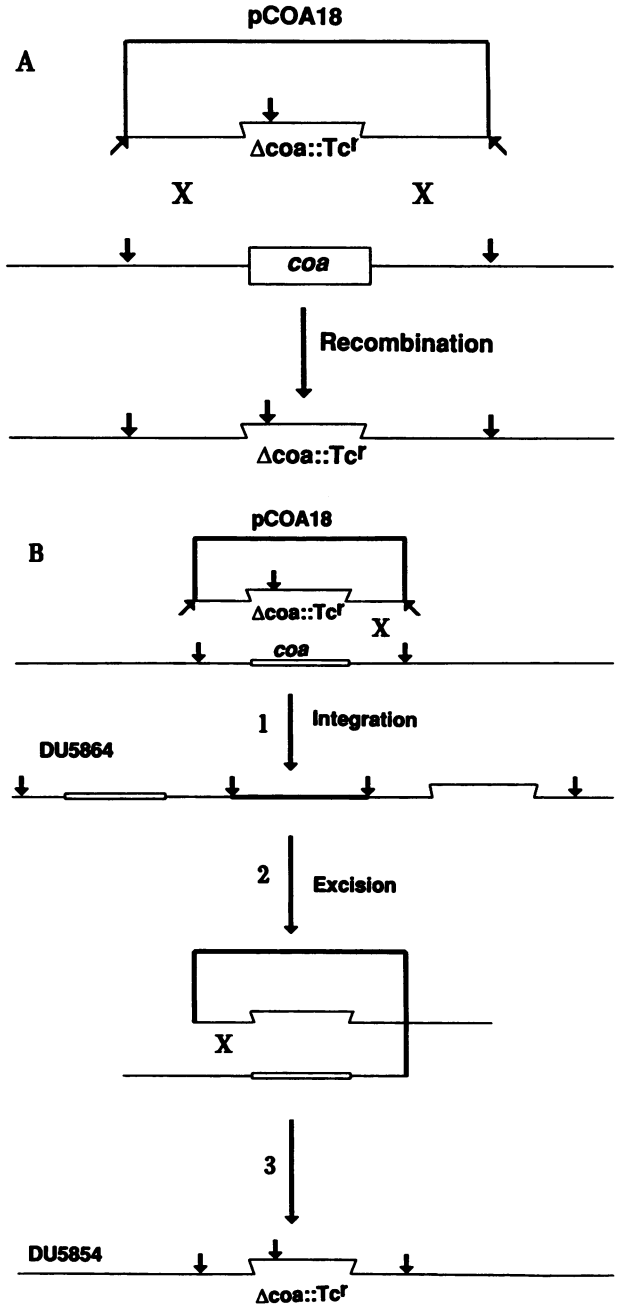

FIG. 3. Recombination at the *coa* locus. (A) The double recombination event that must occur in direct allelic replacement at the *coa* locus after electroporation of *S. aureus* RN4220 with pCOA18 is shown. (B) The integration of pCOA18 by a single crossover 3′ to the mutational site is shown. The second recombination event occurs 5′ to this site, resulting in excision of the plasmid and replacement of *coa* with Δ*coa*::Tc^r. The arrows indicate *Kpn*I sites.

Southern hybridization analysis. Genomic DNA of the parental Newman Coa$^+$ strain, strain DU5854 (Newman Δ*coa*::Tc^r), and a putative single-copy pCOA18 integrant (DU5864, RN4220 *coa*::pCOA18) were cleaved with *Kpn*I and probed with nick-translated pCOA14 DNA (pCOA14 carries an intact *coa* gene on a 5.2-kb *Kpn*I fragment). Some

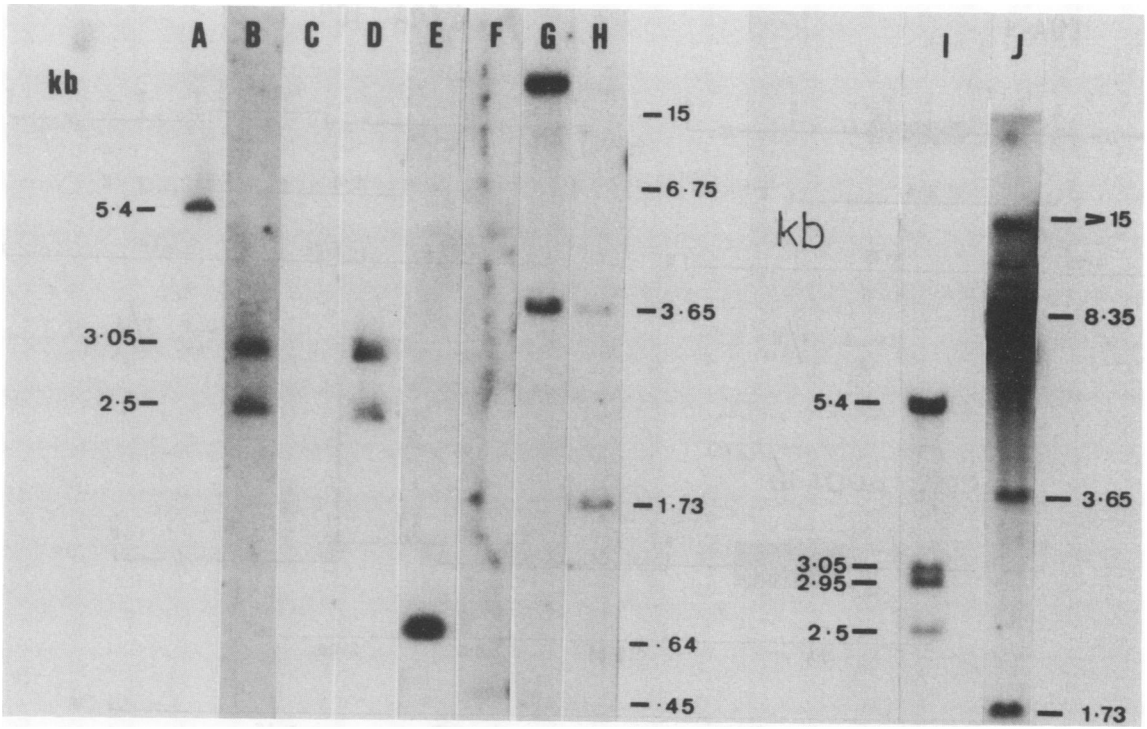

FIG. 4. Southern hybridization. Chromosomal DNA from wild-type strain Newman was cleaved with *Kpn*I (lanes A and C), *Hin*dIII and *Taq*I (lane E), and *Eco*RV (lane G), while chromosomal DNA from strain Newman Δ*coa*::Tc^r (DU5854) was cleaved with *Kpn*I (lanes B and D), *Hin*dIII and *Taq*I (lane F), and *Eco*RV (lane H). DNA from the plasmid integrant strain DU5864 was cleaved with *Kpn*I (lane I) and *Eco*RV (lane J). Samples in lanes A, B, G, H, I, and J were probed with labelled pCOA14, samples in lanes C and D were probed with labelled pCW59, and samples in lanes E and F were probed with the labelled 640-bp 3′ *Hin*dIII-*Taq*I fragment from pCOA5.

samples were also probed with pCW59 (carrying the Tc^r fragment used in constructing the Δ*coa*::Tc^r mutation). A single 5.4-kb fragment hybridized to the pCOA14 probe in the Newman Coa$^+$ sample (Fig. 4, lane A). A fragment of the same size occurred in RN4220 Coa$^+$ (data not shown). In contrast, Newman Δ*coa*::Tc^r has fragments of 3.05 and 2.5 kb (Fig. 4, lane B). This is explained by the presence of a *Kpn*I site within the Tc^r fragment inserted into the *coa* locus (Fig. 5). Consistent with this is the observation that the Tc^r probe also hybridized to fragments of the same size (Fig. 4, lane D). No hybridization occurred with the Tc^r probe in the Coa$^+$ sample (Fig. 4, lane C).

The samples were also cleaved with *Eco*RV, an enzyme that cuts within the Tc^r insert as well as within the *coa* gene *Kpn*I fragment (Fig. 5). Two fragments (>15 and 3.65 kb) hybridized to the *coa* probe in the Newman wild-type sample (Fig. 4, lane G), while three fragments (>15, 3.65, and 1.73 kb) hybridized to the *coa* probe in the putative Δ*coa*::Tc^r mutant (Fig. 4, lane H). The >15-kb fragment is composed partly of Tc^r sequences and partly of *coa* sequences in contrast to the >15-kb fragment from the wild type (Fig. 5). The same results were obtained with the putative allele replacement mutant isolated directly by electroporation of RN4220 with pCOA18 DNA (data not shown).

A RN4220 pCOA18 Tc^r transformant that expressed low-level resistance was also examined by Southern hybridization. Genomic DNA was cut with *Kpn*I and hybridized with the *coa* probe (Fig. 4, lane I). Two of the fragments (2.5 and 3.05 kb) were also present in the allelic replacement mutant DU5854. The two other hybridizing bands were 5.4 kb (the size of the *Coa* gene *Kpn*I fragment in the wild-type strain) and 2.95 kb (the size of the pBluescript plasmid vector) (Fig. 5). Four *Eco*RV fragments hybridized with the *coa* probe (>15, 8.35, 3.65, and 1.73 kb). Three of these fragments were also present in the allele replacement mutant. The unique 8.35-kb fragment can be explained if pCOA18 integrated 3′ to the *Hin*dIII site in the *coa* gene (Fig. 3 and 5).

To verify the structure of the 3′ part of the mutated *coa* gene in Δ*coa*::Tc^r, a 640-bp *Hin*dIII-*Taq*I fragment from pCOA5 was purified and used as a probe for DNA sequences located at the 3′ end of the *coa* gene. When cleaved with *Taq*I and *Hin*dIII, a fragment the same size as the probe hybridized in the Newman Coa$^+$ sample (Fig. 4, lane E) while a fragment of 450 bp hybridized in the Δ*coa*::Tc^r mutant (Fig. 4, lane F). The intensity of this fragment was lower probably because it contains only about 160 bp of DNA that is complementary to the probe.

Expression of clumping factor. The two allelic replacement mutants of *S. aureus* Newman (DU5854 and DU5856) were completely devoid of coagulase activity (Table 3). In contrast, when titrated with human fibrinogen, both strains expressed the same level of clumping factor as the parental strain. This strongly suggests that coagulase cannot be clumping factor. This conclusion was reinforced by the expression of the cloned Newman coagulase gene when it was introduced into the Coa$^-$ 8325-4 strain DU5809. Wild-type 8325-4 expressed low levels of both coagulase and clumping factor. Plasmid pNCOA5, which carries the cloned Newman coagulase gene, was transferred into DU5809. The level of coagulase expressed was increased to that expressed by strain Newman (Table 3). However, the clumping factor

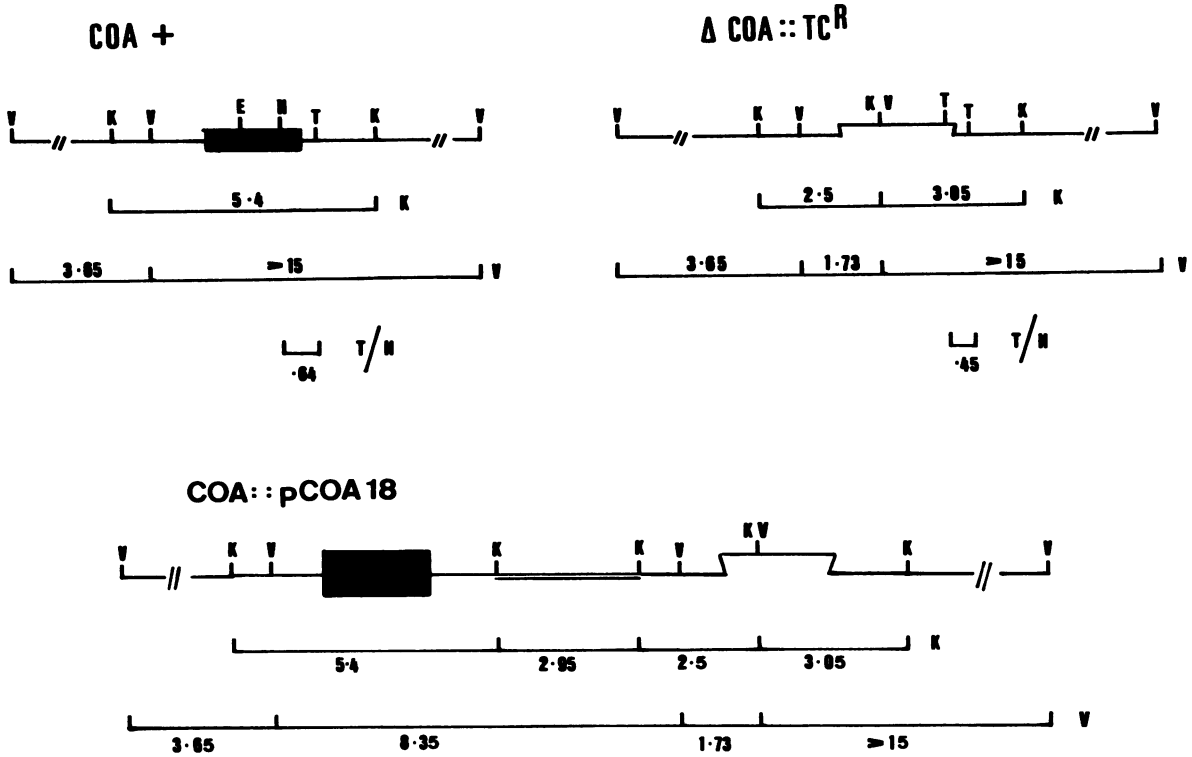

FIG. 5. Schematic diagram showing structure of the *coa* loci. Restriction maps of the *coa* regions of Newman Coa$^+$ and DU5854 (Newman Δ*coa*::Tc^r) and of DU5864 (pCOA18 integrated in RN4220) are shown. The lower three lines indicate restriction fragments that hybridize to the probes used in Fig. 4. The sizes of the fragments are given. Restriction endonuclease sites are abbreviated as follows: V, *Eco*RV; K, *Kpn*I; T, *Taq*I; E, *Eco*RI; H, *Hin*dIII.

titer was not raised. Thus, there was no correlation between the coagulase and clumping factor titers.

Adherence to solid-phase fibrinogen. The ability of *S. aureus* Newman and the Coa$^-$ mutants DU5854 and DU5856 to adhere to solid-phase fibrinogen is shown quantitatively in Fig. 6. Coverslips which did not contain fibrinogen (albumin controls) bound $<4 \times 10^3$ CFU. Adherence was promoted by fibrinogen adsorbed on PMMA. This increased linearly as a function of the amount of fibrinogen adsorbed. The dose-response curves of strain Newman and the Coa$^-$ mutants were indistinguishable. This clearly demonstrates that coagulase cannot be responsible for the adherence of *S. aureus* Newman to solid-phase fibrinogen.

Analysis of fibrinogen-binding proteins. Fibrinogen-binding proteins expressed by *S. aureus* Newman were investigated by Western affinity blotting (4). Proteins extracted from washed cells and proteins from culture supernatants of the wild-type Coa$^+$ strain and the Coa$^-$ mutant DU5854 (Δ*coa*::Tc^r) were compared. Both supernatants and cell extracts contained several polypeptides that reacted with fibrinogen, as was detected by using a monoclonal antifibrinogen antibody (Fig. 7, lanes C and D). The largest and most intensely stained band was 87 kDa, the size previously reported for coagulase (4). Several of the smaller bands may be proteolytic fragments of the 87-kDa protein, namely, the bands that were missing in the Coa$^-$ mutant sample (Fig. 7, lanes B and E). Similarly sized bands also reacted with anticoagulase serum (Fig. 7, lanes G and H). The other band that reacted with fibrinogen is a 19-kDa polypeptide. This protein was not labelled when fibrinogen was omitted (Fig. 7, lane A), and it did not react with the anti-Newman coagulase serum (Fig. 7, lanes F to I). Bodén and Flock also identified a 19-kDa fibrinogen-binding protein (4).

A 60-kDa protein was identified as protein A. It was labelled when fibrinogen was omitted from a control blot (Fig. 7, lane A).

Localization of the fibrinogen-binding region of coagulase. In an effort to locate the fibrinogen-binding region on the coagulase of strain Newman, two deletions were constructed in the cloned Newman *coa* gene which resulted in the expression of truncated coagulase molecules. In plasmid pNCOA6, DNA encoding the C-terminal 177 amino acids was removed by subcloning in pUCKan18. Knowledge of the DNA sequence of the closely related 8325-4 coagulase gene (28) suggested that a protein of 57 kDa would be expressed. The shuttle plasmid pNCOA6 was transferred into *S. aureus* 8325-4 Coa$^-$, and coagulase activity was detected in the culture supernatants. Western blotting with anticoagulase serum revealed a major protein of 65 kDa (Fig. 8, lane A) that was absent in the control experiment with the plasmid-free host strain (data not shown). This protein failed to react with fibrinogen in the Western blotting assay (Fig. 8, lane B). Additional polypeptides were observed in the pNCOA6 sample and in Newman *coa*$^+$ (Fig. 8, lanes A and C). These were probably due to proteolysis.

A *lacZ-coa* fusion was constructed by cloning DNA 3′ to the *Hin*dIII site in *coa* into pUCKan19. An in-frame fusion with *lacZ* was constructed, resulting in the expression of a protein comprising the N-terminal 13 residues of β-galactosidase and the C-terminal 177 residues of coagulase. Indeed, a polypeptide of 25 kDa was detected in the cytoplasm of *E. coli* carrying pNCOA8 in Western blots probed with both

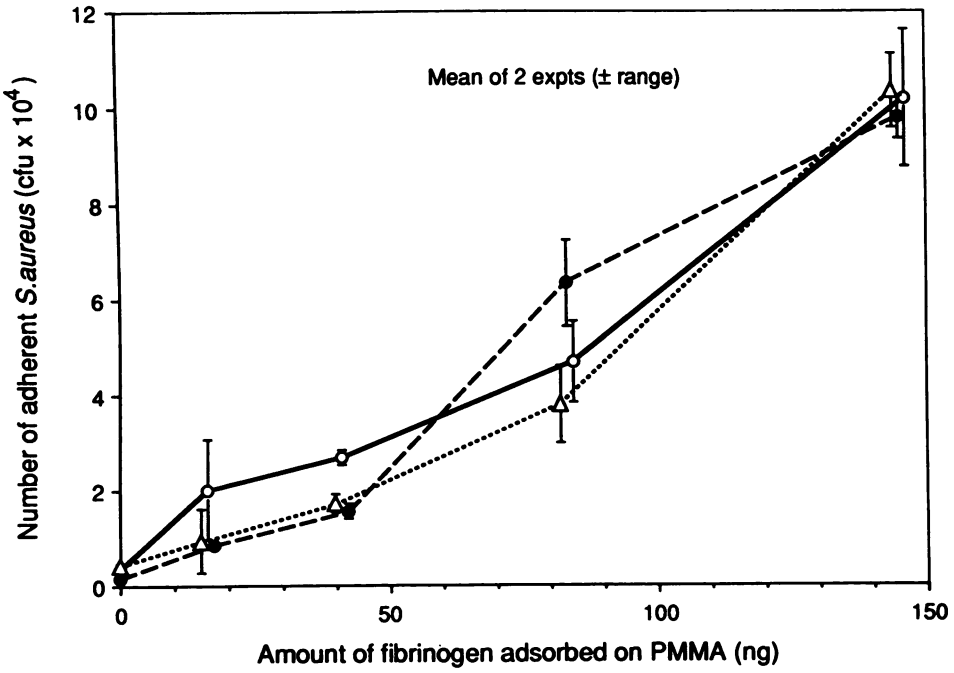

FIG. 6. Adherence of *S. aureus* to fibrinogen-coated PMMA coverslips. The number of adherent bacteria is shown as a function of fibrinogen adsorbed on the coverslip. Symbols: ○, *S. aureus* Newman Coa$^+$; ●, DU5854 (Newman Δ*coa*::Tc^r); Δ, DU5856 (Newman Δ*coa*::Em^r). For the points where bars indicating standard errors of the means are not visible, the bars are smaller than the symbols.

anticoagulase serum (Fig. 8, lane F) and fibrinogen (Fig. 8, lane E). In both cases, no reactions were detected in plasmid-free control samples. These data suggest that coagulase binds fibrinogen at a site that is distinct from the well-documented N-terminal prothrombin-binding region (17, 18, 27) and that binding to fibrinogen can occur independently of prothrombin binding.

DISCUSSION

Most strains of *S. aureus* adhere avidly to fibrinogen in solution and form macroscopic clumps. For this reason, the fibrinogen receptor on the surface of *S. aureus* cells is called clumping factor. This receptor may also be responsible for adherence of *S. aureus* cells to the fibrinogen or fibrin that is deposited on implanted medical devices and intravascular catheters. Clumping factor may be an important virulence factor of *S. aureus*.

The nature of clumping factor is not clear. Several groups have described fibrinogen-binding proteins of 55 to 60 kDa (5, 40, 41), but convincing biochemical data were lacking. A recent article (4) showed that coagulase bound to fibrinogen and, moreover, that a fraction of this normally extracellular protein was firmly associated with the cell wall. It was suggested that this bound form of coagulase was the clumping factor, in contrast to the earlier assertion of Duthie that they are distinct entities (7).

In this article, we have confirmed the reports of Bodén and Flock (3, 4) that coagulase binds to fibrinogen. This was demonstrated in a Western blotting affinity binding experiment where cell-surface-associated or supernatant proteins were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with purified fibrinogen. The bound fibrinogen was recognized by a monoclonal antibody specific for the alpha-chain. A fibrinogen-binding protein of 87 kDa was identified as coagulase because it was missing in the Coa$^-$ mutants and because a protein of the same size reacted with anticoagulase serum. A number of smaller polypeptides (between 73 and 87 kDa) also reacted with fibrinogen. These are probably proteolytic fragments of coagulase. It is well-known that coagulase is susceptible to proteolysis at the C terminus (17–19), particularly those molecules isolated from stationary-phase cultures where extracellular proteases are likely to be more active (12).

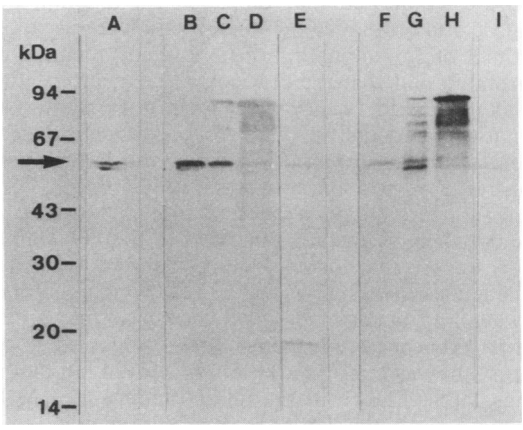

FIG. 7. Western blotting analysis of coagulases and fibrinogen-binding proteins of *S. aureus* Newman. Samples from *S. aureus* Coa$^+$ cell extracts (lanes A, C, and G), *S. aureus* Coa$^-$ cell extracts (lanes B and F), *S. aureus* Coa$^+$ supernatants (lanes D and H), and *S. aureus* Coa$^-$ supernatants (lanes E and I) were fractionated by SDS-PAGE. Filters were probed with purified fibrinogen and then by a monoclonal antifibrinogen antibody and peroxidase-conjugated goat anti-mouse immunoglobulin G (lanes B to E) or with anticoagulase serum and then protein A peroxidase (lanes F to I). In lane A, fibrinogen was omitted. The arrow indicates protein A.

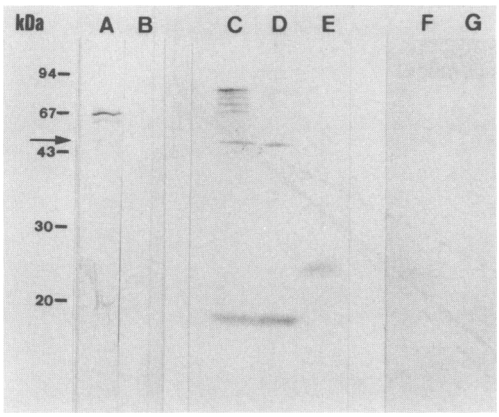

FIG. 8. Western blotting analysis of truncated coagulases. Samples from *S. aureus* cell extracts (lanes A to D) and lysates of *E. coli* (lanes E to G) were fractionated by SDS-PAGE. Filters were probed with anticoagulase serum (lanes A, F, and G) or with fibrinogen and then with monoclonal anti-fibrinogen antibodies (lanes B to E). The arrow indicates protein A which was identified by control experiments where the primary labelling reagent was not included. Samples were from DU5809(pNCOA6) (lanes A and B), Newman Coa$^+$ (lane C), Newman Δ*coa*::Tc^r (lane D), *E. coli*(pNCOA8) (lanes E and F), and *E. coli* TB1 (plasmid free) (lane G).

However, the biological significance of this reaction between coagulase and fibrinogen is not clear. The Coa$^-$ mutants were completely devoid of plasma clotting activity but clumped normally in fibrinogen solution and still adhered avidly to solid-phase fibrinogen. Furthermore, when the cloned Newman *coa* gene was transferred into 8325-4, a strain which expressed a low coagulase titer and which clumped poorly in fibrinogen, the clumping titer did not increase concomittantly with the coagulase titer. Clearly, coagulase cannot be clumping factor. Presumably, the fibrinogen-binding region of cell-bound coagulase is not surface exposed and cannot react with fibrinogen.

It could be argued that the apparent reaction between coagulase and fibrinogen demonstrated by affinity blotting is an artifact of contamination of fibrinogen preparations with prothrombin and that the reaction of coagulase with fibrinogen is mediated by a coagulase-prothrombin complex. Alternatively, coagulase could react with fibrinogen independently of prothrombin. We favor the latter hypothesis because the region of coagulase required for fibrinogen binding appears to be located in the C terminus of the molecule whereas prothombin binds to the N terminus. We have expressed two fusion proteins derived from the Newman coagulase. One protein of 25 kDa which comprises the first 13 amino acids of β-galactosidase at the N terminus fused to the carboxy-terminal 177 residues of coagulase. This protein was expressed in *E. coli*. It did not clot plasma (because it lacks the prothrombin-binding region) but reacted with both the anticoagulase serum and fibrinogen. Conversely, a deletion mutant lacking DNA coding for the C-terminal 177 residues expressed an immunoreactive truncated protein that failed to react with fibrinogen but retained plasma clotting activity.

In this report, we have also demonstrated that it is feasible to isolate allelic replacement mutants in a single step by electroporating a nonreplicating suicide plasmid into *S. aureus* RN4220. The transformation frequency obtained by electroporation was sufficiently high for double recombinants to be selected. However, a more frequent outcome of introducing a suicide plasmid was integration at the site of chromosomal homology by a single crossover event (Campbell insertion). Another approach that does not depend on high-frequency plasmid transformation is to introduce the mutation into *S. aureus* on a temperature-sensitive vector and then to select for integration into the chromosome by elevating the growth temperature (12). We have confirmed an earlier finding made with a temperature-sensitive plasmid integrated in the *tst* locus (34) that it is possible to resolve the cointegrate by transduction to a wild-type recipient (Fig. 3). Resolution occurred by recombination in the duplicated region on the opposite side of the mutation to that which led to plasmid integration. We also noted that amplification of the integrated pCOA18 occurred frequently, generating derivatives that expressed a higher level of tetracycline resistance. Southern blotting experiments have shown that this occurs by tandem duplication and multimerization (22a).

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