Genetic Evidence that Bound Coagulase of \textit{Staphylococcus aureus} Is Not Clumping Factor

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\textit{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 thrombin-like activity which converts 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 \textit{coa} gene was isolated by allelic replacement. Allelic replacement either was directly selected by electrotransformation of \textit{S. aureus} R3N4220 with a nonreplicating suicide plasmid, pCOA18, carrying the \textit{\Delta}coa:Te\textsuperscript{*} mutation or occurred after transduction of the integrated pCOA18 plasmid. The \textit{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 \textit{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 \textit{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.

\textit{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 terminal. 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 \textit{coa} mutation, which lacked 0.9 kb of DNA internal to the \textit{coa} gene, and a deletion isolated here which lacked all but about 50 bp of the \textit{coa} gene were introduced into \textit{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. \textit{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. \textit{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.
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. Haebeler, 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 K2HPO4, 0.01 M KH2PO4, 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 × 109 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 surfacemodified 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 ascertainment 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/cm²) when a 3H-labelled preparation of fibrinogen was used (35). Fibrinogen-coated coverslips were then incubated with 4 × 109 CFU of 3H thyminelabelled S. aureus per ml for 60 min at 37°C, as previously described (42). The adherence medium was PBS with 1 mM Ca2+ and 0.5 mM Mg2+, 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 coagulate activity. Coagulate 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 semidyblotter (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. Fibrinectin 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-32P]dATP (New England Nuclear).

Transfer of DNA. Plasmid DNA was transformed into E. coli cells made competent by CaCl2 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 Δcoa::Te’. The coa gene of strain 8325-4 codes for a single HindIII site and a single EcoRI site (Fig. 1). A deletion of DNA located between these sites was involved in the formation of the coagulate mutation Δcoa::Em’ (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 EcoRI site and 3’ to the HindIII 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 EcoRI and HindIII and then treating it with DNA polymerase I Klenow fragment to fill in the single-stranded
TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Properties</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td></td>
<td></td>
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<tr>
<td>Newman</td>
<td>coa +</td>
<td>High level of clumping factor</td>
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<tr>
<td>DUS809</td>
<td>Δcoa::Em'</td>
<td>Mutant of strain 8325-4 defective in coagulase</td>
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<tr>
<td>DUS856</td>
<td>Δcoa::Em'</td>
<td>Mutant of strain Newman defective in coagulase; transduction from DUS809</td>
<td>This study</td>
</tr>
<tr>
<td>RN4220</td>
<td>Δcoa::Tc'</td>
<td>Mutant of 8325-4 capable of stably maintaining shuttle plasmids</td>
<td>20</td>
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<tr>
<td>DUS861</td>
<td>Δcoa::Tc'</td>
<td>Mutant of RN4220 defective in coagulase; transduction from DUS861</td>
<td>This study</td>
</tr>
<tr>
<td>DUS864</td>
<td>coa::pCOA18</td>
<td>RN4220 with integrated pCOA18</td>
<td>This study</td>
</tr>
<tr>
<td>DUS854</td>
<td>Δcoa::Tc'</td>
<td>Mutant of strain Newman defective in coagulase; transduction from DUS854</td>
<td>This study</td>
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<tr>
<td>E. coli</td>
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<td></td>
<td></td>
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<tr>
<td>TB1</td>
<td>ara thi rpeL kspR</td>
<td></td>
<td>44</td>
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<tr>
<td></td>
<td>Δlac-proXIII</td>
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<td>ϕ80lacZΔM15</td>
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TABLE 2. Plasmids

<table>
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<th>Marker(s)</th>
<th>Relevant properties</th>
<th>Source or reference</th>
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<tr>
<td>pCW59</td>
<td>S. aureus</td>
<td>Cm' Tc'</td>
<td>Cloning vector</td>
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</tr>
<tr>
<td>pSK265</td>
<td>S. aureus</td>
<td>Cm'</td>
<td>Cloning vector</td>
<td>14</td>
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<td>pUC19</td>
<td>E. coli</td>
<td>Ap'</td>
<td>Cloning vector</td>
<td>44</td>
</tr>
<tr>
<td>pUCKan19</td>
<td>E. coli</td>
<td>Ka'</td>
<td>Cloning vector; polylinker sites in opposite orientation to pUCKan19</td>
<td>29</td>
</tr>
<tr>
<td>pUCKan18</td>
<td>E. coli</td>
<td>Ka'</td>
<td>Cloning vector</td>
<td>29</td>
</tr>
<tr>
<td>pBluescript</td>
<td>E. coli</td>
<td>Ap'</td>
<td>Cloning vector</td>
<td>32</td>
</tr>
<tr>
<td>pBluescript(E-H)</td>
<td>E. coli</td>
<td>Ap'</td>
<td>pBluescript lacking EcoRI and HindIII sites</td>
<td>This study</td>
</tr>
<tr>
<td>pCOA5</td>
<td>E. coli</td>
<td>Ap'</td>
<td>5.2-kb KpnI fragment from 8325-4 carrying coa in pUC19</td>
<td>27</td>
</tr>
<tr>
<td>pCOA14</td>
<td>E. coli</td>
<td>Ap'</td>
<td>5.2-kb KpnI fragment from 8325-4 carrying coa in pUC19; opposite orientation by pCOA5</td>
<td>27</td>
</tr>
<tr>
<td>pCOA17</td>
<td>E. coli</td>
<td>Ap'</td>
<td>5.2-kb KpnI coa fragment of 8325-4 in pBluescript (E-H)</td>
<td>This study</td>
</tr>
<tr>
<td>pCOA18</td>
<td>E. coli</td>
<td>Ap' Tc'</td>
<td>pBluescript (E-H) with Δcoa::Tc' mutation</td>
<td>This study</td>
</tr>
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<td>pNCOA1</td>
<td>E. coli</td>
<td>Ap'</td>
<td>7.7-kb XbaI coa fragment from strain Newman in pUC19</td>
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<td>pNCOA2</td>
<td>E. coli</td>
<td>Ka'</td>
<td>4.5-kb KpnI strain Newman coa fragment from pNCOA1 in pUCKan18</td>
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<td>pNCOA3</td>
<td>E. coli</td>
<td>Ka'</td>
<td>3.3-kb KpnI-HindIII Newman coa region in pUCKan18</td>
<td>This study</td>
</tr>
<tr>
<td>pNCOA5</td>
<td>Shuttle</td>
<td>Ka' Cm'</td>
<td>pNCOA2 + pSK265; linked at BamHI sites</td>
<td>This study</td>
</tr>
<tr>
<td>pNCOA6</td>
<td>Shuttle</td>
<td>Ka' Cm'</td>
<td>pNCOA3 + pSK265; linked at KpnI sites</td>
<td>This study</td>
</tr>
<tr>
<td>pNCOA8</td>
<td>E. coli</td>
<td>Ka'</td>
<td>1.3-kb HindIII-BamHI coa fragment from pNCOA2 in pUCKan19</td>
<td>This study</td>
</tr>
</tbody>
</table>
A lacZ-coa in-frame fusion expressing only the C-terminal 177 residues of coagulase was constructed by cutting pNCOA2 with HindIII (in coa) and BamHI (in the polycloning site adjacent to the KpnI site used in constructing pNCOA2), treating it with Klenow polymerase, and ligating it into the HindII site of pUCKan19. Ka' 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 Δcoa::Emr 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 Δcoa::Emr mutation had previously been constructed in vitro by replacing DNA between the EcoRI and HindIII 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 Δcoa::Emr 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 Emr Newman transductant (DU5856) was completely deficient in coagulase activity but retained clumping factor activity (Table 3), suggesting that coagulase and clumping factor are distinct.
TABLE 3. Coagulase and clumping titers

<table>
<thead>
<tr>
<th>Strain</th>
<th>Coagulase titer</th>
<th>Clumping titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newman</td>
<td>512</td>
<td>4,096</td>
</tr>
<tr>
<td>DU5856 Δcoa::Em'</td>
<td>&lt;2</td>
<td>4,096</td>
</tr>
<tr>
<td>DU5854 Δcoa::Te'</td>
<td>&lt;2</td>
<td>4,096</td>
</tr>
<tr>
<td>8325-4</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td>DU5809 Δcoa::Em'</td>
<td>&lt;2</td>
<td>256</td>
</tr>
<tr>
<td>DU5809(pNCOA5)</td>
<td>512</td>
<td>256</td>
</tr>
</tbody>
</table>

Newman Δcoa::Te'. To eliminate the possibility that the clumping phenotype of Newman Δcoa::Em' was due to expression of remaining coa coding sequences, a larger deletion mutant (Δcoa::Te') was constructed by removing DNA 5' to the EcoRI site and 3' to the HindIII site in coa (Fig. 2).

The plasmid carrying Δcoa::Te', pCOA18, does not have a replication system that functions in S. aureus. For the Te' 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 assumed to have acquired the Δcoa::Te' 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 Te' 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 Δcoa::Te' mutation by allelic replacement. One transductant (DU5872, Newman Δcoa::Te') was kept for further analysis.

The remaining Te' 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 Te' 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 Te' transductants were selected in Newman Coa+. In one experiment, 6 of 31 transductants were coagulase deficient and expressed low-level Te', suggesting that plasmid excision had occurred, leaving the Δcoa::Te' mutation in the coa locus (Fig. 3). One such transductant, strain DU5854, was kept for further analysis.

Southern hybridization analysis. Genomic DNA of the parental Newman Coa+ strain, strain DU5854 (Newman Δcoa::Te'), and a putative single-copy pCOA18 integrant (DU5864, RN4220 coa::pCOA18) were cleaved with KpnI and probed with nick-translated pCOA14 DNA (pCOA14 carries an intact coa gene on a 5.2-kb KpnI fragment). Some
samples were also probed with pCW59 (carrying the \(Tc^\prime\) fragment used in constructing the \(\Delta\text{coa}::\text{Tc}^\prime\) 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 \(\Delta\text{coa}::\text{Tc}^\prime\) has fragments of 3.05 and 2.5 kb (Fig. 4, lane B). This is explained by the presence of a \(KpnI\) site within the \(Tc^\prime\) fragment inserted into the coa locus (Fig. 5). Consistent with this is the observation that the \(Tc^\prime\) probe also hybridized to fragments of the same size (Fig. 4, lane D). No hybridization occurred with the \(Tc^\prime\) probe in the Coa\(^+\) sample (Fig. 4, lane C).

The samples were also cleaved with EcoRV, an enzyme that cuts within the \(Tc^\prime\) insert as well as within the coa gene \(KpnI\) 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 \(\Delta\text{coa}::\text{Tc}^\prime\) mutant (Fig. 4, lane H). The \(>15\)-kb fragment is composed partly of \(Tc^\prime\) 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 allelic replacement mutant isolated directly by electroporation of RN4220 with pCOA18 DNA (data not shown).

A RN4220 pCOA18 \(Tc^\prime\) transformant that expressed low-level resistance was also examined by Southern hybridization. Genomic DNA was cut with \(KpnI\) 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 \(KpnI\) fragment in the wild-type strain) and 2.95 kb (the size of the pBluescript plasmid vector) (Fig. 5). Four EcoRV 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 \(3^\prime\) to the HindIII site in the coa gene (Fig. 3 and 5).

To verify the structure of the \(3^\prime\) part of the mutated coa gene in \(\Delta\text{coa}::\text{Tc}^\prime\), a 640-bp HindIII-\(TaqI\) fragment from pCOA5 was purified and used as a probe for DNA sequences located at the \(3^\prime\) end of the coa gene. When cleaved with \(TaqI\) and HindIII, 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 \(\Delta\text{coa}::\text{Tc}^\prime\) 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...
A 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 × 10^5 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 (Δcoa::Tc+) 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 HindIII 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
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. 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+; ●, DU854 (Newman Δcoa::Te); △, DU856 (Newman Δcoa::Em). For the points where bars indicating standard errors of the means are not visible, the bars are smaller than the symbols.

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.
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 concomitantly 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 prothrombin 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 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 tsr 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 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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REFERENCES

12. Igarashi, H. Personal communication.


