

Introduction of the *mec* Element (Methicillin Resistance) into *Staphylococcus aureus* Alters In Vitro Functional Activities of Fibrinogen and Fibronectin Adhesins

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Some methicillin-resistant strains of *Staphylococcus aureus* are defective in the production of major surface components such as protein A, clumping factor, or other important adhesins to extracellular matrix components which may play a role in bacterial colonization and infection. To evaluate the impact of methicillin resistance (*mec*) determinants on bacterial adhesion mediated by fibrinogen or fibronectin adhesins, we compared the in vitro attachment of two genetically distinct susceptible strains (NCTC8325 and Newman) to protein-coated surfaces with that of isogenic methicillin-resistant derivatives. All strains containing an intact *mec* element in their chromosomes were found to be defective in adhesion to fibrinogen and fibronectin immobilized on polymethylmethacrylate coverslips, regardless of the presence or absence of additional mutations in the *femA*, *femB*, or *femC* gene, known to decrease expression of methicillin resistance in *S. aureus*. Western ligand affinity blotting or immunoblotting of cell wall-associated adhesins revealed similar contents of fibrinogen- or fibronectin-binding proteins in methicillin-resistant strains compared to those of their methicillin-susceptible counterparts. In contrast to methicillin-resistant strains carrying a *mec* element in their genomes, methicillin-resistant strains constructed in vitro, by introducing the *mecA* gene on a plasmid, retained their adhesion phenotypes. In conclusion, the chromosomal insertion of the *mec* element into genetically defined strains of *S. aureus* impairs the in vitro functional activities of fibrinogen or fibronectin adhesins without altering their production. This effect is unrelated to the activity of the *mecA* gene.

Bacterial adhesion to host cells or tissues is an important step in the initiation of infection. This process also plays an important role in bacterial colonization of medical devices coated with various cellular and extracellular host components. *Staphylococcus aureus* expresses specific surface proteins called adhesins (11, 12, 22, 33) allowing it to interact specifically with plasma or extracellular matrix proteins associated with normal tissues or adsorbed on biomedical devices. The most important proteins promoting adhesion of *S. aureus* are fibronectin (18, 46, 47, 49, 50), fibrinogen (6, 18, 29), collagen (33–35), vitronectin (7, 26), laminin (18, 27), thrombospondin (17), bone sialoprotein (53), and elastin (52).

Recent molecular studies of major *S. aureus* adhesins allowed identification and characterization of the genes coding for the fibrinogen-binding protein ClfA (clumping factor) (29, 30), the collagen adhesin (34, 36), and two distinct but related fibronectin-binding proteins (FnBPs) encoded by closely linked but separately transcribed genes called *fnbA* and *fnbB* (10, 13, 23). An important aspect of these molecular studies was to demonstrate the functional significance of *S. aureus* adhesins by the production of specific mutants expressing defective in vitro and in vivo attachment to their respective host proteins (13, 14, 29, 32, 35, 51). Thus, both *fnb* genes must be inactivated to eliminate bacterial interactions with fibronectin (13).

Clinical methicillin-resistant *S. aureus* (MRSA) isolates have acquired an additional 30- to 50-kb DNA element of unknown origin that integrates into a specific chromosomal site (21). This DNA element carries among yet-uncharacterized genes the methicillin resistance (*mec*) determinant coding for *mecA*, the structural gene for the low-affinity penicillin-binding protein, PBP2a or PBP2', that mediates methicillin resistance. The level of methicillin resistance is dependent on the genetic background of each strain and is modulated by a number of chromosomal genes termed *fem* (15) or *aux* (8) factors. These factors are involved directly or indirectly in cell wall biosynthesis (3), whereby methicillin resistance is reduced by *femC* or *femD* inactivation (15) or completely abolished by inactivating either *femA* or *femB* (43) without affecting PBP2a production.

We lack precise information for MRSA strains about the molecular mechanisms of their attachment to various host tissues or implanted biomaterials. Detailed knowledge of molecular mechanisms of bacterial adhesion and colonization might help to explain why some strains of MRSA are more epidemic than others. To address this question, we compared the adhesion characteristics of isogenic methicillin-resistant derivatives of two well-defined laboratory strains of *S. aureus*. Strains were tested for in vitro adhesion to fibrinogen- and fibronectin-coated artificial surfaces and expression of adhesins by Western ligand affinity blotting or immunoblotting. The potential contribution of active or inactive *fem* factors which modulate the phenotypic expression of methicillin resistance was also evaluated.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1.

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TABLE 1. Bacterial strains or plasmids used^a

Strain or plasmid	Relevant genotype	Phenotype	Source and/or reference
Strains			
BB255	8325; parent strain	Mc ^s	(2)
BB859	<i>mec</i>	Mc ^r Em ^r	This study; by transformation of <i>mec</i> (EK872) ^b into BB255
BB270	8325, <i>mec</i>	Mc ^r	By transduction of <i>mec</i> (EK142) ^c into BB255 (1)
BB308	8325, <i>mec</i> Ω2003(<i>femA</i> ::Tn551)	Mc ^s Em ^r	BB270 insertionaly inactivated by Tn551 (28)
BB331	8325, Ω2003(<i>femA</i> ::Tn551)	Mc ^s Em ^r	This study; by transduction of Ω2003(<i>femA</i> ::Tn551) into BB255
BB814	8325, <i>mec</i> Ω2006(<i>femB</i> ::Tn551)	Mc ^s Em ^r	BB270 insertionaly inactivated by Tn551 (16)
BB589	8325, <i>mec</i> Ω2005(<i>femC</i> ::Tn551)	Mc ^s Em ^r	BB270 insertionaly inactivated by Tn551 (15)
BB906	8325, Ω2005(<i>femC</i> ::Tn551)	Mc ^s Em ^r	BB255 insertionaly inactivated by Tn551 (15)
Newman	Parent strain	Mc ^s	
BB1003	Newman, <i>mec</i>	Mc ^r	This study; transformation of Newman with DNA of strain BB270
BB749	8325, pBBB79	Mc ^r Cm ^r	Transduction of pBBB79 into BB255 (39)
BB702	8325, pBBB21	Mc ^r Cm ^r	Transduction of pBBB21 into BB255 (44)
Plasmids			
pBBB21	pCA44 <i>mecA</i> insert (44)	Amp ^r <i>Escherichia coli</i> , Cm ^r Mc ^r in <i>S. aureus</i>	Recombinant plasmid carrying a 6.2-kb insert from chromosomal DNA of <i>Staphylococcus epidermidis</i> carrying <i>mecA</i> (44)
pBBB79	pGC2 <i>mecA</i> insert	Amp ^r in <i>E. coli</i> , Cm ^r Mc ^r in <i>S. aureus</i>	4.2-kb <i>Hind</i> III fragment carrying <i>mecA</i> subcloned from pBBB21 (44) into the <i>Hind</i> III site of pGC2 (39)

^a Abbreviations: Amp, ampicillin; Em, erythromycin; Cm, chloramphenicol; Mc, methicillin.

^b EK872 is an MRSA clinical isolate from 1992.

^c EK142 is an MRSA clinical isolate from 1967.

Bacterial adhesion assay. In vitro attachment of *S. aureus* to protein-coated polymer surfaces was measured by a previously described adhesion assay with polymethylmethacrylate (PMMA) coverslips coated in vitro with purified fibrinogen (29, 31) or fibronectin (13, 14). Human fibrinogen and fibronectin were purchased from Imco (Stockholm, Sweden) and Chemicon (Temecula, Calif.), respectively, as previously described (13, 29). Trace amounts of contaminating fibronectin were removed from fibrinogen by gelatin adsorption (38). To optimize adsorption of fibronectin from concentrations below 1 µg/ml, the PMMA coverslips were precoated with gelatin followed by rinsing in phosphate-buffered saline (PBS) as previously described (13, 14, 51).

The two-sided protein coating of PMMA was achieved by incubating for 60 min at 37°C native or gelatin-precoated coverslips with fibrinogen or fibronectin, respectively, at three different concentrations (0.25, 0.5, and 1 µg/ml) of either protein in PBS, followed by rinsing in PBS as previously described (13, 14, 29). PMMA surfaces were shown to be coated in a dose-dependent manner with amounts of human fibrinogen ranging from 41 to 145 ng per coverslip (28 to 101 ng/cm²) when radiolabeled fibrinogen (by reductive methylation with sodium borotrihydride [42, 48]) was used. A similar linear coating of PMMA surfaces with [³H]fibronectin ranging from 46 to 190 ng per coverslip (31 to 128 ng/cm²) was recorded.

The adhesion characteristics of the different strains of *S. aureus* were evaluated by incubating the protein-coated coverslips with 4 × 10⁶ CFU (8 × 10⁴ cpm) of washed log-phase cultures, metabolically radiolabeled with [³H]thymidine during growth for 4 h at 37°C in Mueller-Hinton broth as previously described (average specific radioactivity, 0.02 cpm/CFU) (50). The protein-coated coverslips were incubated with radiolabeled bacteria for 60 min at 37°C in PBS with 1 mM Ca²⁺ and 0.5 mM Mg²⁺ supplemented with 5 mg of human serum albumin per ml, which prevented nonspecific adhesion of *S. aureus* (14, 29, 50). At the end of the attachment period, the fluids containing unbound bacteria were removed, the coverslips were rinsed, and radioactivity on the coverslips was counted as previously described (50). To compare under normalized conditions adhesion of the different strains whose cell-associated contents and viable counts differed slightly, the amount of radioactivity remaining on the coverslips divided by the number of radiolabeled bacteria initially added to the system (expressed as a percentage) was first evaluated. For each strain, the percentage of attached radiolabeled bacteria was used to estimate the number of adherent CFU per coverslip normalized to a fixed inoculum of 4 × 10⁶ CFU/ml. Albumin-coated and gelatin-coated PMMA coverslips were used as controls of nonspecific adhesion to fibrinogen- and fibronectin-coated surfaces, respectively (13, 29, 50, 51).

Each experiment was performed at least three times, and the results were expressed as means ± standard errors of the means. Comparison of bacterial adhesion of methicillin-resistant mutants with their isogenic methicillin-susceptible parents was performed by one-way analysis of variance and *t* tests under analysis of variance for comparison of pairs of groups, with the Bonferroni correction for multiple comparisons (37). Statistical significance was evaluated for each of the three concentrations of fibrinogen or fibronectin immobilized on PMMA coverslips, and data were considered significant when *P* was <0.05 by using two-tailed significance levels.

Measurement of cell clumping. Cell clumping was measured quantitatively in microtiter trays by a previously described procedure (29) with slight modifications. Suspensions of washed cells logarithmically grown for 4 h at 37°C were concentrated 100-fold in PBS. Cell clumping was tested by mixing 10 µl (10⁸ CFU) of washed cells with 50 µl of fibrinogen (Imco), serially diluted twofold from a starting concentration of 160 µg/ml in PBS. The lowest concentration (range, 0.25 to 128 µg/ml) of fibrinogen which induced bacterial clumping was scored.

Preparation of cell wall-associated protein extracts. Cell wall-associated proteins of *S. aureus* were prepared by growing organisms without shaking in 100 ml of Mueller-Hinton broth for 4 h at 37°C. After two washes in PBS, the bacterial cultures were suspended in 1 ml of PBS with 1 mM Ca²⁺ and 0.5 mM Mg²⁺ supplemented with 1.1 M sucrose and proteinase inhibitors (Complete; Boehringer, Mannheim, Germany). Protoplast formation was achieved by adding to the concentrated cell suspension 100 µg of lysostaphin (Ambicin; Applied Microbiology, Inc., Brooklyn, N.Y.) per ml for 5 min at 37°C. After removal of whole cells and debris by low-speed (2,000 × *g*, 10 min) centrifugation, the surface proteins solubilized from enzymatically digested cell walls were separated from intact protoplasts which were sedimented at 8,000 × *g* for 30 min. The cell wall-extracted proteins were precipitated from the protoplast-free supernatants with 10% trichloroacetic acid for 18 h at 4°C. After extraction of trichloroacetic acid with a 1:1 mixture of ethanol and chloroform followed by centrifugation at 12,000 × *g* for 15 min, the sedimented cell wall-associated proteins were suspended in a 100-µl volume of PBS and their concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.).

SDS-PAGE and Western ligand affinity blotting or immunoblotting. Cell wall-associated protein amounts equivalent to 8 µg per strain were solubilized in sodium dodecyl sulfate (SDS) sample buffer and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4 to 15% acrylamide gradient (25). Proteins were transferred to an Immobilon membrane (Millipore) by a liquid transfer system (Bio-Rad Laboratories). The membrane was blocked in 10 mM Tris-HCl (pH 8.0)–500 mM NaCl–0.1% Tween 20 (Fluka) (TBST) containing 2.5% bovine serum albumin.

The ClfA protein was detected by incubation with 2 µg of immunoglobulin G antibodies per ml in TBST, purified from rabbit serum against recombinant ClfA protein (30). After being rinsed several times in TBST, the filters were incubated with peroxidase-conjugated protein A (1:10,000 dilution; Amersham). Detection was by enhanced chemiluminescence (Amersham).

FNBPs were detected by Western ligand affinity blotting by incubation with pure human fibronectin (30 µg/ml) in TBST. The membrane was rinsed several times in TBST and incubated with monoclonal antibody 1936 raised against the N terminus of fibronectin (1:5,000; Chemicon) followed by peroxidase-conjugated anti-mouse immunoglobulin G (1:10,000). Detection was by enhanced chemiluminescence as described above.

Genetics methods. Transformation of the *mec* determinant was done by the CaCl₂ method, selecting for transformants growing on 5 µg of methicillin per ml. Transduction of chromosomally integrated Tn551 was with phage 80α with selection for transductants on 20 µg of erythromycin per ml (43).

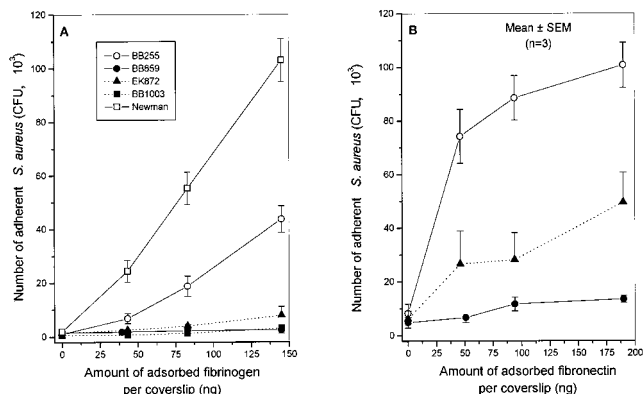


FIG. 1. Adhesion to fibrinogen-coated (A) or fibronectin-coated (B) coverslips of methicillin-resistant transformants of *S. aureus* BB255 (closed symbols) and Newman (open symbols). Susceptible strain BB255, Mc^r mutant strain BB859 derived from BB255; susceptible strain Newman, Mc^r mutant strain BB1003 derived from Newman. Strain EK872 is the methicillin-resistant clinical isolate donor of the *mec* element to BB255. SEM, standard error of the mean.

RESULTS

Defective adhesion and clumping of *S. aureus* transformed with the methicillin resistance determinant. To test the effects of the *mec* element on the adhesion properties of *S. aureus* for fibrinogen or fibronectin, two series of strains that varied in the presence or absence of the *mec* element were constructed. Two MRSA strains, EK142 (isolated in 1967) and EK872 (isolated in 1992), served as donors of the *mec* element. The recipients of these *mec* elements were strain BB255, a derivative of the laboratory strain NCTC8325, and *S. aureus* Newman. Both recipients are genetically distinct strains differing from each other by their chromosomal *Sma*I restriction patterns (data not shown). The *mec* elements were either transduced or transformed into these strains as described in Table 1. The sizes of both elements were approximately 30 kb as seen by the *Sma*I restriction patterns of the Mc^r transformant BB859 (*mec* EK872) when compared with susceptible parent BB255 (data not shown). Both elements seemed identical at least over a region of 14 kb when probed with fragments covering part of the *mec* element, except for some restriction site polymorphism at the right chromosomal junction fragment (data not shown) and cotransformation of erythromycin resistance with *mec*EK872.

In vitro attachment of methicillin-resistant transformants of strain BB255 or Newman to increasing amounts of immobilized fibrinogen is shown in Fig. 1A. In contrast to the methicillin-susceptible parental strains, whose attachment was dose dependently promoted by fibrinogen, methicillin-resistant transformants of strain BB255 (BB859) or Newman (BB1003) showed completely defective attachment to fibrinogen (Fig. 1A), as illustrated by the lack of dose response to increasing amounts of fibrinogen and background levels of attached bacteria ($\leq 10^3$ CFU/coverslip). At the two higher levels of immobilized fibrinogen (83 and 145 ng/coverslip), the reductions in attachment of strains BB859 and EK872 were highly significant ($P < 0.01$) compared to that of strain BB255. This was also the case for the methicillin-resistant transformant (BB1003) of strain Newman, compared to its parent, which showed significant ($P < 0.01$) differences in adhesion at all levels of immobilized fibrinogen. Finally, the clinical MRSA strain EK872, which served as a donor of the *mec* element to strain BB859, also exhibited very low attachment to fibrinogen.

Measurement of cell clumping confirmed the lack of the

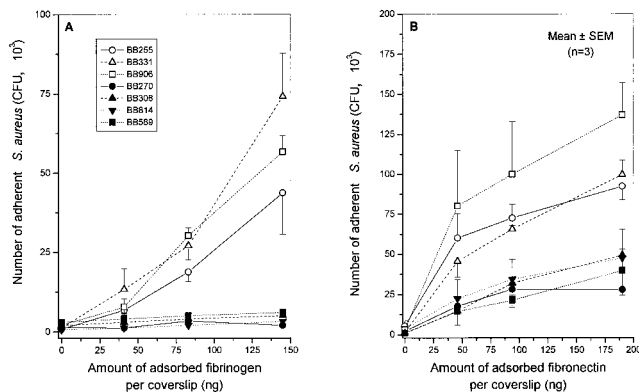


FIG. 2. Adhesion to fibrinogen-coated (A) or fibronectin-coated (B) coverslips of *fem* mutants of either the methicillin-susceptible strain BB255 of *S. aureus* (open symbols) or its Mc^r mutant BB270 (closed symbols). Susceptible strain BB255, *femA* mutant BB331 of strain BB255 and *femC* mutant BB906 of strain BB255; Mc^r strain BB270, *femA* mutant BB308 of strain BB270, and *femB* mutant BB814 of strain BB270, and *femC* mutant BB589 of strain BB270. At points where bars for standard errors of the means (SEM) are not visible, the bars are smaller than the symbols.

fibrinogen-binding ClfA protein-mediated agglutination in the methicillin-resistant transformants. Whereas the parental strain BB255 was clumped by 8 μ g of fibrinogen per ml, strain BB859 remained clumping negative up to 128 μ g/ml. The loss of clumping activity was even more impressive with the methicillin-resistant transformant of strain Newman, known to produce higher amounts of ClfA protein than strain 8325-4 or 8325 (29, 30). Whereas strain Newman was clumped by 0.5 μ g of fibrinogen per ml, its methicillin-resistant transformant BB1003 lost clumping activity even in the presence of 128 μ g of fibrinogen per ml.

In vitro attachment of the methicillin-resistant transformant of strain BB255 (BB859) to increasing amounts of immobilized fibronectin is shown in Fig. 1B. Compared to the methicillin-susceptible parent whose attachment was dose dependently promoted by fibronectin, the methicillin-resistant transformant showed a major reduction in attachment to fibronectin, which was highly significant ($P < 0.001$) at all levels of immobilized fibronectin. Figure 1B also shows that the clinical MRSA strain EK872, which was the donor of the *mec* element to strain BB859, exhibited a moderate attachment to fibronectin, which was significantly ($P < 0.01$) lower than that of strain BB255.

The methicillin-resistant transformant of strain Newman could not be tested for defective attachment to surface-bound fibronectin because of the weak affinity of the parental strain itself to this immobilized protein, as previously described (51).

Influence of *fem* mutations on adhesion properties of methicillin-susceptible or methicillin-resistant strains. Dose-dependent attachment of various *fem*-inactivated mutants of either strain BB255 or its *mec* determinant-bearing transductant BB270 to fibrinogen- and fibronectin-coated coverslips is shown in Fig. 2A and B, respectively. Both *femA* and *femC* mutants of the methicillin-susceptible strain BB255 showed dose-response profiles of adhesion to solid-phase fibrinogen (Fig. 2A) or fibronectin (Fig. 2B) that were similar to or slightly, but not significantly, higher than those of their parent. On the other hand, *femA*, *femB*, or *femC* mutants of strain BB270 were all as defective in attachment to either fibrinogen or fibronectin as was the methicillin-resistant parent. The differences between the group of strain BB270 and its *fem* mutants (BB308, BB814, and BB589) and the group of BB255 and

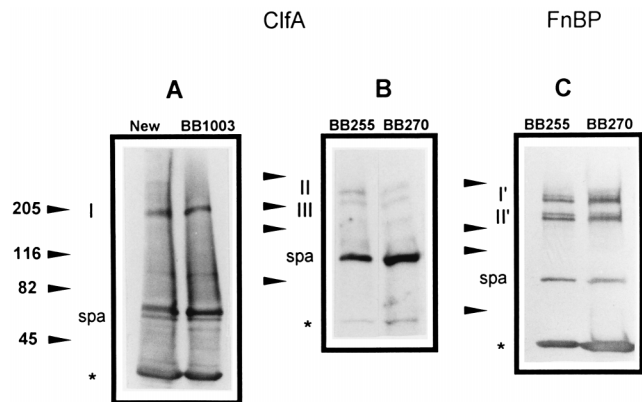


FIG. 3. Visualization of the ClfA protein (A and B) and FnBPs (C) by Western immunoblots and ligand affinity blots, respectively, in equivalent amounts (8 μ g) of cell wall-associated protein extracts from methicillin-susceptible strains of *S. aureus* and their isogenic methicillin-resistant mutants. Positions of protein size markers (in kilodaltons) are shown at the left of each panel. Band I corresponds to native ClfA protein. Bands II and III represent truncated ClfA protein. Bands I' and II' correspond to native and truncated FnBPs, respectively. spa is identified as protein A. Asterisks indicate the fronts of the gels. (A) Lane 1, Newman; lane 2, Mc^r strain BB1003 derived from Newman. (B) Lane 1, BB255; lane 2, Mc^r strain BB270 derived from BB255. (C) Lane 1, BB255; lane 2, Mc^r strain BB270.

its *fem* mutants (BB331 and BB906) were highly significant ($P < 0.01$) at the two higher levels of immobilized fibrinogen (83 and 145 ng/cover-slip) or fibronectin (94 and 190 ng/cover-slip).

Thus, the activity of *fem* factors played no significant role in the adhesion phenotypes of strain BB255 or its methicillin-resistant derivative BB270.

Expression of fibrinogen and fibronectin adhesins by methicillin-susceptible and methicillin-resistant strains. Western immunoblotting or ligand affinity blotting of the ClfA protein or FnBPs, respectively, was performed to evaluate the level of expression of each adhesin in the wild-type and methicillin-resistant derivatives of strain Newman or BB255.

Fibrinogen adhesins were detected by Western immunoblotting with anti-ClfA protein antibodies. Figure 3A shows the presence of a predominant immunoreactive protein band (band I) of 180 to 190 kDa in parental strain Newman and its Mc^r derivative BB1003 (lane 2). This band corresponds to the previously described native ClfA protein of 185 kDa (30). Unexpectedly, strains Newman and BB1003 exhibited equivalent amounts of cell wall-associated ClfA protein despite their widely different adhesion phenotypes.

Expression of the ClfA protein by strain BB255 is quite low compared to that by strain Newman. A previous study performed with *S. aureus* 8325-4, which is closely related to strain BB255, failed to detect immunoreactive proteins in whole-cell lysostaphin extracts (30). In contrast, whole-cell extracts of strain 8325-4 carrying the *clfA* gene of Newman on the multi-copy plasmid pCF4 revealed proteins of 150 and 130 kDa which were assumed to be proteolytic fragments of the native 185-kDa ClfA protein (30). In this study, enrichment of cell wall-associated proteins allowed detection of immunoreactive proteins of 150 to 160 kDa (band II) and 130 to 140 kDa (band III) in strain BB255 and its methicillin-resistant derivatives. These sizes are similar to those reported for strain 8325-4(pCF4). Regardless of their different adhesion phenotypes, the methicillin-susceptible parental strain BB255 (Fig. 3B, lane 1), the methicillin-resistant transductant strain BB270 (Fig. 3B, lane 2), and transformant strain BB859 (data not shown) ex-

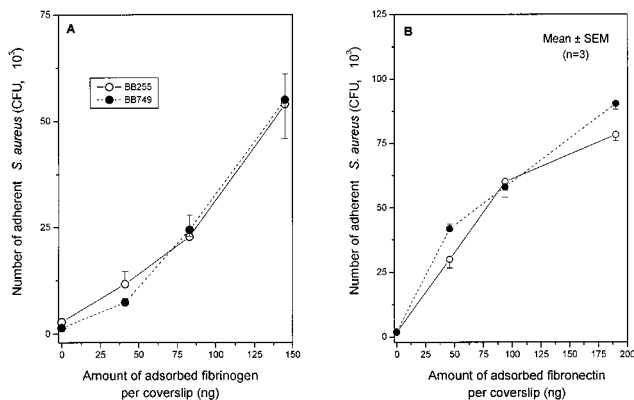


FIG. 4. Adhesion to fibrinogen-coated (A) or fibronectin-coated (B) coverslips of the methicillin-susceptible strain BB255 of *S. aureus* (open symbols) or its methicillin-resistant transformant (closed symbols) carrying plasmid pBBB79 with a *mecA* insert leading to heterogeneous expression of methicillin resistance. SEM, standard error of the mean.

hibited similar amounts of the proteolytic fragments of the cell wall-associated ClfA protein.

FnBPs were detected by Western ligand affinity blotting with fibronectin and antifibronectin monoclonal antibodies. Figure 3C shows the presence of two groups of FnBPs, whose sizes are 160 to 170 and 120 to 130 kDa, respectively. Each group is composed of similarly sized bands. The 160- to 170-kDa doublet (band I') may represent the mixture of native FnBPA and FnBPB, whereas the 120- to 130-kDa doublet (band II') may represent proteolytic fragments of the two FnBPs, according to their previously described patterns in the closely related strains 8325-4 (13) and ISP794 (4). Regardless of their different adhesion phenotypes, the methicillin-susceptible parental strain BB255 (Fig. 3C, lane 1), the methicillin-resistant transductant strain BB270 (Fig. 3C, lane 2), and transformant strain BB859 (data not shown) exhibited similar amounts of wall-associated FnBPs.

Control experiments showed that the 56-kDa protein band present in the Western blots is protein A as previously described (4).

Influence of the plasmid-located *mecA* gene on the adhesion phenotype. To evaluate whether the presence and/or activity of the *mecA* gene contributed to the defective attachment of *S. aureus* to fibrinogen or fibronectin, we analyzed a methicillin-resistant strain constructed in vitro, by introducing into strain BB255 the plasmid pBBB79 (39) having a 4.2-kb *Hind*III fragment carrying the *mecA*-encoding region on the shuttle vector pGC2 (39). Strain BB749 transduced with the *mecA*-carrying plasmid pBBB79 expressed heterogeneous resistance to methicillin as previously described (39). However, neither this heterogeneous Mc^r strain nor the control transductant complemented with the shuttle vector pGC2 lacking the *mecA* insert (39) (data not shown) showed any reduction in attachment to fibrinogen (Fig. 4A) or fibronectin-coated coverslips (Fig. 4B) compared to that by the methicillin-susceptible parental strain BB255.

Additional experiments were performed with another methicillin-resistant transductant (BB702) of strain BB255, carrying the pBBB21 plasmid (44) with a larger 6.2-kb insert covering the *mecA* gene, the direct repeat (*dru*) element, and the insertion sequence-like element IS431. The methicillin-resistant strain BB702 also failed to demonstrate any significant reduction in attachment to fibrinogen or fibronectin compared

to that of its methicillin-susceptible parent BB255 (data not shown).

These data indicated that expression of methicillin resistance in *S. aureus* by the low-affinity *mecA*-encoded PBP2' did not contribute to the adhesion defects exhibited in vitro by methicillin-resistant mutants containing a chromosomally inserted *mec* element.

DISCUSSION

This study provides evidence that chromosomal insertion of methicillin resistance determinants into genetically defined susceptible strains of *S. aureus* alters functional surface expression of fibrinogen and fibronectin adhesins. Both *mec* elements, which were isolated independently, produced the same effect regardless of the genetic background (NCTC8325 or Newman) into which they were introduced. Since at least three genetically distinguishable *mec* elements have been described elsewhere (20, 21), it remains to be defined whether the adhesion defects observed in vitro are a general property of all chromosomally inserted *mec* elements or of only some of them.

The adhesion defects of methicillin-resistant transformants or transductants of *S. aureus* NCTC8325 or Newman were not influenced by their level of methicillin resistance and thus are unrelated to the presence or activity of the *mecA* locus. Furthermore, mutations affecting the *femA*, *femB*, or *femC* locus, which interfere with peptidoglycan precursor formation and lower expression of methicillin resistance, did not modify the defective adhesion phenotypes of strains bearing the *mec* determinant. Comparisons of the normal adhesion phenotypes exhibited by methicillin-resistant transductants carrying *mecA* on plasmids with the defective phenotypes of transductants or transformants having chromosomally inserted resistance determinants indicate that the genetic determinants of the adhesion defects may be found within the additional 30 kb of the *mec*-associated DNA or may be due to interruption of the chromosomal locus at the *mec* attachment site or to a polar effect of the *mec* element.

Several studies have described the defective in vitro expression of various surface proteins by clinical isolates MRSA. The most frequently reported defects in MRSA clinical isolates are lower contents of protein A and clumping factor (9, 19, 24, 41, 45). These phenotypic defects seem to occur more frequently in epidemic than in nonepidemic strains of MRSA (24, 41, 45). Unfortunately, most of the studies describing phenotypic alterations in MRSA clinical isolates failed to include for comparison an equivalent group of representative isolates of methicillin-susceptible *S. aureus*. Direct comparison of quantitative amounts of specific surface proteins expressed by methicillin-resistant versus methicillin-susceptible clinical isolates of *S. aureus* has not been reported and may be of limited significance in view of their different genetic backgrounds.

Several different mechanisms explaining the defective functional activities of fibrinogen and fibronectin adhesins in methicillin-resistant transformants or transductants of *S. aureus* are possible: (i) decreased biosynthesis of ClfA protein and FnBPs in methicillin-resistant compared to methicillin-susceptible strains; (ii) normal biosynthesis but defective cell wall sorting of ClfA protein and FnBPs in the methicillin-resistant strains, leading to decreased amounts of cell wall-anchored adhesins (40); and (iii) functional inhibition of ClfA protein and FnBPs which are synthesized and cell wall anchored in normal amounts but prevented from interacting with fibrinogen and fibronectin, respectively, by some unknown mechanisms.

Determination of the amounts of fibrinogen and fibronectin

adhesins by Western ligand affinity blotting or immunoblotting, respectively, required the development of more elaborate procedures than those previously available (13, 29, 30). Initial studies (not shown) performed with whole-cell lysostaphin extract of strain BB255 and its methicillin-resistant derivatives failed to detect significant levels of the ClfA protein in this group of strains. These findings were similar to those previously reported with the closely related strain 8325-4 (29, 30). The preparation of cell wall-associated protein extracts allowed detection of significant amounts of ClfA protein and FnBPs in this group of strains by Western immunoblotting. Both fibrinogen and fibronectin adhesins were present in equivalent amounts in the cell wall protein extracts of the parental and methicillin-resistant strains. Further support for these observations was obtained by comparing the relative contents of ClfA in strain Newman, a high producer of this protein, with those of its methicillin-resistant transformants. All these different strains yielded equivalent contents of the fibrinogen adhesin regardless of their methicillin resistance.

The mechanisms leading to defective adhesion phenotypes of the isogenic methicillin-resistant mutants of *S. aureus* are difficult to explain in view of the normal amounts of ClfA protein and FnBPs recovered on their surface. We have to speculate that anchoring of the functionally defective adhesins to peptidoglycan (40) may occur in a conformationally inactive way or that some additional cell wall or surface protein component(s) specifically expressed by methicillin-resistant mutants may interfere with adhesin functions. Arguments for the second hypothesis were provided by two recent studies indicating the presence of a 230-kDa cell wall protein in certain methicillin-resistant clinical isolates of *S. aureus* which was not detected on the surfaces of other staphylococci (19, 24). The presence of this 230-kDa protein was assumed to contribute to negative agglutination results in commercial assays used to identify *S. aureus* clinical isolates, although the molecular mechanisms by which this inhibition may occur are not elucidated (19). More-extensive molecular studies are needed to characterize the complete primary structure of the 230-kDa protein and to identify the genetic loci responsible for its production by some methicillin-resistant isolates but not others. The relationship of the 230-kDa protein with the native ClfA protein and FnBPs, whose apparent molecular masses on SDS-PAGE are close to 200 kDa, needs also to be clarified.

The epidemiological and clinical significance of functional defects found in vitro for two major adhesins in methicillin-resistant mutants of the laboratory strains 8325 and Newman of *S. aureus* is difficult to evaluate. Significant defects in adhesin expression should be expected to prevent the emergence and sometimes epidemic spreading of certain MRSA isolates. However, since the adhesins expressed in laboratory conditions by isogenic methicillin-resistant mutants of *S. aureus* are also present in a significant proportion of MRSA clinical isolates (9, 19, 24, 41, 45), we believe that the in vivo relevance of our experimental in vitro data deserves to be further explored in future studies. Adhesin expression by *S. aureus* strains expressing different antibiotic resistance patterns also needs to be studied in experimental conditions more closely approaching the complex in vivo environment. Indeed, recent studies indicate that expression of some adhesins is under the control of internal and external signals (4, 5). Expression of FnBPs is regulated to some extent by the global regulators *sar* and *agr*, which are themselves responsive to the growth phase (5). Expression of bacterial adhesins is also altered by the combined presence of antibiotics and antibiotic resistance determinants, as shown by a recent report which documented the increased expression of FnBPs by fluoroquin-

olone-resistant mutants of *S. aureus* exposed to subinhibitory concentrations of ciprofloxacin (4). Further studies are planned to evaluate the functional surface expression of fibrinogen and fibronectin adhesins by laboratory or clinical methicillin-resistant strains of *S. aureus* exposed to subinhibitory levels of various antimicrobials. They may potentially help to evaluate how *S. aureus* adhesins contribute to bacterial attachment and colonization under clinically relevant conditions of antibiotic pressure and resistance determinants.

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