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Insertional Inactivation of the Gene for Collagen-Binding Protein Has a Pleiotropic Effect on the Phenotype of *Staphylococcus aureus*

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This report describes phenotypical changes caused by the insertional inactivation of the gene for the collagen-binding protein in *Staphylococcus aureus* PH100. Insertional inactivation resulted in reductions in the amount of fibronectin-binding protein in PH100 and the ability of intact cells to aggregate in the presence of fibronectin. However, the capacity of PH100 to adhere to immobilized fibronectin remained the same.

Recombinant-DNA technology to create isogenic mutants deficient in a single gene product has been widely used to investigate protein function. It has been assumed that mutants with inactivated target genes will show only phenotypic changes that are associated with the targeted gene and its protein. However, recent reports suggest that the insertional inactivation of genes encoding cell surface proteins in streptococci had pleiotropic effects on a number of other cell surface polypeptides (7, 8). The work presented here was therefore aimed at describing the phenotypic changes caused by the insertional inactivation of the gene for the collagen-binding protein (CnBP) in *Staphylococcus aureus* PH100 and comparing them with phenotypes of the parent, Phillips; the mutant PHC (PH100 expressing CnBP on a plasmid); and 12 clinical isolates naturally differing in the expression of CnBP. Phillips and PH100 were kindly supplied by M. Höök (Department of Biochemistry, Texas A&M University, Houston), and 12 clinical isolates from patients with complicated septicemia were obtained from B. Christensson (Department of Infectious Diseases, Lund University Hospital, Lund, Sweden). Bacteria were grown in Luria-Bertani medium or brain heart infusion. Strains were radiolabelled and tested for their ability to adhere to immobilized collagen (Cn) or fibronectin (Fn) as described by Flock et al. (5). Phillips and PH100 differed in their adherence properties only when Cn was used (Fig. 1), consistent with the report that originally described them (15). The reintroduction of the *cna* gene on a plasmid (pDirect kit; Clontech, Palo Alto, Calif.) in PH100 yielded a mutant, PHC, with an ability to bind to Cn that was significantly increased compared with that of PH100 but still lower than that of the Phillips strain (Fig. 1).

Following solubilization of binding proteins from cell suspensions by using lysostaphin according to a method described earlier (6), crude digests were adjusted to equal protein concentrations by a protein assay (Bio-Rad Laboratories, Richmond, Calif.). Cell digests or supernatant protein precipitates were electrophoresed according to the Laemmli polyacrylamide gel electrophoresis system (9) and transferred to polyvinylidene difluoride membranes (Bio-Rad) in a semidry blot cell (Bio-Rad). Membranes were blocked with skim milk in phosphate-buffered saline (PBS) and incubated overnight at 4°C in a solution containing 10^5 cpm of ^{125}I-labelled human type I Cn (Southern Biotechnology Associates, Inc., Birmingham, Ala.), Fn (Sigma), or rat serum diluted 1:1,000 in PBS. Iodination of Fn and Cn was conducted by a modified chloramine-T method using Iodobeads (Pharmacia, Uppsala, Sweden) (11). Membranes preincubated with rat serum were washed and incubated with ^{125}I-labelled sheep anti-rat antibodies (Amersham Corp.) to quantify protein A. Unbound ligand was removed by extensive washing with PBS. Membranes were air dried and scanned, and the amount of bound ligand was quantified by using a Fuji Bio-imaging Analyzer (model BAS2000; Fuji, Tokyo, Japan). The CnBP is reported to have a molecular mass of 133 kDa, as deduced from the nucleotide sequence (16). Cn did not bind to the digest from PH100 (Fig. 2A). A His-CnBP fusion protein containing the A binding domain of CnBP (14) (a gift from J. Patti, Department of Biochemistry, Texas A&M University) which was run in parallel with the digests gave a single band corresponding to its expected molecular mass of 60 kDa, whereas a zz-FnBP (4) fusion protein did not show any binding (data not shown). Analysis of the Fn-binding activity revealed that the two strains yielded different quantities of FnBP. In the extracts of Phillips, a 20- to 30-fold larger amount of FnBP, relative to the amount in PH100, was consistently present. Binding proteins had molecular masses ranging from 100 to 200 kDa (Fig. 2B). Fn was also bound by the zz-FnBP fusion protein but not by the His-CnBP fusion protein (data not shown). The ratio of FnBPs released into the supernatants of the two strains was the same as the ratio found in the cell digests. The reduction of the level of FnBPs in PH100 cannot be explained by a difference in digestion rates of the two strains, since the levels of protein A were identical (data not shown). In addition, extraction of FnBP with lithium chloride, by a method described by Liang et al. (10), yielded results equivalent to those obtained by lysostaphin digestion of cells. Such an extraction effect would also fail to explain the reduced levels of FnBP in the supernatant of PH100. Levels of FnBP were consistently lower in PH100 throughout the microorganism's growth cycle; therefore, it seems unlikely that increased shedding of the proteins can account for the reduction in the amount of FnBP in PH100.

A surface protein (60 and 72 kDa), such as that described by McGavin et al. (12), which could bind both Cn and Fn could not be identified in this assay.

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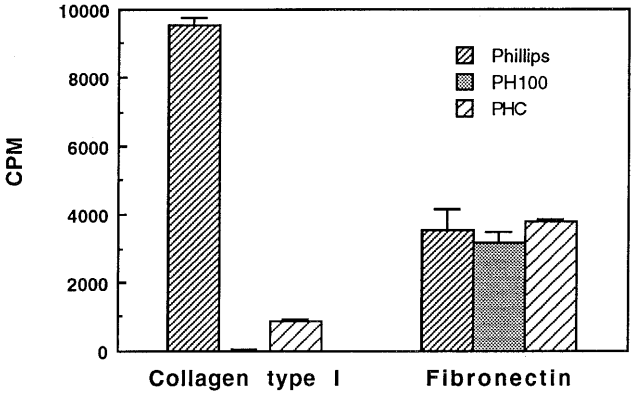

FIG. 1. Binding of *S. aureus* Phillips, PH100, and PHC to immobilized Cn type 1 and Fn. Proteins were applied to tissue culture plates, and 1.6×10^6 CFU of radiolabelled bacteria was added. Adherent bacteria were quantitated after removal with sodium dodecyl sulfate. Means ± standard deviations (error bars) from three experiments are shown.

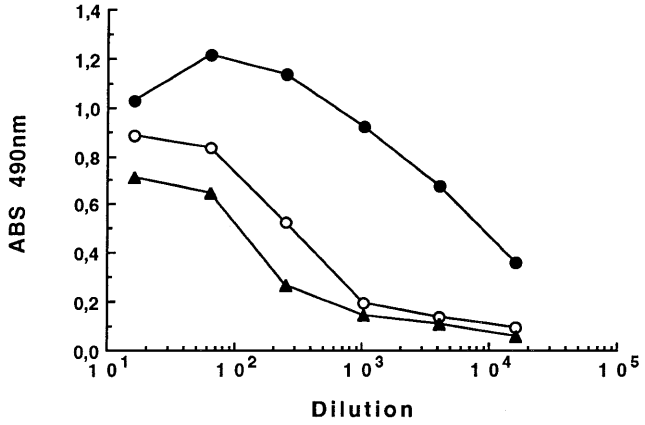

FIG. 3. Quantification of FnBPs by a capture ELISA. Cell digests from Phillips (closed circles), PH100 (open circles), and PHC (triangles) were allowed to bind to immobilized Fn in microtiter wells. FnBP was then detected with rat anti-FnBP antibodies. ABS, absorbance.

The results from phosphorimaging were substantiated by a capture enzyme-linked immunosorbent assay (ELISA). Briefly, Fn (Sigma) was applied at 5 μg/ml to polystyrene microtiter plates (Costar, Cambridge, Mass.). Plates were then coated with 2% bovine serum albumin (BSA) fraction V (Sigma) in PBS, washed, and incubated with serially diluted cell digest or extract. Adherent FnBP was detected with rat anti-zz-FnBP and goat anti-rat horseradish peroxidase conjugate (Sigma) in a Bio-Rad ELISA reader. The results again showed a striking reduction in the amount of extractable FnBP in strain PH100 (Fig. 3). However, FnBP was not restored to the level seen in Phillips in strain PHC. This observation might in part be due to the fact that PHC's capability to bind to Cn is only about 10% that of Phillips. The discrepancy between adherence to immobilized Fn and the level of FnBP in the strains was surprising. Since Phillips, PH100, and PHC adhered to immobilized Fn to the same extent, it is conceivable that binding does not improve with an increase in the level of FnBPs and that a critical threshold level of FnBPs on the surface of the microorganism is sufficient for adherence. This notion was supported by the fact that there was no correlation between adherence ability and the amount of FnBP among 12 clinical isolates (data not shown). In addition, we were able to demonstrate that the ability of intact cells of PH100 to aggregate in the presence of Fn was diminished (Fig. 4). This was tested by adding 100 μl of Fn at different concentrations to 800 μl of overnight cultures from both strains that had been adjusted to the same optical density. Aggregation was measured as a function of decreasing optical density. The results suggest that the microorganism's ability to aggregate is dependent on the number of FnBPs and their extracellular accessibility.

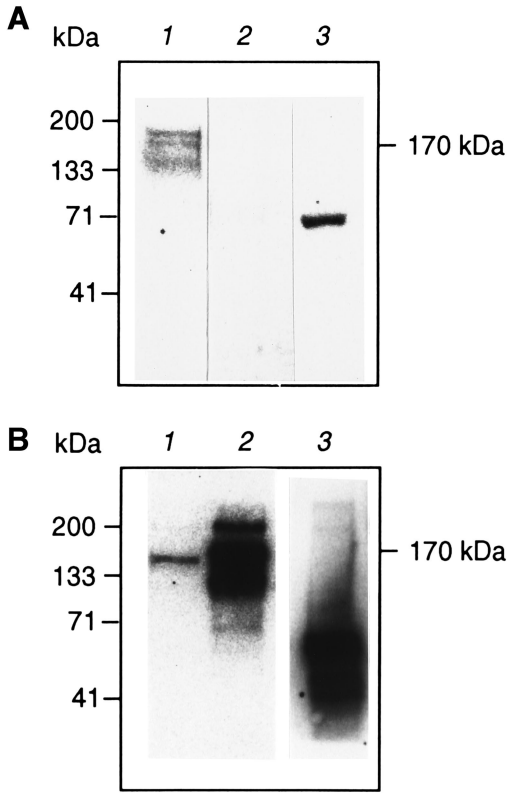

FIG. 2. Cell digests were run on a sodium dodecyl sulfate-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and incubated with ^{125}I-labelled Cn (A) or Fn (B). His-CnBP (A) and zz-FnBP (B) were included as controls (lanes 3). (A) Lanes: 1, Phillips; 2, PH100. (B) Lanes: 1, PH100; 2, Phillips.

Furthermore, one has to consider that the insertional inactivation of *cna* could possibly have a regulatory effect on FnBP and other proteins. A direct disturbance of the integrity of the *fnb* genes is highly improbable, since PH100 was generated by replacing the *cna* gene of Phillips through homologous recombination with an inactivated copy of the cloned *cna* gene, but it is conceivable that some effects of the *cna* disruption are due to altered depression of downstream genes. On the basis of phenotypical analysis, it appears unlikely that such genetic regulation takes place, since protein A levels remain the same in PH100. Altered protein A levels would be expected if the pleiotropic effect extended to regulatory genes such as *sar*, *agr*, or a new locus recently identified by Cheung et al. (1–3, 19). It must be noted, as additional evidence, that no changes were detected in the expression of alpha-toxin or coagulase activity (data not shown), both of which one would have expected to change in combination with an effect on those regulatory systems. The fact that there was no correlation between the presence of *cna* and the ability to bind to Fn among the 12 clinical isolates argues against a possible regulatory effect of *cna* on FnBP.

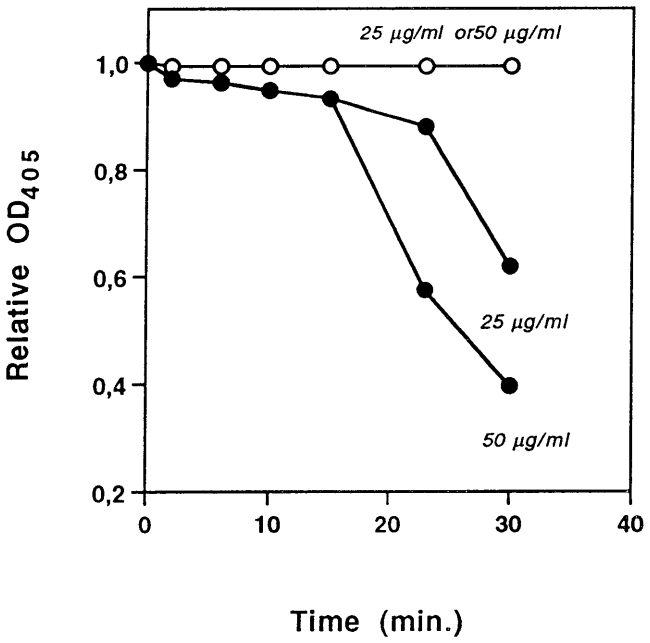

FIG. 4. Cultures of Phillips (closed circles) and PH100 (open circles) were compared for their ability to aggregate in the presence of different concentrations of Fn. OD, optical density.

The absence of CnBP expression might contribute to a general destabilization of the cell surface through the abolition of specific interactions or a disturbance in cell wall sorting (13, 18), which in turn could influence the hydrophobicity of the bacteria and the level of FnBP. However, we were not able to demonstrate any significant change in hydrophobicity, as determined by a method described by Rosenberg et al. (17), and showed that the presence of the *cna* gene has no influence on hydrophobicity among the 12 clinical isolates. Nor was there a correlation between the level of FnBP and hydrophobicity or CnBP phenotype in these isolates.

In conclusion, our findings suggest that the insertional inactivation of *cna* had pleiotropic effects on the level of FnBP and the ability of intact cells to aggregate in the presence of Fn. The fact that other reports likewise describe multiple changes in mutants after insertional inactivation (7, 8) underscores the importance of screening for alterations of phenotype that were not targeted by the inactivation.

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