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The Fibronectin-Binding Proteins of *Staphylococcus aureus* May Promote Mammary Gland Colonization in a Lactating Mouse Model of Mastitis

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The fibronectin-binding proteins (FnBPs) of *Staphylococcus aureus* are believed to be implicated in the pathogen's adherence to and colonization of bovine mammary glands, thus leading to infectious mastitis. In vitro studies have shown that FnBPs help the adhesion of the pathogen to bovine mammary epithelial cells. However, the importance of FnBPs for the infection of mammary glands has never been directly established in vivo. In this study with a mouse model of mastitis, the presence of FnBPs on the surface of *S. aureus* increased the capacity of the bacterium to colonize mammary glands under suckling pressure compared to that of a mutant lacking FnBPs.

The most costly disease in the dairy industry, bovine mastitis, is characterized by inflammation of the mammary gland. This inflammation is frequently caused by a microbial infection. *Streptococcus* spp., primarily *Streptococcus agalactiae*, coagulase-negative staphylococci, and *Staphylococcus aureus* may account for more than 75% of intramammary infections (30). Nearly 19% of these infections are caused by *S. aureus* (30), and this bacterium causes the mastitis cases that are the most difficult to cure (7). This pathogen possesses specific adhesins that can bind a diversity of host proteins mainly in the host extracellular matrix, such as collagen, fibrinogen, and fibronectin (5, 18). The fibronectin-binding proteins A and B (FnBP-A and FnBP-B), encoded by the *fnbA* and *fnbB* loci, respectively, were identified as the main adhesins that bind purified fibronectin (10, 24). At least one of the FnBP genes is found in the vast majority of *S. aureus* strains (25). Pathogen adherence to the teat canal of the udder is believed to be the first step of mammary gland infection, and FnBPs have been proposed as virulence factors in bovine mastitis (27). To date, there is no direct evidence concerning the importance of FnBPs in the colonization of the mammary gland.

Various studies have reported the role of FnBPs in bacterial adherence to eukaryotic cells in vitro (28), to ex vivo biomaterials (6, 29), and to airway epithelia in a xenograft model (17). As reviewed and noted elsewhere (9), the role of FnBPs as virulence factors has never been clearly demonstrated in an in vivo model of infection. In a rat model of endocarditis, transposon mutagenesis mutants impaired in their ability to adhere to fibronectin showed a reduction in the colonization of heart valves (11). The importance of FnBPs in endocarditis has been questioned, however, by another study in which no difference was detected in the virulence of an isogenic mutant of *S. aureus* 8325-4 that lacked FnBPs (4). In the same infection model, the poorly pathogenic bacterium *Lactococcus lactis* was found to cause more severe endocarditis when expressing *S. aureus* FnBP-A (21). Despite such a result, it remains unclear whether other *S. aureus* adhesins may compensate in vivo for the absence of FnBPs. Surprisingly, a recent study showed that the absence of FnBPs accentuated the virulence of *S. aureus* in a rat model of pneumonia (16).

In regard to bovine mastitis, the significance of FnBPs in the disease is unknown but in vitro results suggest a role for these adhesins in the colonization of the mammary gland. For example, *S. aureus* strains isolated from animals with clinical mastitis adhered, in a strain-dependent manner, to primary cultures of bovine mammary epithelial cells (8). By using a well-defined strain of bacteria and a well-defined cell lineage, an isogenic mutant lacking FnBPs was shown to have a 40% decrease in the capacity to adhere to the MAC-T bovine mammary gland epithelial cell line compared to the wild-type laboratory strain *S. aureus* 8325-4 (3). Moreover, using the same wild-type strain, its isogenic FnBP mutant, and a flow chamber recreating the physiological shear stress found in blood vessels, Reddy and Ross showed that the FnBPs help the adherence of *S. aureus* to bovine aortic endothelial cells under flow (22). The higher capacity of *S. aureus* 8325-4 than that of the mutant lacking FnBPs to adhere to and stay on the cells under flow was observed only when the buffer circulation was stopped for 30 min to allow bacteria to settle before restarting the flow. Under continuous circulation of buffer, no adherence was observed for either of the two strains. Similarly, the flow of milk is not continuous during lactation, thus potentially giving the bacteria the opportunity to adhere between milkings.

In addition to the shear stress caused by suckling or milking, the presence of milk and serum proteins in vivo may represent additional factors influencing the expression of virulence genes and bacterial adherence to the mammary gland. For example, clinical strains of *S. aureus* isolated from animals with mastitis were shown to adhere more efficiently to mammary gland epithelial cells in vitro when grown in a medium composed of milk whey instead of tryptic soy broth (TSB) (8, 14). However,

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TABLE 1. Characteristics of mouse mammary glands and CFU counts resulting from different inocula of *S. aureus* 8325-4 (with FnBPs) and DU5883 (without FnBPs) bacteria mixed in equal proportions, with or without suckling pressure, after 20 h of infection

Inoculum (CFU)	Nonsuckled glands (R4)				Suckled glands (L4)			
	No. of glands	Avg no. of CFU (log)	Avg wt (g)	% DU5883 bacteria	No. of glands	Avg no. of CFU (log)	Avg wt (g)	% DU5883 bacteria
4.5×10^1	3	6.2	0.98	29.0	4	5.0	0.45	22.7
4.5×10^2	3	7.2	0.84	48.1	4	4.7	0.36	35.8
4.5×10^3	3	7.2	0.80	34.2	4	4.6	0.36	33.4
4.5×10^4	3	6.8	0.86	44.3	3	4.9	0.61	22.0
Combined results[a]	12	6.9 (1.0)[b]	0.87 (0.17)[b]	41.2 (33.8 and 44.4)[c]	15	4.8 (0.8)[b]	0.43 (0.2)[b]	28.9 (20.0 and 35.4)[c]

[a] Individual data for all R4 or L4 glands were grouped.
[b] Average (standard deviation); $P < 0.001$; Student *t* test.
[c] Median (lower and upper quartiles); $P < 0.05$; Mann-Whitney U test

growth in human ex vivo peritoneal dialysate reduced the fibronectin-binding capacity of *S. aureus* (15), suggesting that different in vivo growth environments may have different consequences for adherence. It should be noted that inflammation of the mammary gland increases the serum protein content in cow milk. Recently, *S. aureus* genes which are specifically expressed during growth in milk or serum were identified (12, 31). Even if valuable data can be obtained from adherence studies performed in vitro, it is clear that these observations should be taken with care when extrapolated to apply to the in vivo context (28). Consequently, there is a need to study adherence in animal models that represent the disease under investigation.

A mouse model of mastitis was used in the present work to directly evaluate the importance of *S. aureus* FnBPs for colonization and infection of the mammary gland during lactation. The bacteria used here are the laboratory strain *S. aureus* 8325-4 and an isogenic strain with mutations in the *fnbA* and *fnbB* genes, *S. aureus* DU5883 (6). Numerous in vitro and in vivo studies have used these strains to evaluate the role of *S. aureus* FnBPs (3, 4, 6, 13, 16, 17, 20, 22), and in our study, similar to what has previously been shown (3), *S. aureus* DU5883 demonstrated a 30% reduction in the ability to adhere to cultured MAC-T bovine mammary epithelial cells compared to the wild-type 8325-4 strain (data not shown). The inactivation of *S. aureus* DU5883 *fnb* genes was achieved by the insertion of genes for resistance to erythromycin and tetracycline, and the present study took advantage of such genetic markers to selectively identify, on agar plates, tetracycline-resistant bacteria present in mixtures containing both 8325-4 and DU5883 strains. The bacteria used for infection were prepared by using overnight cultures diluted 1:50 in fresh TSB medium and incubated for another 2 h at 35°C to reach the exponential growth phase and thus maximum FnBP expression (23). The cells were then centrifuged at $3,000 \times g$ for 10 min, washed twice, and suspended in cold endotoxin-free phosphate-buffered saline (PBS). Both suspensions of *S. aureus* (8325-4 and DU5883) were adjusted to exactly the same optical densities. The two *S. aureus* suspensions were then mixed in equal proportions and diluted to the desired number of CFU per milliliter. By using this procedure, it was possible to get mixtures of bacteria with proportions that did not differ by more than 1.6% between the strains. To evaluate the number of CFU per milliliter and the relative percentage of *S. aureus* DU5883 bacteria, mixtures of bacterial suspensions were serially diluted and plated onto tryptic soy agar medium, with or without 1 μg of tetracycline/ml. The percentage of *S. aureus* DU5883 bacteria has been calculated by dividing the number of CFU from the plates with tetracycline by the number of CFU from the plates without antibiotic and multiplying the result by 100. When a half-and-half mixture of *S. aureus* 8325-4 and DU5883 was incubated and grown in TSB, the proportions of both strains remained the same for all phases of the growth curve (data not shown).

Intramammary inoculation was carried out as described by Chandler (2), with some modifications. Briefly, the 13- to 14-day-old pups were removed from lactating CD1 mice (Charles River, St.-Constant, Canada) 1 h before inoculation. The lactating mice were anesthetized by using ketamine and xylazine at 87 and 13 mg/kg of body weight, respectively, and buprenorphine at 0.075 mg/kg as an analgesic. Using a 100-μl glass syringe and a 33-gauge blunt needle, the mammary glands (fourth on the right [R4] and fourth on the left [L4]) were inoculated with 100 μl of bacterial suspension containing equal amounts of *S. aureus* 8325-4 and DU5883. To allow precise injection into the mammary ducts, the near ends of the teats were aseptically removed before inoculation. Immediately after the injection, the R4 glands were ligatured and referred to as the nonsuckled glands while the L4 glands were allowed to be suckled. The lactating mice were then kept alone for 1 h and 30 min before the return of the pups to allow suckling of the L4 glands. After 20 h of infection, the R4 and L4 mammary glands of each mouse were aseptically removed, weighed, and homogenized in PBS at a final volume of 3 ml. Homogenates diluted 1/10 in PBS containing 20% glycerol were kept at −80°C until used for counting of CFU. To evaluate the CFU content and the proportion of *S. aureus* DU5883 bacteria, the homogenates were plated onto tryptic soy agar with and without tetracycline as described above. The guidelines of the Canadian Council on Animal Care were respected during all the procedures (19).

Experiments were carried out to evaluate the number of CFU required for a moderate infection in both suckled (L4) and nonsuckled (R4) glands. Four different mixed inocula containing from 10^1 to 10^4 CFU were used for intramammary infection of three or four lactating mice per group. Table 1 shows the weights of glands recorded 20 h after inoculation

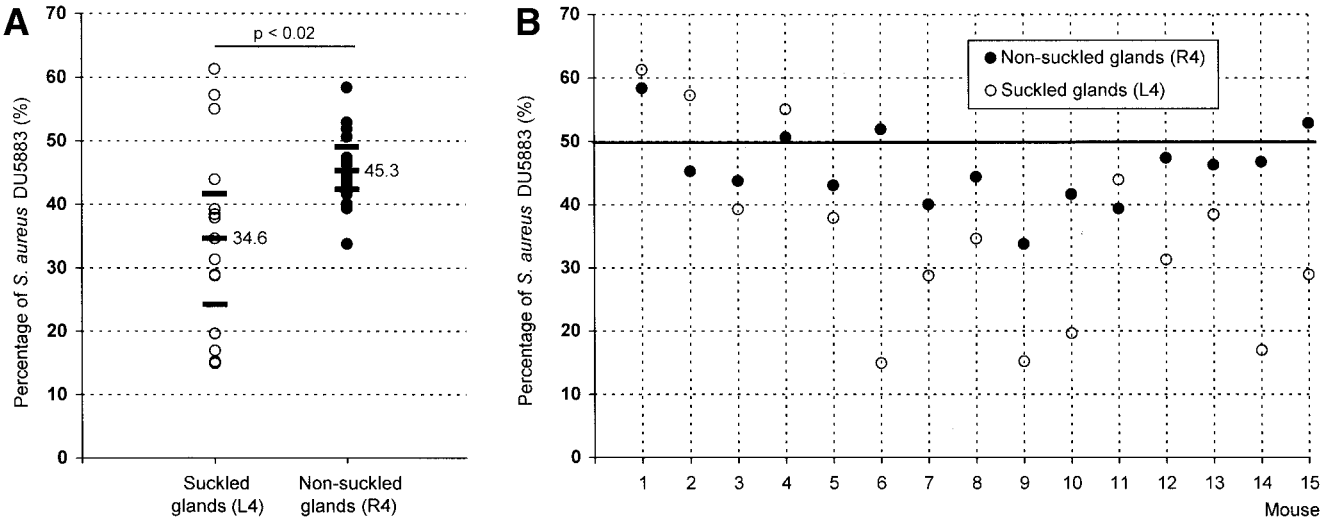

FIG. 1. Percentages of *S. aureus* DU5883 bacteria present in infected mammary glands. Mammary glands of CD1 lactating mice were inoculated with a half-and-half mixture of *S. aureus* 8325-4 bacteria (with FnBPs) and *S. aureus* DU5883 bacteria (without FnBPs) at 6.4×10^2 CFU/gland. The percentages for individual glands are represented by circles. (A) Glands were grouped as suckled and nonsuckled. The medians (indicated values) and the first and third quartiles are represented by bars. By using the Mann-Whitney U test, the difference in the percentages of *S. aureus* DU5883 bacteria in L4 and R4 glands was found to be statistically significant ($P < 0.02$). (B) For each mouse, results for the suckled and nonsuckled glands are paired to allow comparison of the proportions of *S. aureus* DU5883 bacteria found within each animal. The bold line indicates the percentage of DU5883 bacteria in the initial inoculum.

with *S. aureus*. For the three smaller bacterial inoculum amounts, the difference between the average weights of suckled and nonsuckled glands clearly indicated that milk was indeed removed from the suckled glands since the L4 glands weighed approximately 50% less than the R4 glands. A 50% weight difference between suckled and nonsuckled glands was also previously reported for the Compton White lactating mouse strain (1). We however observed a smaller weight difference when a very large inoculum amount was used (Table 1). In regard to the resulting infection of the glands, Table 1 reports the logarithms of total numbers of CFU obtained according to the inoculum administrated. Similar counts were obtained in all the R4 (or L4) glands despite the wide range in the numbers of CFU injected. In fact, the counts reached their maximum levels at 20 h in both R4 and L4 glands when an inoculum of at least 4.5×10^2 CFU/gland was used. Likewise, maximum bacterial counts were observed at 24 h of infection with similar amounts (1×10^2 to 4×10^2 CFU/gland) of inoculum containing the *S. aureus* strain Newbould 305, originally isolated from a clinical case of bovine mastitis (E. Brouillette, B. G. Talbot, G. Grondin, and F. Malouin, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol. 2002, abstr. Z-56, 2002). The average bacterial counts determined for the R4 and L4 glands were different by more than 2 log ($P < 0.001$), indicating that suckling contributes to a reduction in colonization. Also shown in Table 1, and of greater interest, the percentages of *S. aureus* DU5883 bacteria found in the L4 gland homogenates for all inocula were significantly different from those found in the R4 gland homogenates (nonparametric Mann-Whitney U test; $P < 0.05$). In other terms, *S. aureus* DU5883 bacteria lacking FnBPs were present in a lower proportion, as determined by the amount of DU5883 bacteria relative to the total amount of DU5883 and 8325-4 bacteria, under suckling pressure (L4 glands; 28.9%) than in the absence of suckling pressure (R4 glands; 41.2%).

To confirm the observation that *S. aureus* DU5883 bacteria were present in a lower proportion when the glands were suckled, 15 CD1 lactating mice were then experimentally infected with a unique inoculum (6.4×10^2 CFU/gland) composed of equal amounts of *S. aureus* 8325-4 and DU5883 bacteria. As for the experiment results reported in Table 1, the differences in the weights and the base-10 logarithms of CFU counts for the L4 and R4 glands were found to be statistically significant by using the Student *t* test (P is <0.001 and <0.01, respectively) (data not shown). The percentage of DU5883 bacteria found in the mammary tissues also confirmed the diminished ability of the mutant lacking FnBPs to colonize the glands under suckling pressure (Fig. 1). More precisely, the percentage of DU5883 bacteria in the initial inoculum (49.9%) was reduced to 34.6% in the glands when they were suckled (L4) (Fig. 1A). By using a Mann-Whitney U test, this percentage was found to be statistically different from the value of 45.3% found for the nonsuckled glands (R4) ($P < 0.02$). Therefore, the presence of FnBPs at the surface of the wild-type 8325-4 strain increased the pathogen's capacity to colonize the gland when the offspring were suckling. By plotting the percentage of DU5883 bacteria present in the L4 and R4 glands for each mouse, the collective significant difference between the suckled and nonsuckled glands can be put in perspective (Fig. 1B). In fact, only a few mice did displayed no marked difference in the amounts of the FnBP-lacking bacteria represented in the R4 and L4 glands and, for others, the difference in favor of the wild-type *S. aureus* strain 8325-4 reached 30 to 35%. A factor that may explain the observed differences between mice may be that the number of pups was different for each mouse, i.e., from 8 to 12. Thus, even if the

weight of all 15 individual L4 glands showed suckling by comparison to the R4 glands (data not shown), the turnover volume of milk could individually vary and so could the overall suckling-induced flow.

Using a lactating mouse model of mastitis, this report demonstrates that the FnBPs of *S. aureus* confer a selective advantage to the bacterium in vivo. The results show for the first time that *S. aureus* FnBPs are virulence factors for the colonization of the mammary gland under milk flow. It is reasonable to suggest that adherence to the mammary gland cells conferred by FnBPs prevents *S. aureus* from washing out during suckling. As opposed to in vitro adherence studies, the lactating mouse model of mastitis supplies conditions that better represent the natural environment found during an infection (presence of serum and milk), incorporates physical factors such as suckling, and takes into account the complexity of inflammation and immune reactions that occur during infectious mastitis. In the future, the mouse mammary gland and its model of infection may also allow the use of recently developed microbiological tools (26), such as in vivo bioluminescence, signature-tagged mutagenesis, and DNA microarrays, to study bacterial gene expression in a context relevant to the infectious mastitis disease.

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