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J. Miedzobrodzki
University of Lund, Lund, Sweden

A. S. Naidu
University of Lund, Lund, Sweden

J. L. Watts
Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Homer, Louisiana

Pawel Ciborowski
University of Nebraska Medical Center, pciborowski@unmc.edu

K. Palm
University of Lund, Lund, Sweden

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Effect of Milk on Fibronectin and Collagen Type I Binding to *Staphylococcus aureus* and Coagulase-Negative Staphylococci Isolated from Bovine Mastitis

J. MIEDZOBRODZKI,[1†] A. S. NAIDU,[1] J. L. WATTS,[2*] P. CIBOROWSKI,[1‡] K. PALM,[1] AND T. WADSTRÖM[1]

Department of Medical Microbiology, University of Lund, S-223 62 Lund, Sweden,[1] and Hill Farm Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Homer, Louisiana 71040[2]

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Tryptic soy broth (TSB)-grown cells of *Staphylococcus aureus* isolated from acute and chronic bovine mastitis bound mainly ^{125}I-fibronectin (^{125}I-Fn), whereas strains of nine species of coagulase-negative staphylococci showed a predominant interaction with ^{125}I-collagen (^{125}I-Cn) type I. A particle agglutination assay (PAA) was used to examine the interaction of coagulase-negative staphylococci with ^{125}I-Fn and ^{125}I-Cn immobilized on latex. All 368 coagulase-negative staphylococci demonstrated high ^{125}I-Cn and moderate to low ^{125}I-Fn interactions in the PAA. Cn-PAA reactivity was high among strains of *Staphylococcus xylosus* (84.2%), *Staphylococcus simulans* (77.8%), *Staphylococcus epidermidis* (76.7%), and *Staphylococcus hyicus* (74.3%), whereas all six *Staphylococcus capitis* strains clumped Cn-PAA reagent. Incubating TSB-grown cells in 10% skim milk for 1 h decreased the ^{125}I-Fn- and ^{125}I-Cn-binding affinity in most of the *S. aureus* and coagulase-negative staphylococci, while growth in 10% skim milk for 18 h resulted in more than 90% decrease or complete loss of interaction with these proteins. Decreased ^{125}I-Fn binding in the presence of milk was correlated with protease production but not with ^{125}I-Cn binding.

Staphylococcus aureus and coagulase-negative staphylococci are the most important udder pathogens causing bovine mastitis (1, 23). Earlier studies suggest that the proximity of staphylococci to the test cistern and contact with milk is the critical factor in the development of intramammary infections (1, 17, 26). Furthermore, *S. aureus* strains isolated from bovine mastitis and grown in raw milk or carbohydrate-salt-rich medium produce extracellular factors which cause toxic reactions in bovine skin (3).

Milk, although rich in essential nutrients, is regarded to be bacteriostatic as a result of the presence of immunoglobulins, phagocytic cells, complement components, lysozyme, lactoferrin, and the lactoperoxidase system (20, 21). However, *S. aureus* strains isolated from bovine mastitis demonstrate enhanced growth in whey prepared from the same milk the organism was isolated from (14). It has also been shown that staphylococci grown in milk show a decline in or complete loss of charge and hydrophobic cell surface properties (12).

S. aureus and *Streptococcus agalactiae* strains adhere to ductal epithelial cells (7). Hibbitt and Benians demonstrated that keratin, a cationic basic protein in the test duct, attaches to staphylococcal mastitis strains (9). Fibronectin (Fn), a glycoprotein, exists in a soluble form in serum and biological fluids and in an insoluble form in tissue matrices (15). *S. aureus* and coagulase-negative staphylococci isolated from various human infections bind Fn and collagen (Cn) (10, 25). Such interactions with the host mucosal surface at the molecular level may be important in the pathogenesis of staphylococcal intramammary infections (28). The Fn-binding receptor of *S. aureus* is a protein, susceptible to a wide range of proteases (22). However, the role of proteolytic enzymes produced during growth in milk is not known. In this study, we have explored the influence of skim milk on ^{125}I-Fn and ^{125}I-Cn binding to *S. aureus* and various species of coagulase-negative staphylococci isolated from acute and chronic bovine mastitis.

MATERIALS AND METHODS

Bacterial strains. A total of 80 *S. aureus* and 100 coagulase-negative staphylococcal strains, including 23 *Staphylococcus epidermidis*, 18 *Staphylococcus simulans*, 19 *Staphylococcus xylosus*, 16 *Staphylococcus hominis*, 14 *Staphylococcus cohnii*, 6 *Staphylococcus capitis*, and 4 *Staphylococcus warneri*, isolated from chronic and acute bovine mastitis were kindly supplied by P. Jonsson, National Veterinary Institute, Uppsala, Sweden. Additionally, 171 *Staphylococcus hyicus*, 50 *Staphylococcus epidermidis*, and 47 *Staphylococcus chromogenes* strains were isolated from bovine mastitis at the Mastitis Research Laboratory, Hill Farm Research Station, Homer, La. *S. aureus* was identified by standard procedures (4). Coagulase-negative staphylococci were identified according to the Schleifer-Kloos classification scheme and by Staph-Trac system (Analytab Products, Plainview, N.Y.) (24, 29). The accuracy of Staph-Trac system has been established previously and isolates yielding atypical results were identified by a previously described conventional method (29).

Chemicals. Fn, purified from porcine plasma by the method described by Vuento and Vaheri (27), was a kind gift from BioInvent AB International, Lund, Sweden. Vitrogen 100 collagen (containing 95% type I and 5% type III collagens, lot 87H18.3) was purchased from Collagen Corporation, Palo Alto, Calif. ^{125}Iodine was purchased from Amersham Sweden AB, Solna, Sweden. Iodo-beads (*N*-chloro-benzene sulfonamide-derivated, uniform nonporous polystyrene beads, lot 28666) were obtained from Pierce Chemicals Company, Rockford, Ill., and column PD-10 Sephadex G-25M was obtained

* Corresponding author.
† Present address: Institute of Molecular Biology, Jagiellonian University, 31-120 Cracow, Poland.
‡ Present address: National Institute of Hygiene, 00-791 Warsaw, Poland.

from Pharmacia, Uppsala, Sweden. Bovine serum albumin (lot 41F-0061) and ovalbumin (lot 11F-8055) were purchased from Sigma Chemical Co., St. Louis, Mo. Latex beads with a diameter of 0.8 μm (control 746460) were obtained from Difco Laboratories, Detroit, Mich., and Merthiolate was from KEBO Lab AB, Stockholm, Sweden. All chemicals used for the preparation of buffer solutions were of analytical grade.

Bacteriological media. Blood agar base was from Lab M, Salford, England. Tryptic soy broth (TSB) was from Difco. Standard methods caseinate agar was prepared as described previously (13). Skim milk was kindly supplied by the Swedish Dairies Association, Malmö, Sweden.

Growth conditions. Overnight cultures of staphylococci grown on blood agar were inoculated in TSB, TSB supplemented with 10% skim milk, or 10% skim milk at 37°C for 18 h with constant shaking. Cells were harvested, washed once in 0.02 M potassium phosphate (Pp) buffer (pH 6.8), resuspended in the same buffer to a density of 10^{10} cells per ml, and stored at −80°C until tested.

Proteolytic activity. Proteolytic activity was determined on standard methods caseinate agar (13). The size and type of zone formed as a result of proteolysis were examined after 48 h of incubation.

^{125}I-Fn and ^{125}I-Cn type I binding assay. Fn and Cn were labeled with ^{125}I by the chloramine-T method with Iodobeads (8). Binding of ^{125}I-Fn and ^{125}I-Cn to bacteria was quantitated by the method described by Fröman et al. (6). ^{125}I-labeled portions were diluted to 15,000 to 25,000 cpm in phosphate-buffered saline (pH 7.2) containing 0.05% sodium azide and 0.1% bovine serum albumin. Labeled protein (50 μl) was mixed with 100 μl of staphylococcal cell suspension (10^{10}/ml) in a polystyrene centrifuge tube and kept at room temperature for 1 h. After adding 2 ml of ice-cold phosphate-buffered saline containing 0.05% azide and 0.1% Tween 20, tubes were centrifuged at 4,500 × *g* for 10 min and the supernatant was aspirated. Bound radioactivity of the bacterial pellet was measured in a 1260-Multigamma (LKB-Wallac, Turku, Finland). Laboratory strains of *S. aureus* Cowan 1 and *S. aureus* Newman served as positive controls, and two *Micrococcus luteus* dairy isolates served as negative controls in the binding assay.

Fn and Cn particle agglutination assay (PAA). Fn-PAA and Cn-PAA reagents were prepared by a method described by Naidu et al. (16) for the rapid screening of Fn and Cn receptors on staphylococcal cell surfaces. The agglutination reaction was performed on glass slides by mixing 20 μl of staphylococcal cell suspensions (10^{10} cells per ml of Pp buffer) with an equal volume of Fn- or Cn-PAA reagent. The clumping was scored after 2 min as 1+, 2+, or 3+ reactions and recorded as positive, whereas +/− and − reactions were considered negative. Strains were checked for autoaggregation by mixing a drop of cell suspension with a drop of Pp buffer.

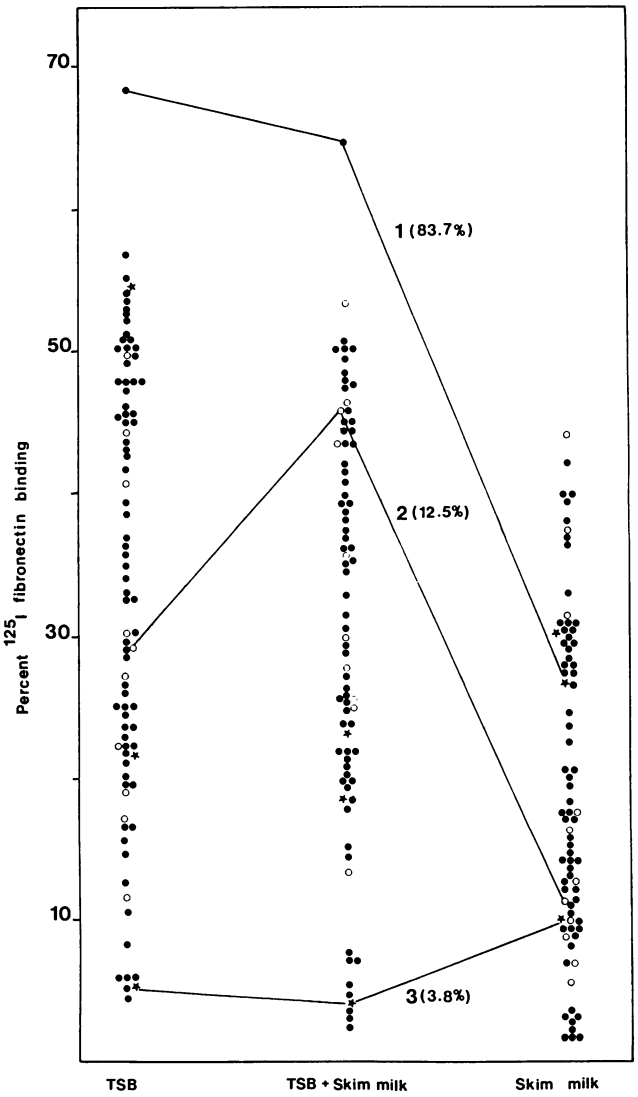

FIG. 1. Effect of milk on ^{125}I-Fn binding to *S. aureus* isolated from acute and chronic bovine mastitis. TSB-grown *S. aureus* cells were incubated in skim milk for 1 h or cells were cultivated in skim milk at 37°C for 18 h and tested for uptake of ^{125}I-Fn and compared with standard binding assay with cells grown in TSB. Three binding patterns were observed. In pattern 1, 67 (83.7%) isolates demonstrated a sizable decrease in binding under both conditions; in pattern 2, 10 (12.5%) isolates showed an increase in binding when grown in skim milk for 1 h but a decrease when grown in skim milk for 18 h; and in pattern 3, 3 (3.8%) isolates showed a slight or no decrease when grown in skim milk for 1 h but an increase when grown in skim milk for 18 h.

RESULTS

TSB-grown cells of *S. aureus* strains isolated from bovine mastitis showed high ^{125}I-Fn and low ^{125}I-Cn binding. TSB-grown *S. aureus* cells, when washed and incubated for 1 h in skim milk, showed certain changes in ^{125}I-Fn-binding properties (Fig. 1). ^{125}I-Fn binding was suppressed in 87.5% of *S. aureus*, while the remaining strains demonstrated an increased binding tendency. Similarly, cultivation of *S. aureus* strains in skim milk at 37°C for 18 h resulted in a sizeable decrease in ^{125}I-Fn binding (Fig. 1). However, a few *S. aureus* strains expressed increased ^{125}I-Fn (3.8%) and ^{125}I-Cn (5.0%) binding.

All nine coagulase-negative staphylococcal species included in the present study showed high ^{125}I-Cn and low ^{125}I-Fn binding. The frequency of ^{125}I-Fn and ^{125}I-Cn binding among 368 coagulase-negative staphylococci isolated from bovine mastitis was tested in a PAA (Table 1). All nine species of coagulase-negative staphylococci grown on blood agar showed a high Cn-PAA and a low Fn-PAA reactivity comparable with that of the ^{125}I-protein binding. *S. hominis* (62.5%) and *S. capitis* (50%) strains showed higher Fn-PAA reactivities. Cn-PAA reactivity was high among *S. xylosus* (84.2%), *S. simulans* (77.8%), *S. epidermidis* (76.7%), and *S. hyicus* (74.3%) strains. All six *S. capitis* strains agglutinated

TABLE 1. Fn and Cn type I particle agglutination of coagulase-negative staphylococci isolated from bovine mastitis[a]

Species	No. of isolates tested	No. positive (%)		
		Fn-PAA	Cn-PAA	AA[b]
S. hyicus	171	37 (21.6)	127 (74.3)	1
S. epidermidis	73	26 (35.6)	56 (76.7)	4
S. chromogenes	47	4 (8.5)	32 (68.1)	1
S. simulans	18	4 (22.2)	14 (77.8)	0
S. capitis	6	3 (50.0)	6 (100.0)	0
S. hominis	16	10 (62.5)	10 (62.5)	3
S. warneri	4	1 (25.0)	2 (50.0)	0
S. xylosus	19	5 (26.3)	16 (84.2)	0
S. cohnii	14	5 (35.7)	9 (64.3)	0

[a] Strains were grown on blood agar at 37°C for 18 h. Cells were washed once in Pp buffer and resuspended in the same buffer to a cell density of 10^{10}/ml.
[b] AA, Autoaggregating strains.

Cn-PAA reagent. Coagulase-negative staphylococcal cells showed no high tendency to autoaggregate when harvested from blood agar; however, cells of a few isolates of *S. hominis* (18.8%) and *S. epidermidis* (5.5%) autoaggregated.

^{125}I-Cn binding of all coagulase-negative staphylococcal species, except *S. hyicus*, was suppressed after 1 h of incubation of TSB-grown cells in skim milk (Fig. 2). Skim milk-grown cells of coagulase-negative staphylococci demonstrated a considerable decrease in ^{125}I-Cn binding.

The proteolytic activity of *S. aureus* and coagulase-negative staphylococci was compared with the ^{125}I-Fn- and ^{125}I-Cn-binding ability. Proteolytic strains of *S. aureus* showed a decrease in ^{125}I-Fn binding when grown in skim milk, whereas the ^{125}I-Fn binding to nonproteolytic strains was not affected by growth conditions in skim milk (Table 2). This phenomenon was observed mostly among *S. aureus* strains with greater than 10% binding of ^{125}I-Fn.

TABLE 2. Proteolytic activity of *S. aureus* strains isolated from bovine mastitis as related to ^{125}I-Fn binding of cells grown in TSB and skim milk[a]

^{125}I-Fn binding	Protease production			
	No. positive (%)		No. negative (%)	
	TSB	Skim milk	TSB	Skim milk
>10%	19 (90.5)	12 (57.1)	51 (100)	42 (82.4)
<10%	2 (9.5)	9 (42.9)	0 (0)	9 (17.6)

[a] Proteolytic activity was determined on SMCA agar by the method described by Martley et al. (13). ^{125}I-Fn binding assay was performed as described in Materials and Methods.

Of 70 coagulase-negative staphylococcal strains from bovine mastitis, only five strains bound ^{125}I-Fn to a degree greater than 10% or more. These isolates produced extracellular proteases and lost their ability to bind ^{125}I-Fn when grown in skim milk. However, protease production did not show any correlation to ^{125}I-Cn binding by *S. aureus* and coagulase-negative staphylococci in any of the growth conditions tested.

DISCUSSION

The adherence, survival, and multiplication of staphylococci on the udder epithelium are the decisive events in the pathogenesis of bovine mastitis (1). Frost et al. (7) demonstrated the adhesion of staphylococci to the ductular epithe-

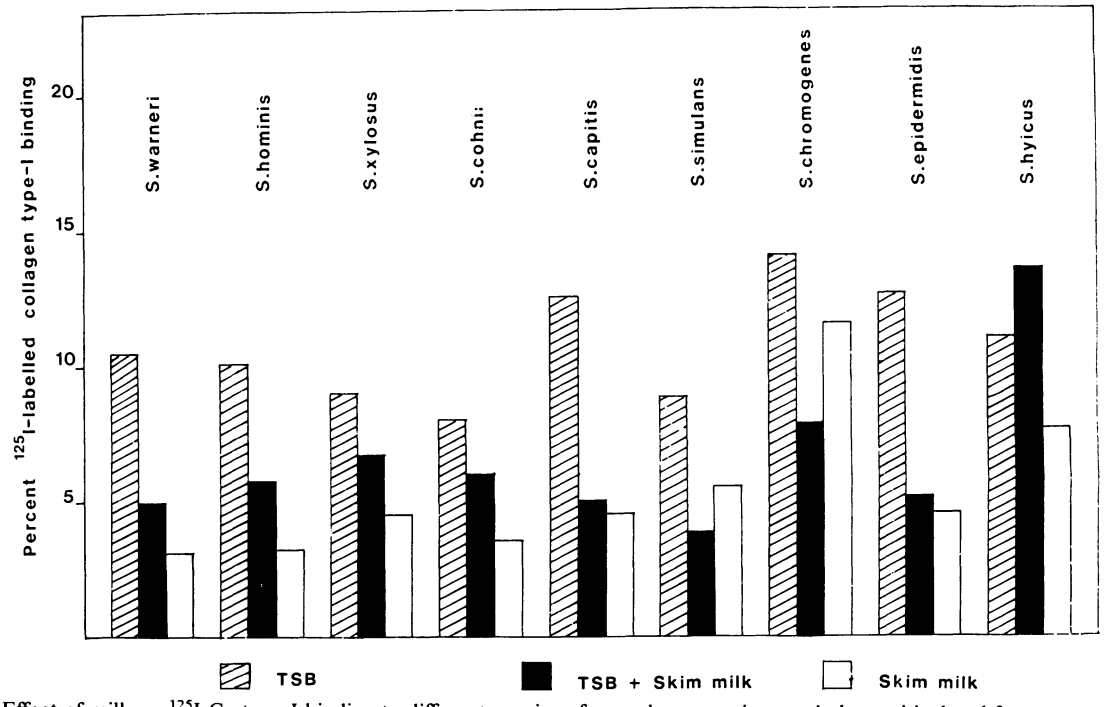

FIG. 2. Effect of milk on ^{125}I-Cn type I binding to different species of coagulase-negative staphylococci isolated from acute and chronic bovine mastitis. Histograms represent median values of ^{125}I-Cn type I binding to *S. capitis* (6 strains), *S. warneri* and *S. saprophyticus* (4 strains each), and 10 strains from each of the remaining coagulase-negative staphylococcal species. Except for *S. hyicus*, TSB-grown cells of different coagulase-negative staphylococcal species showed a decrease in ^{125}I-Cn type I binding after incubation in skim milk for 1 h. Except for strains of *S. simulans* and *S. chromogenes*, the decrease in binding was more prominent when cells were grown in skim milk at 37°C for 18 h.

lial cells in vitro. However, the events preceding establishment of intramammary infection by staphylococci are not well defined. Contact of bacterial cells with milk has been described as the critical factor in the survival and multiplication of staphylococci during intramammary infection (17, 26). Binding of bacterial cells to serum and tissue components, such as Fn, Cn, and fibrinogen, has been suggested as the initial stage in the development of intramammary infection (11). Therefore, we determined the ^{125}I-Fn or ^{125}I-Cn binding to staphylococci after incubation of cells for 1 h or overnight in milk.

In the present study, *S. aureus* isolated from acute, as well as chronic, mastitis bound mainly ^{125}I-Fn. Conversely, different coagulase-negative staphylococcal species predominantly interacted with ^{125}I-Cn. However, this binding ability remarkably decreased in *S. aureus* and coagulase-negative staphylococcal strains during contact with milk.

The majority of TSB-grown, washed *S. aureus* cells demonstrated decreased ^{125}I-Fn binding after incubation in milk for 1 h; however, a few strains either showed an increase or no change in ^{125}I-Fn binding. Except for five *S. hyicus* strains, coagulase-negative staphylococci showed a decrease in ^{125}I-Cn binding after cells were incubated in milk for 1 h. Although milk contains Fn but not Cn (15), decreased ^{125}I-Fn binding to *S. aureus* as a result of cold Fn present in milk is less likely, as this does not account for the reduced ^{125}I-Cn binding to coagulase-negative staphylococci. Furthermore, antibodies of the immunoglobulin G class to a cloned Fn-binding protein were not detected in skim milk by using a recently developed enzyme-linked immunosorbent assay (A. S. Naidu et al., manuscript in preparation). Our recent observation that *S. aureus* has a different, serologically distinct Fn-binding protein does not completely exclude the possible immune blocking of ^{125}I-Fn binding to mastitis strains of *S. aureus* (Naidu et al., in preparation). The interaction of hydrophobic milk components, such as casein and various lipids, with the staphylococcal cell surface is also not known. Although the interference of immunoglobulin A in microbial adhesion has been suggested (5, 30), its role in Fn and Cn binding has not been elucidated.

Interestingly, only proteolytic strains of *S. aureus* showed a remarkable decrease in Fn binding when grown in milk in comparison to nonproteolytic strains. Similar tendencies were also observed with Cn binding to coagulase-negative staphylococcal strains. Staphylococci produce increased levels of proteases in casein-rich medium (2). Moreover, Fn and Cn binding to staphylococci has been shown to be sensitive to trypsin, pepsin, and proteinase K, suggesting the protein nature of cell surface receptors (22, 25). Further experiments have suggested that Fn and Cn binding to staphylococci is a protease-dependent property (P. Aleljung et al., manuscript in preparation). The effect of milk on the staphylococcal interaction with serum and connective tissue proteins during the development of intramammary infections may be important. Fn binding may also be important in presentation of bacterial cells to the host immune system (18, 19). Whether this milk effect is a protective phenomenon for the bovine mammary gland preventing adhesion and colonization of staphylococci or an adaptive mechanism of the organism to evade phagocytic killing (15) and the host immune defense system awaits a detailed characterization.

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