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Bovine Lactoferrin Binding to Six Species of Coagulase-Negative Staphylococci Isolated from Bovine Intramammary Infections

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Bovine lactoferrin (BLf), an acute-phase iron-binding secretory protein present in secretions of the bovine udder, was demonstrated to bind to the following staphylococcal species associated with bovine intramammary infections: *S. epidermidis*, *S. warneri*, *S. hominis*, *S. xylosus*, *S. hyicus*, and *S. chromogenes*. The degree of ^{125}I-labeled BLf uptake significantly varied among the blood agar-grown cells of all six species of coagulase-negative staphylococci tested. Isolates identified as *S. xylosus* demonstrated the highest (mean, $35.1 \times 10^6 \pm 13.3 \times 10^6$ nmol) and *S. hyicus* the lowest (mean, $10.7 \times 10^6 \pm 5.9 \times 10^6$ nmol) binding to ^{125}I-BLf. BLf binding was optimum at an acidic pH, with time-dependent binding saturation ranging from 70 min for *S. warneri* to 240 min for *S. hominis*. The BLf-binding mechanism was specific, with affinity constants (K_a values) ranging between 0.96×10^6 and 11.90×10^6 liters/mol. The numbers of BLf-binding sites per cell, as determined by using Scatchard analysis, were as follows: *S. epidermidis*, 3,600; *S. warneri*, 1,900; *S. hominis*, 4,100; *S. xylosus*, 4,400; *S. hyicus*, 6,100; and *S. chromogenes*, 4,700. ^{125}I-BLf binding to all species was inhibited by unlabeled BLf and unlabeled human lactoferrin, whereas none of the various plasma, connective tissue, or mucosal secretory proteins or carbohydrates tested caused significant interference. BLf-binding receptors of the six coagulase-negative staphylococcal species demonstrated marked differences in patterns of susceptibility to proteolytic or glycolytic enzyme digestion and to heat or periodate treatment. These data suggest that the BLf-binding components in *S. epidermidis* and *S. warneri* are proteins containing glycosidyl residues. In the remaining four species, the proteinaceous nature of the BLf-binding component was evident, but the involvement of glycosidyl residues was not clear. Results of this study establish the presence of specific binding components for BLf on coagulase-negative staphylococci isolated from bovine intramammary infections.

Coagulase-negative staphylococci frequently colonize the bovine udder skin, streak canal, and interior of the mammary gland (11, 51). These organisms are frequently isolated from intramammary infections (IMIs) in first-lactation cows (7), resulting in milk alterations as well as decreased milk production (16, 33, 50). Coagulase-negative staphylococci, which were previously considered to be of low pathogenicity (49), cause mild inflammatory reactions that result in elevated milk somatic cell counts (18). IMIs caused by coagulase-negative staphylococci have been suggested to prevent subsequent IMIs by other mammary gland pathogens (6, 14, 22).

Bovine lactoferrin (BLf), a 92.1-kilodalton iron-binding protein secreted mainly by polymorphonuclear leukocytes, occurs in milk and various biological secretions (21, 26, 27, 58). BLf concentrations vary from 0.1 to 0.3, 2 to 5, and 20 to 30 mg/ml in normal milk, colostrum, and secretions collected during glandular involution, respectively, depending on the physiologic status of the bovine mammary gland (43, 46, 59). During acute bovine mastitis, BLf levels in the lacteal secretions increase 30-fold, corresponding to the severity of infection (15). As a major component of the specific granules of polymorphonuclear leukocytes, lactoferrin (Lf) contributes to both hydrogen peroxide-dependent and -independent bacterial killing (1, 3, 12). By limiting iron availability, Lf causes bacteriostasis (2, 36) which is enhanced by further cooperation with immunoglobulin A (IgA) from human milk or IgG1 from bovine colostrum (48). As an acute-phase protein, Lf amplifies the inflammatory response (37) and stimulates the phagocytic and cytotoxic properties of macrophages (8, 9). Lf is also a potent inhibitor of granulocyte-monocyte colony-stimulating factor and regulates myelopoiesis (10). Specific binding of iron-saturated human Lf to the membrane of mouse peritoneal macrophages has been described previously (52). A mannose-sensitive, calcium-dependent, specific binding of human Lf to the human adherent mononuclear cells has also been reported (4).

The susceptibility of the bovine mammary gland to IMI depends upon its physiologic transitions (28). Changes in the BLf:citrate molar ratio have been suggested to be indicators of the functional transition of the bovine mammary gland (35, 47). Apart from antibacterial activity, BLf may be important in antigen processing by the cells of the reticuloendothelial system and in antibody production (9). Thus, the interaction of BLf with bacteria associated with bovine mammary glands may be decisive in the various events of host defense. We have recently shown a calcium- and mannose-independent, specific high-affinity binding of BLf to *Staphylococcus aureus* associated with IMIs (A. S. Naidu, M. Andersson, J. Miedzobrodzki, J. L. Watts, and A. Forsgren, submitted for publication). The frequency of

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BLf binding was high among bovine mastitis isolates of *S. aureus*.

The purpose of this study was to examine the interaction of BLf with six different species of coagulase-negative staphylococci commonly associated with bovine IMIs. Evidence for specific BLf binding to strains of coagulase-negative staphylococci and characterization of the BLf-binding mechanism among the representative strains of the respective species are presented.

MATERIALS AND METHODS

Bacterial strains. A total of 150 coagulase-negative staphylococci isolated from chronic and acute infections of the bovine mammary gland were tested for BLf-binding capacity. These isolates were identified as follows: *S. epidermidis* (22 isolates), *S. hominis* (11 isolates), *S. warneri* (15 isolates), *S. chromogenes* (36 isolates), *S. xylosus* (20 isolates), and *S. hyicus* (46 isolates). All isolates were identified by using the STAPH-Trac system (Analytab Products, Plainview, N.Y.) (57). The accuracy of the STAPH-Trac system has been established in an earlier study (57), and isolates yielding atypical results were identified by a previously described conventional method (44). Bacterial strains were stored in glycerol at −80°C, subcultured, and subsequently cultivated on blood agar at 37°C for 18 h. For ^{125}I-labeled BLf-binding experiments, blood agar-grown bacterial cells were harvested and washed in phosphate-buffered saline (PBS; pH 7.2), and the density was adjusted to ~10^{10} cells per ml optically at an A_{540}, and cell counts were made under a phase-contrast microscope by using a modified Burker chamber (depth, 0.01 mm).

Chemicals. BLf from colostrum and human Lf were purchased from U.S. Biochemical Corp., Cleveland, Ohio. BLf, which was purified from bovine milk whey, was kindly provided by Hans Burling, Swedish Dairies Association, Malmö, Sweden. Fibronectin was purified from bovine plasma by the method of Veunto and Vaheri (54). Collagen type I (lot 87H18.3) was obtained from Collagen Corp., Palo Alto, Calif. IgG was obtained from Kabi Vitrum, Stockholm, Sweden. The following proteins, carbohydrates, enzymes, and enzyme inhibitors (purchased from Sigma Chemical Co., St. Louis, Mo.) were tested for their effect on BLf binding: transferrin (lot 67F.9457); fibrinogen (from bovine plasma; lot 58F-9484); hemin (bovine, type I; lot 104F.0090); fetuin (from fetal calf serum; lot 28F.9570); albumin (from bovine serum; lot 38F.0020); mannose, fucose, ribose, sorbitol, and sialic acid type VIII (from sheep submaxillary glands; lot 28F.1046); *N*-acetyl-D-galactosamine (lot 77F.5053); *N*-acetyl-D-glucosamine (lot 87F.0329); trypsin (type XIIS; lot 78F.6804); pepsin (from porcine stomach mucosa; lot 88F.8010); proteinase K (from *Tritirachium album*; type XIS; lot 89F.6828); pronase E (from *Streptomyces griseus*; type XXV; lot 48F.057015); papain (from papaya latex; type IV; lot 78F.8140); peptidase (from porcine intestinal mucosa; lot 80F.80111); β-glucosidase (from almonds; lot 29F.4000); β-galactosidase (from *Escherichia coli*; grade VI; lot 19F.6835); trypsin inhibitor (from soybean; lot 96F.8115); phenylmethylsulfonyl fluoride; and iodoacetamide. All chemicals used for the preparation of buffer solutions were of analytical grade.

^{125}I-BLf-binding assay. BLf was labeled according to a modified chloramine T method with Na (^{125}I) (specific activity, 629 GBq/mg; DuPont Scandinavia AB, Stockholm, Sweden) by using Iodobeads (Pierce Chemical Co., Rockford, Ill.) (25). Binding assays were performed as described earlier by Naidu et al. (31). Briefly, 10^9 bacterial cells (in a volume of 100 μl of PBS) were mixed with 100 μl of ^{125}I-BLf (radioactivity was adjusted to 2×10^4 to 2.5×10^4 cpm, i.e., approx. 8 ng, when diluted in cold PBS). After 1 h of incubation at room temperature, 2 ml of ice-cold PBS (containing 0.1% Tween 20) was added to the tubes. The suspension was centrifuged at 4,500 × *g* for 15 min at 4°C, and the supernatant was aspirated. The radioactivity that was retained in the bacterial pellet was measured in a gamma counter (Clingamma 1271; LKB Wallac, Turku, Finland). The residual background radioactivity from incubation mixtures containing no bacteria was 2.5%, whereas the nonspecific ^{125}I-BLf binding in the presence of an excess of unlabeled BLf was approximately 7.5% of the total labeled protein added. Thus, a binding value of below 10% of the total ^{125}I-BLf added was considered to be a negative result during binding measurements. Samples were always tested in triplicate, and each experiment was repeated at least two times unless stated otherwise.

Selection of isolates for characterization of BLf binding. The following isolates representing each of the six coagulase-negative staphylococcal species were selected for further characterization of BLf binding: *S. epidermidis* AF-9, *S. hominis* AF-93, *S. warneri* AF-101, *S. chromogenes* AD-1, *S. xylosus* AG-12, and *S. hyicus* AC-166. Blood agar-grown cells were used for the characterization studies.

Saturation time and optimum pH determination for BLf binding. A concentration of ~8 ng (100 μl) of ^{125}I-BLf was added to 10^9 cells (100 μl) of each test strain. Tubes were mixed, and binding was measured at different time intervals. Samples were tested in quadruplicate and repeated two times. The time required for the saturation of bacterial cells with the ligand was set as the incubation time for that individual test strain during further characterization studies. The optimum pH of different isolates for BLf binding was tested in the pH range of 3.0 to 9.0. Sodium citrate at a pH range of 3.0 to 7.5 or with sodium barbitone at a pH range of 7.0 to 9.0 was used as a 50 mM buffer solution during the experiment.

Displacement and competitive inhibition of ^{125}I-BLf binding. Increasing amounts of unlabeled BLf (in 100-μl volumes diluted in PBS) were mixed with ~8 ng of ^{125}I-BLf (in 100-μl volumes). Approximately 10^9 cells of the test strain were added to this mixture (final volume, 300 μl) and incubated at the appropriate binding saturation time.

The competitive inhibitory effect of the following different proteins on ^{125}I-BLf binding to bacteria was tested: human Lf, transferrin, hemin, bovine fibronectin, bovine fibrinogen, IgG, collagen type I, bovine fetuin, and bovine serum albumin. The following carbohydrates were also tested: D-(+)-mannose, L-(−)-fucose, D-(−)-ribose, D-(−)-sorbitol, sialic acid type VIII, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine. A volume of 100 μl of protein or carbohydrate solution (concentration, 1 mg/ml) was mixed with approximately 10^9 cells of the test strain (volume, 100 μl) and kept at room temperature for 30 min. Finally, 100 μl of ^{125}I-BLf was added, and the mixture (final volume, 300 μl) was incubated at the binding saturation time. ^{125}I-labeled protein binding measurements were made as described above.

Treatment of bacteria with enzymes. Blood agar-grown cells (100 μl containing 10^9 bacteria) of the representative coagulase-negative staphylococcal strains were treated with proteases or glycosidases (in a 100-μl volume). Trypsin (25,000 U/ml) hydrolysis was performed in 0.15 M PBS (pH 7.4), and the reaction was blocked with soybean trypsin

TABLE 1. Binding of ^{125}I-BLf to coagulase-negative staphylococci

Organism	No. of isolates tested	^{125}I-BLf binding[a]	
		No. (%) positive	Mean ± SD binding (nmol [10^6])
S. epidermidis	22	11 (50.0)	13.9 ± 8.3
S. hominis	11	8 (72.7)	19.8 ± 10.1
S. warneri	15	8 (53.3)	14.5 ± 6.6
S. chromogenes	36	29 (80.6)	16.5 ± 7.1
S. xylosus	20	19 (95.0)	35.1 ± 13.3
S. hyicus	46	18 (39.1)	10.7 ± 5.9

[a] ^{125}I-BLf binding to individual coagulase-negative staphylococcal isolates is shown in Fig. 1.

inhibitor. Pepsin (10,000 U/ml) treatment was performed in 100 mM sodium acetate buffer (pH 4.5), and the hydrolysis was stopped by increasing the pH of the reaction mixture to 7.4. Proteinase K (100 U/ml) digestion was carried out in 40 mM potassium phosphate buffer (pH 7.5), and the reaction was inhibited by the addition of 0.5 M phenylmethylsulfonyl fluoride. Peptidase (1 U/ml), pronase E (5 U/ml), and papain (10 U/ml) hydrolysis reactions were performed in 30 mM Tris hydrochloride at pH 7.1, 7.5, and 6.2, respectively; and all three reactions were terminated with 0.5 M iodoacetamide. Glycolytic digestion of the bacterial cells with β-galactosidase (100 U/ml) or β-glucosidase was performed in 30 mM Tris hydrochloride buffer at pH 7.3 and 5.0, respectively.

After 1 h of incubation, bacterial cells were washed thoroughly with PBS. During the enzymatic treatments described above bacterial sedimentation was prevented by gentle rotation. Bacteria were washed carefully, suspended in 100 μl of PBS, and incubated with ^{125}I-BLf (100 μl) for the desired incubation times. The ^{125}I-BLf-binding assay was performed as described above. Untreated cells suspended in appropriate buffers served as controls during the calculation of percent susceptibility values.

Heat and periodate treatment of bacteria. One milliliter of bacterial suspension (10^{10} cells per ml) was heated at 80 or 100°C for 1 h, with gentle shaking in a water bath. Furthermore, 1 ml of bacterial suspension (10^{11} cells) was mixed with 5 mg of sodium-*p*-periodate (lot 58F.0310; Sigma). The mixture was protected from light, kept in a cold room, and gently agitated. After 24 h of incubation, the mixture was centrifuged at 500 rpm (150 × *g*) for 5 min. Bacterial cells in the supernatant were aspirated and dialyzed against PBS for 24 h. Finally, the density of the sodium periodate free cell suspension was adjusted to 10^{10} cells per ml. Cells obtained from the two treatments described above were tested for ^{125}I-BLf uptake as described above.

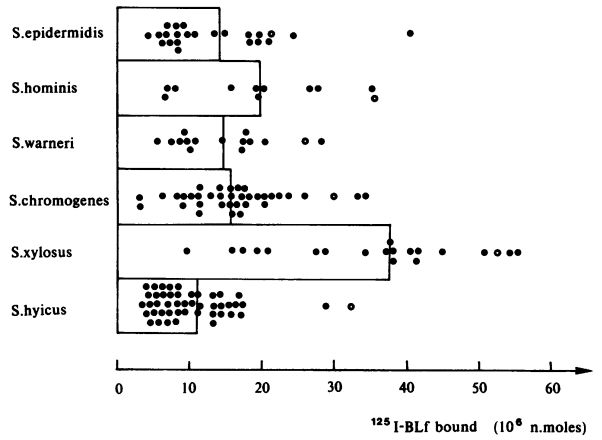

FIG. 1. Distribution of isolates according to ^{125}I-BLf-binding capacity. A high-binding isolate (O) was selected from each species for a detailed characterization of the bacterium-BLf interaction. The bars represent mean binding values for each species.

Data analysis. A single standard deviation was used for calculations. During Scatchard analysis, linear regression analysis was performed when necessary.

RESULTS

Of the 150 coagulase-negative staphylococci tested, 93 (62.0%) isolates were positive for BLf binding (Fig. 1). Differences in BLf binding between species were observed, with *S. xylosus* yielding the highest mean binding values ($35.1 \times 10^6 \pm 13.3 \times 10^6$ nmol) and *S. hyicus* the lowest ($10.7 \times 10^6 \pm 5.9 \times 10^6$ nmol) (Table 1). BLf-binding values, number of binding sites per cell, binding affinity constants, saturation times, and optimum pH ranges for binding of isolates representing each coagulase-negative staphylococcal species are presented in Table 2. All isolates exhibited optimum binding at acidic pH. BLf binding was time dependent and ranged from 70 min for *S. warneri* to 240 min for *S. hominis*. ^{125}I-BLf uptake was displaced by unlabeled BLf in a dose-dependent manner, with marked variation between isolates (Fig. 2). The following concentrations of unlabeled BLf were required for 50% displacement: *S. epidermidis*, 501.2 ng; *S. hominis*, 1,000 ng; *S. warneri*, 177.8 ng; *S. chromogenes*, 562.3 ng; *S. xylosus*, 177.8 ng; and *S. hyicus*, 891.3 ng. These data indicate that the bacteria recognize both forms of ligand with specificity.

The number of saturable BLf-binding receptor sites was determined for each species by using Scatchard analysis

TABLE 2. Characteristics of BLf binding by six species of coagulase-negative staphylococci

Organism	Bound BLf (nmol [10^6])	No. of binding sites/cell[a]	Affinity constant (K_a [liters/mol; 10^6])	Saturation time (min)	Optimum binding range (pH)
S. epidermidis AF-9	21.2	3,600	11.90	90	4.5–6.5
S. hominis AF-93	35.4	4,100	3.80	240	5.0–6.0
S. warneri AF-101	27.3	1,900	3.10	70	5.5–6.5
S. chromogenes AD-1	29.8	4,700	2.50	180	4.0–6.0
S. xylosus AG-12	54.0	4,400	3.30	210	4.5–7.0
S. hyicus AC-166	32.2	6,100	0.96	180	4.5–5.5

[a] Blood agar-grown cells were adjusted to a density of 10^{10} cells per ml, and bacterial cells were counted by using a modified Burker chamber. A molecular weight of 92,100 for BLf was used, as described by Weiner and Szuchet (58), during analyses of Scatchard plots (41).

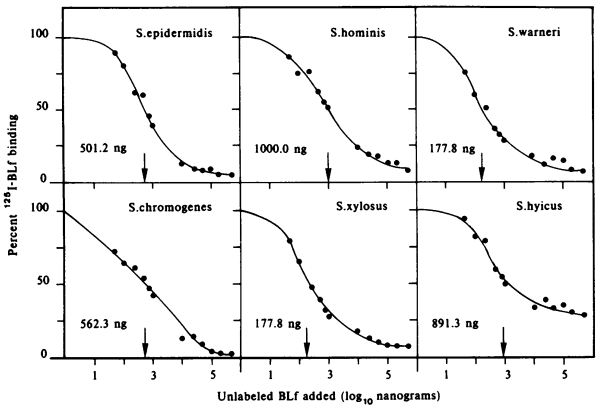

FIG. 2. Displacement of ^{125}I-labeled BLf binding to cells as a function of unlabeled BLf added. Arrows indicate 50% displacement values.

(Fig. 3). *S. warneri* had the lowest number of binding sites (1,900 per cell), and *S. hyicus* had the highest (6,100 per cell). BLf-bacterial cell affinities were high, with values ranging from 0.96×10^6 liters/mol for *S. hyicus* to 11.90×10^6 liters/mol for *S. epidermidis* (Table 2).

The inhibitory effects of various proteins or carbohydrates on BLf binding are presented in Table 3. In competitive inhibition tests, unlabeled human Lf inhibited ^{125}I-BLf binding to a similar degree as unlabeled BLf did. The other proteins tested, including hemin, fibronectin, fibrinogen, IgG, collagen type I, fetuin, and bovine serum albumin, had modest effects on BLf uptake. Similar results were obtained with the carbohydrates that were tested.

The effect of enzymatic or physicochemical treatment on BLf binding varied among the different coagulase-negative staphylococcal species (Table 4). The BLf-binding sites of *S. epidermidis*, *S. warneri*, and *S. hominis* were highly susceptible to proteinase K treatment; this enzyme had only a moderate effect on BLf binding by *S. hyicus* or *S. chromogenes*. Susceptibility to pepsin was high for *S. epidermidis* and moderate for *S. warneri*, *S. hominis*, and *S. hyicus*. Pepsin treatment increased BLf binding of *S. xylosus* cells and had no effect on *S. chromogenes* binding. Of the two glycosidases tested, only β-glucosidase caused a decrease in BLf binding. Heat treatment of cells resulted in moderate decreases in BLf binding. Periodate treatment decreased BLf binding to *S. epidermidis* and *S. warneri*, did not alter BLf binding to *S. hominis* and *S. xylosus*, and increased BLf binding to *S. chromogenes*.

DISCUSSION

The bacteriostatic properties of bovine milk have been attributed to a variety of factors, including immunoglobulins, phagocytic cells, complement components, lysozyme, lactoperoxidase, and the Lf system (40). During an inflammatory response, somatic cells, mainly polymorphonuclear leukocytes, infiltrate the bovine mammary gland and provide the primary host defense (13). Leukopenic cows are more prone to contract mastitis (19, 20). However, a rise in somatic cell count levels in milk to 200×10^3 to 880×10^3 cells per ml is protective against infection by common mammary gland pathogens (34, 39). Furthermore, experimental induction of inflammatory reactions and elevation of cell counts with sterile saline, endotoxin, or intramammary polyethylene devices seem to provide prophylaxis against IMIs (5, 38, 42). Secondary granules of polymorphonuclear leukocytes release increased amounts of Lf during an inflammatory response or endotoxic shock and cause hyposideremia (53). BLf, an important bacteriostatic factor in the prevention of udder infections (15), increases following a mild irritation of the bovine udder by minor pathogens such as coagulase-negative staphylococci. However, the interaction of BLf with coagulase-negative staphylococci is not well understood. Furthermore, the presence of specific Lf receptors on reticuloendothelial system cells (4, 52) warrants further studies on whether specific Lf receptors exist on coagulase-negative staphylococci. Thus, complexing of bacteria to reticuloendothelial system cells by Lf may promote the uptake and phagocytic processing of bacteria. Results of this study indicate that specific receptors for BLf are present on the cell surface of six species of coagulase-negative staphylococci associated with IMIs. Some variation in the BLf-binding capacity among the six species of coagulase-negative staphylococci was noted. However, the pathobiological significance of such variation is unclear.

The staphylococcal cell surface is a complex chemical mosaic and may allow various interactions with different biological substances. Therefore, competitive inhibition experiments were performed to define the specificity of BLf binding and also to elucidate interference or steric hinderance, if any, by various plasma, connective tissue, and mucosal secretory proteins. The inhibition profiles of all the six species varied markedly. The binding of ^{125}I-labeled BLf to all six species was inhibited by BLf or human Lf, suggesting that cells recognize the proteins of both mammalian species. However, in displacement experiments with homologous or heterologous combinations with ^{125}I-labeled BLf or human Lf against unlabeled BLf or human Lf, a stronger displacement was observed with BLf than with human Lf (data not shown). Unlike staphylococci, human isolates of *Neisseria meningitidis* demonstrate specific binding to human Lf with no competitive blocking with Lf from other mammalian species (45). Another iron-binding protein, transferrin, with functional and evolutionary similarities to Lf (29), caused moderate inhibition of BLf binding in *S. hyicus* and *S. hominis*. Bacterial interaction with components of the udder subepithelial matrix may be important in the pathogenesis of IMIs (55). Different species of coagulase-negative staphylococci demonstrate binding of fibronectin and collagen types I and II (23, 30, 56). In the present study, bovine fibronectin showed less than 20% interference to BLf binding in any of the species, while collagen type I caused 30% inhibition only in *S. chromogenes* and *S. epidermidis*. This suggests that BLf binding to coagulase-negative staphylococci is distinct from fibronectin and collagen binding. Fibrinogen, IgG, fetuin, or bovine serum albumin showed less than 30% interference in BLf binding to the six species tested. Conversely, hemin enhanced BLf uptake in the six species and enhanced uptake more strongly in *S. hyicus*.

Several mammalian receptors mediate glycoprotein clearance through recognition of different terminal carbohydrate units, in particular, *N*-acetyl-D-glucosamine, mannose, and fucose in the mononuclear-phagocyte system (17). Of the two fucosyl residues in the Lf molecule, the one between the α1-3 linkage with *N*-acetyl-D-glucosamine could be a potential binding determinant (17, 29). Moreover, the binding of human adherent mononuclear cells to human Lf is mannose dependent (4). In the present study, none of the sugars, including L-(−)-fucose or D-(+)-mannose, inhibited BLf binding to any of the six species. Interestingly, in *S. hyicus*

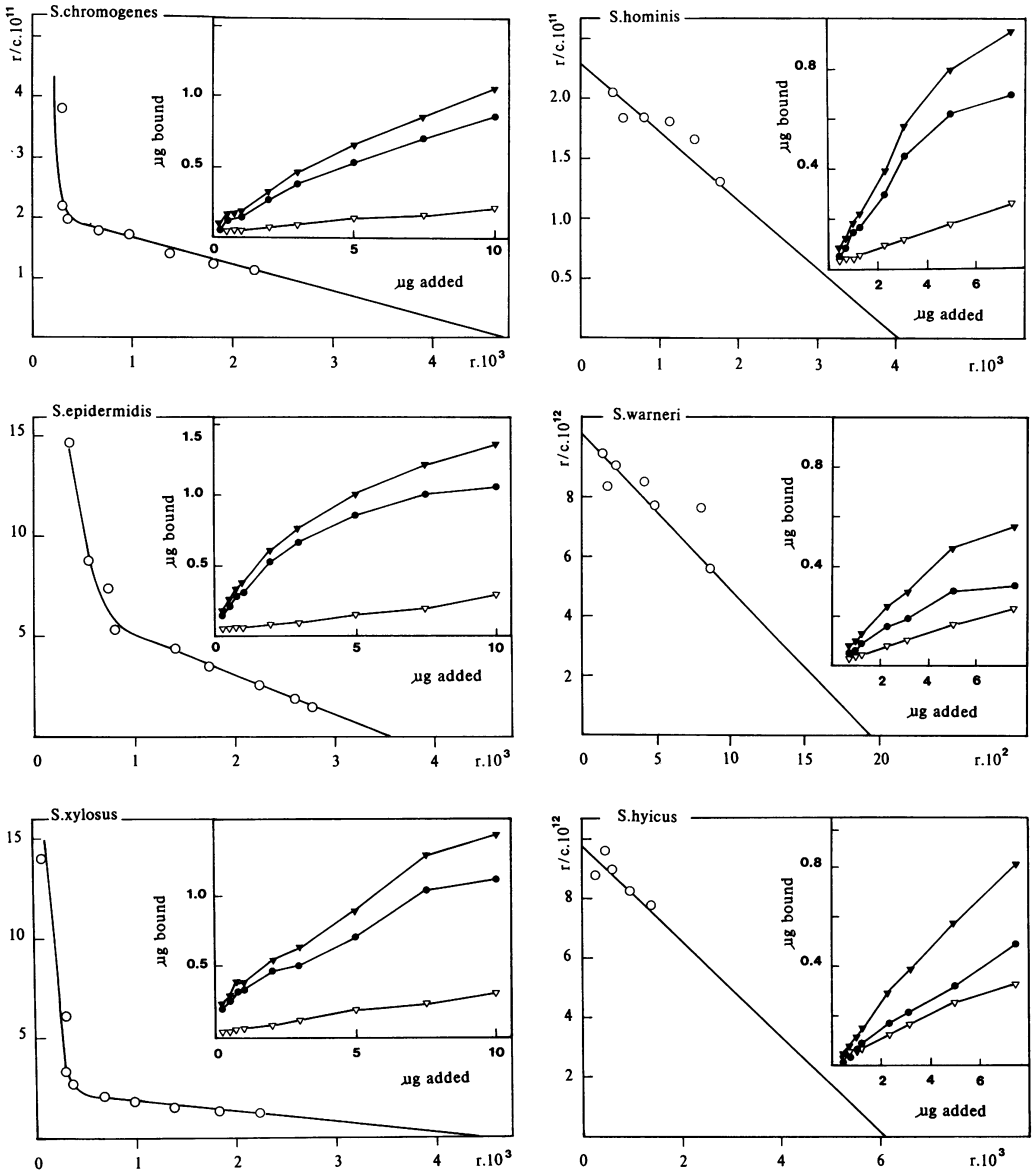

FIG. 3. Scatchard plot ($r/c = nK_a - rK_a$) of the binding of ^{125}I-BLf to cells of coagulase-negative staphylococci. The molecules of BLf bound to one bacterial cell (r) and the molecules of BLf free in the medium (c) were calculated by using a molecular weight of 92,100 for BLf. The intercept on the x axis represents the number of receptors per cell (n). The slope of the line represents the effective association constant (K_a), in liters per mole, (Insets) Binding of increasing amounts of ^{125}I-labeled BLf, as indicated, to bacteria was performed in the presence (▼; total binding) or absence (▽; nonspecific binding) of unlabeled BLf (50 times excess). Specific binding (●) was calculated by subtracting the nonspecific BLf uptake values from total binding values.

all of the sugars increased the ^{125}I-BLf uptake, probably via electrostatic bridging.

Staphylococcal cell surface receptors involved in the binding of serum or connective tissue proteins are mostly proteinaceous in nature (32; A. S. Naidu, C. Schalén, J.-I. Flock, I. Nilsson, J. Miedzobrodzki, and T. Wadström, *in* T. Wadström, I. Eliasson, I. Holder, and Å. Ljungh, ed., *Pathogenesis of Wound and Biomaterial-Associated Infections*, in press). Furthermore, protein-rich media, such as milk, induce protease production in staphylococci and cause alterations in the physicochemical or the binding properties of the cell surface (24, 30). Cells from each representative species of coagulase-negative staphylococci were subjected to a variety of proteolytic digestions under similar controlled conditions. Cell surface components involved in the BLf-binding mechanism of all six species in general, were susceptible to proteinase K, a broad-spectrum proteolytic enzyme. However, with other proteases, the susceptibility patterns differed among species. For instance, trypsin and peptidase hydrolysis reduced BLf binding to a moderate to low degree in *S. epidermidis* (which is highly susceptible to pepsin), *S. hyicus*, *S. hominis*, *S. warneri*, and *S. chromogenes*. However, the same treatment enhanced BLf binding to *S. xylosus*. Pronase E treatment of cells caused a moder-

TABLE 3. Inhibitory effects of various unlabeled proteins and carbohydrates on binding of ^{125}I-BLf to six different species of coagulase-negative staphylococci[a]

Inhibitor	% ± SD Inhibition of ^{125}I-BLf binding[b]					
	S. epidermidis AF-9	*S. hominis* AF-93	*S. warneri* AF-101	*S. chromogenes* AD-1	*S. xylosus* AG-12	*S. hyicus* AC-166
Proteins						
BLf	91.5 ± 1.0	91.3 ± 2.4	92.7 ± 2.0	87.8 ± 5.1	89.3 ± 5.1	78.9 ± 6.1
Human Lf	78.5 ± 2.4	84.1 ± 2.8	84.7 ± 4.4	75.8 ± 1.0	79.9 ± 0.7	79.5 ± 6.8
Transferrin	7.0 ± 5.6	24.7 ± 5.9	3.9 ± 1.9	2.0 ± 1.6	7.2 ± 1.3	48.4 ± 20.8
Hemin	16.6 ± 4.7	−18.7 ± 7.4	−2.0 ± 2.9	−31.4 ± 4.4	−15.1 ± 2.9	−90.4 ± 4.2
Fibronectin	10.0 ± 2.6	16.9 ± 12.9	9.9 ± 2.6	19.3 ± 1.5	15.7 ± 2.8	−17.7 ± 7.9
Fibrinogen	13.4 ± 3.0	28.4 ± 3.7	19.4 ± 5.1	18.3 ± 3.8	22.8 ± 3.4	26.1 ± 7.4
IgG	2.2 ± 1.4	0.6 ± 2.3	−2.0 ± 2.2	11.2 ± 5.2	3.4 ± 0.1	−37.5 ± 13.4
Collagen type I	30.0 ± 0.9	20.5 ± 0.9	19.2 ± 2.1	45.9 ± 1.7	19.2 ± 4.2	−11.0 ± 7.1
Fetuin	10.2 ± 1.4	−0.5 ± 4.8	−4.7 ± 1.3	4.6 ± 2.9	7.1 ± 3.1	28.2 ± 11.0
Bovine serum albumin	4.1 ± 1.9	10.2 ± 3.1	7.8 ± 8.6	28.9 ± 4.9	6.2 ± 1.1	−17.8 ± 12.1
Carbohydrates						
D-(+)-Mannose	6.9 ± 4.7	19.1 ± 7.3	−2.8 ± 1.1	9.2 ± 3.6	1.7 ± 0.5	−30.5 ± 13.3
L-(−)-Fucose	11.8 ± 5.6	9.7 ± 3.0	4.5 ± 2.0	4.1 ± 2.2	2.1 ± 0.5	−36.7 ± 0.0
D-(−)-Ribose	4.5 ± 0.8	16.5 ± 13.4	8.6 ± 1.6	12.6 ± 5.2	3.7 ± 1.3	−52.8 ± 15.8
D-(−)-Sorbitol	10.1 ± 3.9	17.6 ± 6.9	5.3 ± 1.1	10.0 ± 9.3	1.9 ± 0.4	−20.9 ± 15.2
Sialic acid type VIII	7.1 ± 2.0	12.5 ± 5.5	−3.2 ± 1.7	3.5 ± 2.0	6.8 ± 2.1	−39.1 ± 3.8
N-Acetyl-D-galactosamine	7.7 ± 5.9	18.4 ± 1.0	5.6 ± 3.3	7.7 ± 0.2	4.0 ± 3.4	−50.2 ± 15.9
N-Acetyl-D-glucosamine	6.4 ± 1.5	12.6 ± 6.1	8.9 ± 1.7	12.4 ± 3.5	4.3 ± 5.6	−38.5 ± 14.6

[a] A 100-μl bacterial suspension containing approximately 10^9 cells was mixed with 100 μg of protein or carbohydrate (in a 100-μl volume) and incubated at room temperature for 1 h. ^{125}I-BLf was added, and the binding experiment was performed as described in the text.
[b] Percent inhibition values were from ^{125}I-BLf binding to cells in PBS in the absence of inhibitor.

ate decrease in BLf binding to all the species except *S. hominis*, which demonstrated increased binding. Such enhancement of BLf binding in certain species after a proteolytic treatment may be due to uncoiling of the receptor molecule or the exposure of additional binding domains.

The involvement of glycosidyl residues in the BLf-binding cell surface component of coagulase-negative staphylococci species was tested by two different carbohydrases and by physicochemical treatments. β-Glucosidase (which has an enzymatic mechanism similar to that of lysozyme) elicited a moderate decline in BLf binding, whereas β-galactosidase caused no decrease. After heating, cells of all the six species were moderately affected in their BLf-binding capacities. Finally, sodium periodate treatment affected BLf binding only in *S. epidermidis* and *S. warneri*. These data suggest that the bacterial cell surface components involved in the binding mechanism of BLf are probably proteinaceous in nature containing glycosidyl residues among isolates of *S. epidermidis* and *S. warneri*. In the remaining four species, the proteinaceous nature of the BLf binding component is evident; however, the involvement of glycosidyl residues is unclear.

TABLE 4. Effect of enzyme or physicochemical treatment of bacteria on ^{25}I-BLf binding

Treatment[a]	% ± SD Inhibition of ^{125}I-BLf binding[b]					
	S. epidermidis AF-9	*S. hominis* AF-93	*S. warneri* AF-101	*S. chromogenes* AD-1	*S. xylosus* AG-12	*S. hyicus* AC-166
Proteinases						
Pepsin	84.3 ± 6.9	45.2 ± 4.6	24.9 ± 11.6	12.0 ± 4.9	−26.6 ± 1.5	62.6 ± 12.3
Trypsin	37.5 ± 1.1	14.7 ± 5.9	50.0 ± 1.6	42.1 ± 7.3	0.9 ± 1.1	45.9 ± 3.8
Proteinase K	87.2 ± 2.3	88.8 ± 1.0	91.4 ± 1.9	74.4 ± 2.6	82.8 ± 2.7	70.2 ± 3.2
Pronase E	22.1 ± 5.1	−32.4 ± 12.6	13.6 ± 4.8	29.9 ± 6.7	57.4 ± 2.6	34.9 ± 7.2
Peptidase	31.6 ± 5.9	16.2 ± 11.6	48.3 ± 5.0	43.1 ± 3.3	−25.7 ± 7.2	47.3 ± 11.1
Glycosidases						
β-Glucosidase	49.8 ± 7.6	56.3 ± 1.9	67.8 ± 2.9	59.8 ± 2.8	25.8 ± 3.4	49.5 ± 0.5
β-Galactosidase	−2.4 ± 1.9	−4.0 ± 3.1	−4.0 ± 3.1	4.9 ± 3.1	−7.6 ± 5.7	11.9 ± 3.8
Heating						
80°C for 1 h	29.0 ± 2.0	32.7 ± 3.1	19.0 ± 3.7	12.0 ± 2.5	52.0 ± 2.0	31.8 ± 4.4
100°C for 1 h	54.2 ± 3.2	37.7 ± 2.4	47.4 ± 1.9	40.5 ± 3.6	38.2 ± 2.3	37.8 ± 5.6
Periodate	38.7 ± 0.7	−9.3 ± 4.4	25.9 ± 4.1	−24.3 ± 1.7	−17.9 ± 1.7	−18.2 ± 4.9

[a] Enzymes used: pepsin (EC 3.4.23.1); trypsin (EC 3.4.21.4); proteinase K (from *Tritirachium album*); pronase E (type XXV); peptidase (from porcine intestinal mucosa); β-glucosidase (EC 3.2.1.21); and β-galactosidase (EC 3.2.1.23). Enzymes were used at the concentrations given in the text. After treatment procedures, bacteria were washed in PBS and the cell density was readjusted to 10^{10} cells per ml. The ^{125}I-BLf-binding assay was performed as described in the text.
[b] Percent inhibition values were from ^{125}I-BLf binding to cells in PBS in the absence of inhibitor.

In summary, specific receptor-mediated binding of BLf to coagulase-negative staphylococci associated with IMIs was demonstrated. This BLf binding seems distinct from the previously described binding mechanisms for fibronectin and collagen. Carbohydrate inhibition profiles indicate that the interaction of BLf with coagulase-negative staphylococci is different from the BLf binding to cells of the reticuloendothelial system.

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PHYSIOLOGY AND MANAGEMENT

Comparison Between Lactoferrin and Subepithelial Matrix Protein Binding in *Staphylococcus aureus* Associated with Bovine Mastitis

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ABSTRACT

Staphylococcus aureus strains (n = 100) isolated from bovine mastitis were classified according to the presence of capsular polysaccharide serotype 5 (n = 46), type 8 (n = 26), and non-5/8 (n = 28). Strains from each type were tested for protein interaction in a ^{125}I-labeled ligand binding assay. A majority of type 5 and type 8 strains showed a higher degree of binding to lactoferrin, fibronectin, and IgG than the non-5/8 strains. Fibrinogen binding was low in all serotypes. Most of the type 5 and non-5/8 strains bound less than 10% laminin, whereas type 8 strains bound laminin in the 11 to 20% range. Non-5/8 strains significantly differed from type 5 in lactoferrin, fibronectin, fibrinogen, and IgG and also from type 8 in fibrinogen and IgG binding. The differences in protein binding between type 5 and type 8 were nonsignificant. The degree of lactoferrin binding in all types positively correlated with laminin binding. Lactoferrin and fibrinogen bindings were correlated in type 5 and type 8 strains. Lactoferrin and fibronectin bindings were correlated only in type 5 strains. These data suggest that bovine lactoferrin binding is common and associated with subepithelial matrix protein interactions in certain serotypes of *S. aureus*. (Key words: lactoferrin, matrix proteins, capsular polysaccharide, *Staphylococcus aureus*)

Abbreviation key: bFg = bovine fibrinogen, bFn = bovine fibronectin, bLf = bovine lactoferrin, CP = capsular polysaccharide, hLf = human lactoferrin, Lf = lactoferrin, Lm = laminin, MAb = monoclonal antibody, PBS = phosphate-buffered saline.

INTRODUCTION

Lactoferrin (Lf) is an important component of various mammalian host defense processes. It is an iron-binding, acute-phase protein secreted by exocrine glands (14). A role for Lf has been suggested in the amplification of inflammatory response, phagocytosis, regulation of granulocyte-monocyte colony-stimulating factors, and antibody production (2). Furthermore, cells of the reticuloendothelial system possess specific receptors for Lf (1, 15, 28).

Bovine Lf (bLf) levels significantly rise during intramammary infections (11). Bovine Lf is released by polymorphonuclear leukocytes, the predominant inflammatory cells in milk (6, 17), and the bLf:citrate ratio is critical

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in the development of bovine mastitis (3, 6). However, in certain microorganisms, the antimicrobial effect is dependent on Lf adsorption to the bacterial surface (5).

Staphylococcus aureus is a frequent etiological agent of bovine mastitis and one of the most difficult pathogens to control. Virulence factors of the bacteria as well as the functional transition of the bovine udder contribute to the onset and progression of intramammary infection (24, 27). Capsular polysaccharide (CP) is an extracellular virulence factor of *S. aureus* (25). A majority of *S. aureus* mastitis strains belong to CP serotypes 5 (CP-5) and 8 (CP-8) (26). Specific binding of bLf to strains of *S. aureus* and to six commonly occurring coagulase-negative staphylococci associated with bovine intramammary infections has been reported (20, 21). An Lf-binding protein has been recently isolated from a human clinical isolate of *S. aureus* (19).

Staphylococcus aureus that cause human and animal infections bind to various plasma and subepithelial matrix proteins such as IgG, fibronectin, fibrinogen, and laminin (Lm). These interaction mechanisms have been suggested to anchor bacteria to host tissues (13). However, the distribution of these different bacterial receptors among various CP types has not been reported. In addition, a correlation between bLf and other protein binding has not been described. The purposes of the present study were 1) to determine the degree of binding of bLf, bovine fibronectin (bFn), bovine fibrinogen (bFg), bovine IgG, and Lm among different *S. aureus* CP types and 2) to test for correlations between bLf and other proteins within different CP type strains.

MATERIALS AND METHODS

Bacteria

A total of 100 *S. aureus* strains isolated from acute and chronic bovine mastitis tested for bLf binding in an earlier study (20) were included. Bacterial strains were stored in glycerol at –80°C, revived, and subsequently cultured on blood agar base number 2 (Oxoid Limited, Basingstoke, Engl.) supplemented with 10% human blood at 37°C for 18 h. For ^{125}I-labeled protein binding experiments, bacterial cells grown on blood agar were harvested, washed in phosphate-buffered saline (PBS), pH 7.2, and the density of the suspension was adjusted to ~10^{10} cells/ml by measuring the optical density of the suspension at 540 nm.

Chemicals

Bovine Lf (purified from bovine milk) was kindly provided by Hans Burling, Swedish Dairies Association (Malmö, Sweden). Both bLf (from bovine colostrum) and human Lf (hLf from milk) were purchased from US Biochemicals Corp. (Cleveland, OH). Bovine Lf protein preparations obtained from these two different sources demonstrated similar binding properties. Bovine fibronectin was purified from bovine plasma according to Veunto and Vaheri (29). Bovine fibrinogen (lot 58F-9484) was purchased from Sigma Chemicals Co. (St. Louis, MO). Bovine IgG was obtained from Dakopatts AB (Hägersten, Sweden). Laminin from basement membrane of the Engelbreth-Holm-Swarm transplantable mouse tumor (lot 89-0495) was purchased from Collaborative Research, Inc. (Bedford, MA). Chemicals used in the preparation of buffers were of analytical grade.

Protein Binding Assay

Bovine Lf, bFn, bFg, IgG, and Lm were labeled according to a modified chloramine T method with Na(^{125}I) (specific activity 629 GBq/mg) (DuPont Scandinavia AB, Stockholm, Sweden) using Iodobeads (Pierce Chemicals Co., Rockford, IL) (16). Binding assays were performed as described earlier by Naidu et al. (20, 21). Briefly, 10^9 bacterial cells (in .1 ml of PBS) were mixed with .1 ml of ^{125}I-labeled protein (radioactivity measurement was adjusted in the range of 2 to 2.5 × 10^4 cpm when diluted in cold PBS). After 1-h incubation, 2 ml of ice-cold PBS (containing .05% Tween 20) were added to tubes and centrifuged at 4500 × *g* for 15 min. After aspirating the supernate, the radioactivity bound to the bacterial pellet was measured in a gamma counter (LKB Wallac Clinigamma 1272, Turku, Finland). Samples were always tested in triplicate, and each experiment was repeated at least twice unless stated otherwise.

Capsular Polysaccharide Typing

Staphylococcus aureus isolates from bovine mastitis were cultivated on Columbia agar (Difco Laboratories, Detroit, MI) at 37°C overnight. Bacterial cells were harvested, suspended in 2 ml of PBS, and autoclaved at 121°C for 30 min. After centrifugation, supernate was collected and tested for CP-5 and CP-8 by a two-step inhibition ELISA technique described by Boutonnier et al. (4). Then CP-5 and CP-8 were purified according to Fournier et al. (9, 10), and corresponding monoclonal antibodies (MAb) were raised according to Hochkeppel et al. (12). In brief, the CP typing was performed in flat-bottomed microplates (Nunc, Roskilde, Denmark) coated with purified CP-5 or CP-8 (.1 μg/well in .1-ml volume). After incubation at 37° C for 1 h, plates were washed with PBS containing .05% Tween-20. Free sites in the wells, if any, were blocked with .5% gelatin (Prolabo, Paris, France). After incubation at 37°C for 60 min or at 4°C overnight, plates were washed with PBS-Tween. In the first step, a .1-ml volume of test samples (supernatants of autoclaved bacteria) and .1 ml of MAb (concentration adjusted photometrically to an optical density of .2 or .5 at 492 nm) were added to a gelatin-blocked plate and incubated at 37°C for 1 h and then at 4°C overnight. In the second step, .1-ml volumes of these incubated reaction mixtures were transferred well to well into a fresh gelatin-blocked plate. After incubation at 37°C for 1 h, plates were washed with PBS-Tween, and .1 ml of an anti-mouse peroxidase-conjugated IgG (H and L chain specific) (Diagnostic Pasteur, Marnes La Coquette, France) was added and incubated at 37°C for 45 min. After washing, .1 ml of *o*-phenylenediamine enzyme substrate (.4 mg/ml solution in .1 *M* citrate buffer, pH 5.2, containing .03% hydrogen peroxide) was added. After 10 min at room temperature, enzyme hydrolysis was terminated by adding 3*N* hydrochloric acid (.05 ml per well). Color reaction was measured at 492 nm with an El 311 automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). During the assay, wells receiving only PBS served as negative controls, and a titration of purified individual CP type was performed to determine the sensitivity of the assay. Values corresponding to 50% inhibition or more were defined as CP positive.

TABLE 1. Frequency distribution of *Staphylococcus aureus* strains belonging to different capsular polysaccharide (CP) types according to the magnitude of ^{125}I-labeled protein binding.

Protein	Binding range	Number of strains			
		Total	CP-5	CP-8	CP non-5/8
	(%)	(n = 100)	(n = 46)	(n = 26)	(n = 28)
Lactoferrin	0 to 10	7	2	3	2
	11 to 20	9	2	2	5
	21 to 30	19	8	2	9
	≥ 31	65	34	19	12
Fibronectin	0 to 10	3	0	0	3
	11 to 20	13	1	3	9
	21 to 30	28	15	8	5
	≥ 31	56	30	15	11
Fibrinogen	0 to 10	13	1	1	11
	11 to 20	56	27	16	13
	21 to 30	26	16	8	2
	≥ 31	5	2	1	2
IgG	0 to 10	9	1	2	6
	11 to 20	11	3	3	5
	21 to 30	9	4	2	3
	≥ 31	71	38	19	14
Laminin	0 to 10	55	26	11	18
	11 to 20	45	20	15	10
	21 to 30	0	0	0	0
	≥ 31	0	0	0	0

TABLE 2. ^{125}I-labeled protein binding among mastitis strains of *Staphylococcus aureus* belonging to different capsular polysaccharide (CP) serotypes.

Protein	Percentage binding					
	CP-5		CP-8		CP non-5/8	
	X	SE	X	SE	X	SE
Lactoferrin	37*	1.8	33	2.6	29*	2.6
Fibronectin	37**	1.9	33	2.4	28**	3.0
Fibrinogen	19***	1.0	19**	1.2	16**,***	2.1
IgG	49***	2.4	48**	4.1	33**,***	4.0
Laminin	10	.6	10	.8	9	.8

*$P \leq .05$.

**$P \leq .01$.

***$P \leq .001$.

Data Analysis

Mann-Whitney Wilcoxon U test was used to assess the significance of differences in protein binding among strains belonging to various CP types. For each CP type, the bLf binding also was compared with other protein bindings using the Spearman rank correlation coefficient test. Probability values less than or equal to .05 were considered indicative of significant differences. The Stat View™ (Brain Power Inc., Calabasas, CA) program was used for statistical analysis.

RESULTS

Out of 100 bovine mastitis isolates of *S. aureus* tested, 46 belonged to CP-5 and 26 to CP-8, whereas the remaining 28 isolates did not react with either of the antisera (CP non-5/8). The frequency distribution of various CP types of *S. aureus* according to the magnitude of different ^{125}I-labeled protein binding is shown in Table 1. The bLf, bFn, and IgG binding was greater than 31% for a majority (more than 50%) of isolates belonging to CP-5 or CP-8; bFg binding was less than 20% for all CP types. A majority of CP-5 and CP non-5/8 strains demonstrated Lm binding less than 10%, whereas CP-8 strains were in the 11 to 20% binding range.

The mean percentage of proteins for each CP type is shown in Table 2. Strains belonging to CP-5 significantly differed from CP non-5/8 in bLf, bFn, bFg, and IgG but not in Lm binding. However, CP-8 strains significantly differed from CP non-5/8 only in bFg and IgG binding. No significant differences were found in binding of any of the proteins between CP-5 and CP-8 strains.

Further comparison of the degree of binding of bLf and the other proteins revealed a significant correlation between bLf and Lm in all CP types (Figure 1). Both CP-5 and CP non-5/8 demonstrated the highest correlation coefficient ($P = .49$), followed by CP-8 ($P = .46$). Bovine fibrinogen binding was significantly correlated with bLf binding only in CP-5 ($P = .30$) and CP-8 ($P = .40$) (Figure 2). A significant correlation was found between bLf and bFn binding only with CP-5 strains ($P = .32$) (Figure 3). None of the other comparisons revealed significant positive correlations.

DISCUSSION

The distribution of CP serotypes among bovine mastitis strains of *S. aureus* in the present study was similar to that reported by Poutrel et al. (26). Thus, the material might be considered representative for CP distribution. A majority of strains in all CP types demonstrated high to moderate bLf binding. This degree of binding is comparable with hLf binding in *S. aureus* strains associated with low invasive or localized human skin infections (22). The bLf binding was highest in CP-5. Interestingly, CP-5 has been shown to constitute more than 50% of the bovine mastitis *S. aureus* isolates (26). A similar pattern was observed with bFn binding in these different CP types.

Bovine fibrinogen and IgG binding frequency was significantly higher among CP-5

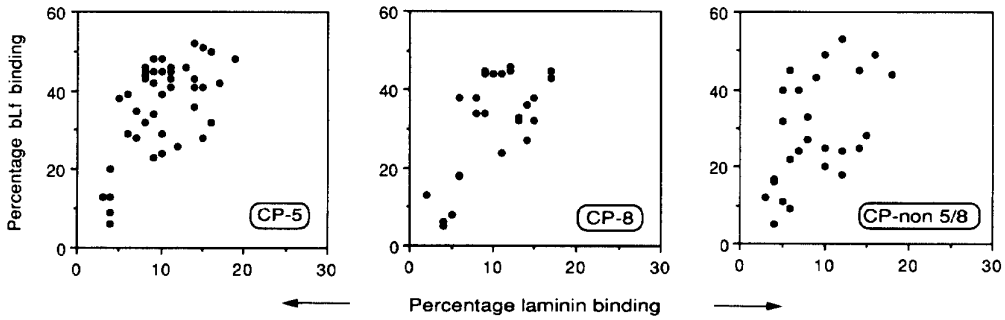

Figure 1. Scatterplot of bovine lactoferrin (bLf) versus murine laminin binding for *Staphylococcus aureus* strains belonging to capsular polysaccharide (CP) groups CP-5, CP-8, and CP-non 5/8. Growth and ^{125}I-labeled protein binding assay conditions were comparable for all strains tested. Scatterplots are shown only for significantly correlated (Spearman rank correlation coefficient test) bindings.

and CP-8 compared with CP non-5/8. However, about 6% of strains from CP-5 and CP-8 showed bFg binding less than 10%. The corresponding value for IgG was about 10%. On the contrary, about 35% of CP non-5/8 strains bound less than 10% bFg; the corresponding value for IgG was about 20%. These two proteins are used in latex agglutination tests for rapid identification of *S. aureus* isolates (7). A threshold of less than 11.5% of relative protein binding has been suggested to elicit a negative agglutination reaction (23). Thus, this rapid identification test is useful for bovine mastitis-associated strains of only CP-5 and CP-8. Fournier et al. (8) reported that a majority of human clinical isolates of *S. aureus* unidentified by similar agglutination methods belong to CP-5. This finding is not in accordance with bovine mastitis strains in the present study. The discrepancy may be due to the differences in methods, such as culture growth conditions that are shown to influence the expression of bacterial cell surface components (18, 23).

Results on Lm interaction further indicated similarities in the expression of binding to plasma and matrix proteins between human and bovine strains of *S. aureus*. The low degree of binding found with this protein is comparable with a previously observed pattern in human clinical isolates of *S. aureus* (22).

Distinct differences were found in correlations between bLf and other protein binding among various CP types. Thus, at phenotypic level, the expression of bLf binding seems to be related to the bFn, bFg, and Lm binding in CP-5; to bFg and Lm in CP-8; and only to Lm in CP non-5/8. Among these glycoproteins, only bLf has antimicrobial properties (14) that might affect the bacterial metabolism and, probably, the expression of cell surface properties. Lactoferrin has been shown to inhibit adherence of *Streptococcus mutans* to hydroxyapatite (30). We have also observed that the fibronectin and collagen binding ability of *S. aureus* is reduced in the presence of milk (18) or bLf (unpublished data). The strains of *S. aureus* that cause bovine mastitis most frequently, i.e., CP-5 and CP-8, show a positive correlation between bLf binding and interaction with subepithelial matrix proteins. This might indicate an important mechanism that

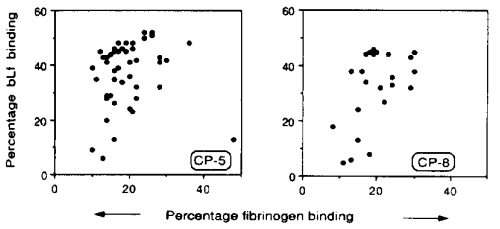

Figure 2. Scatterplot of bovine lactoferrin (bLf) versus bovine fibrinogen binding for *Staphylococcus aureus* strains belonging to capsular polysaccharide (CP) groups CP-5 and CP-8. Growth and ^{125}I-labeled protein binding assay conditions were comparable for all strains tested. Scatterplots are shown only for significantly correlated (Spearman rank correlation coefficient test) bindings.

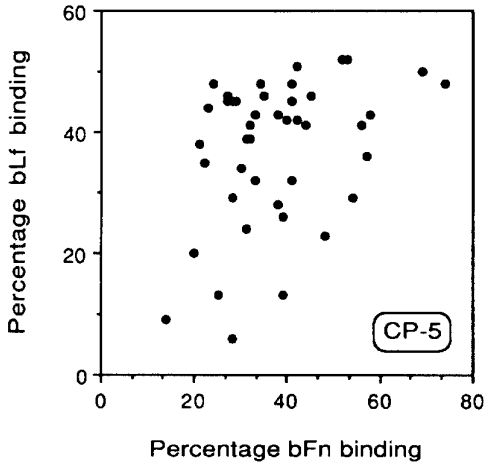

Figure 3. Scatterplot of bovine lactoferrin (bLf) versus bovine fibronectin (bFn) binding for *Staphylococcus aureus* strains belonging to capsular polysaccharide (CP) group CP-5. Growth and ^{125}I-labeled protein binding assay conditions were comparable for all strains tested. Scatterplots are shown only for significantly correlated (Spearman rank correlation coefficient test) bindings.

enables bacteria to adhere to udder tissue in the presence of elevated bLf concentrations during mastitis.

CONCLUSIONS

In summary, bLf binding was common among isolates of *S. aureus* from bovine mastitis. The most frequently encountered CP type in bovine intramammary infections, CP-5, demonstrated higher bLf and bFn binding compared with other CP types. Moreover, the degree of bLf binding in the same CP type was positively correlated with the binding of the subepithelial matrix proteins bFn, bFg, and Lm. The clinical implications of these findings remain to be elucidated.

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