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Specific binding of lactoferrin to *Escherichia coli* isolated from human intestinal infections

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The degrees of human lactoferrin (HLf) and bovine lactoferrin (BLf) binding in 169 *Escherichia coli* strains isolated from human intestinal infections, and in an additional 68 strains isolated from healthy individuals, were examined in a ^{125}I-labelled protein binding assay. The binding was expressed as a percentage calculated from the total labelled ligand added to bacteria. The HLf and BLf binding to *E. coli* was in the range 3.7 to 73.4% and 4.8 to 61.6%, respectively. Enterotoxigenic strains demonstrated a significantly higher HLf binding (median = 19%) than enteropathogenic, enteroinvasive, enterohaemorrhagic strains or normal intestinal *E. coli* isolates (medians 6 to 9). Enteropathogenic strains belonging to serotypes O44 and O127 demonstrated significantly higher HLf binding compared to O26, O55, O111, O119 and O126. No significant differences in the degree of HLf or BLf binding were found between aerobactin-producing and non-producing strains. The interaction was further characterized in a high Lf-binding EPEC strain, E34663 (serotype O127). The binding was stable in the pH range 4.0 to 7.5, did not dissociate in the presence of 2M NaCl or 2M urea, and reached saturation within two h. Unlabelled HLf and BLf displaced the ^{125}I-HLf binding to E34663 in a dose-dependent manner. Apo- and iron-saturated forms of Lf demonstrated similar binding to E34663. Among various unlabelled subepithelial matrix proteins and carbohydrates tested (in 10^4-fold excess) only fibronectin and fibrinogen caused a moderate inhibition of ^{125}I-HLf binding. According to Scatchard plot analysis, 5,400 HLf-binding sites/cell, with an affinity constant (K_a) of 1.4×10^{-7} M, were estimated in strain E34663. These data establish the presence of a specific Lf-binding mechanism in *E. coli*.

Key words: Lactoferrin; *Escherichia coli*; specific binding; gastroenteritis.

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Escherichia coli, a member of the normal intestinal flora (14), is also a frequent aetiological agent of intestinal infections, in particular, infantile gastroenteritis (28, 29, 61). The latter infection has been estimated to affect 500 million children below five years of age, with an annual mortality rate of 1 to 4% worldwide (51, 56). Based on the disease manifestaion, *E. coli* are categorized into enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC) and enterohaemorrhagic (EHEC) groups (29).

Milk is the natural first feed for newborns, and a main ingredient in the diet of many children and also adults. Breast-fed infants are better protected against gastroenteritis than infants fed with cow milk formulae (6, 8, 9), and milk has been found to inhibit the growth of certain *E. coli* (46, 50, 57, 59).

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Milk contains various antimicrobial components, including lactoferrin (Lf) (2 to 7 mg/ml) (36, 48). Lf is also found at the mucosal surface as a secretory product of polymorphonuclear leukocytes and of various exocrine glands (4, 13, 33, 35). This protein is suggested as an important non-specific defence factor and its level rises significantly during inflammation or endotoxic shock (15, 63). Lf has been shown to inhibit the growth of *E. coli* and certain other microorganisms *in vitro* (6, 20, 47, 57). The antimicrobial property is mainly attributed to the iron-chelating capacity of Lf, leading to iron deprivation of bacteria (20, 34). However, some pathogens may overcome Lf-mediated iron deficiency by producing siderophores (7, 38). In *E. coli*, production of the siderophore aerobactin has been suggested to contribute to the bacterial survival in a low-iron acidic environment (19).

Other studies have indicated that the antimicrobial mechanism of Lf action is more complex (3, 16, 60). Lf mediates bacterial uptake and killing during phagocytosis (2, 10, 30). Specific receptor-mediated interaction of Lf with monocytes, macrophages, polymorphonuclear leukocytes, B lymphocytes and activated T lymphocytes has also been described (5, 31, 49, 64). The adsorption of Lf to bacteria has been suggested to enhance the antimicrobial effect (12). Recently, specific receptors for Lf have been described on various extraintestinal pathogens (1, 27, 38, 41, 42, 44, 53, 54). However, our knowledge of the specific interaction of Lf with *E. coli* is limited. *Steel* (58) has reported adsorption of a trace amount of Lf to one enteropathogenic *E. coli* strain incubated with colostral whey. Since this binding did not dissociate with 0.1 M sodium acetate and other whey proteins were absent in immunoelectrophoresis, the author speculated on a probable specific nature of the interaction. However, no clear evidence for a specific binding of Lf to *E. coli* has so far been found.

This study was conducted to investigate and characterize a specific binding of Lf to *E. coli*. In addition, we have sought a correlation between Lf binding and different clinical groups or serotypes of *E. coli*. The possible relationship between aerobactin production and Lf binding was also tested.

MATERIALS AND METHODS

Bacteria

A total of 237 *E. coli* strains belonging to EPEC ($n = 112$), ETEC ($n = 27$), EIEC ($n = 25$) and EHEC ($n = 5$) groups and normal flora ($n = 68$) were tested for binding to ^{125}I-labelled HLf or BLf. Of the above, 90 strains (EPEC, $n = 80$; and ETEC, $n = 10$) were isolated from children (< 5 years of age) admitted with symptoms of acute diarrhoea to the Ethio-Swedish Paediatric Clinic, Addis Abeba, Ethiopia during 1979, and were characterized in a previous study (61). The remaining 79 strains belonging to different clinical groups were from the existing culture collection at the National Institute of Hygiene, Budapest, Hungary. Isolates belonging to normal faecal flora were from Sweden ($n = 23$) and Hungary ($n = 45$). All the isolates represent epidemiologically well-distributed populations. Bacteria were grown on colonization factor antigen (CFA) agar (17) at 37 C for 18 h. Cells were harvested, washed with phosphate-buffered saline (PBS), pH 7.2, and the density was optically (at 540 nm) adjusted to 10^{10} bacteria/ml in PBS.

Chemicals

Human lactoferrin (HLf; lot 63541) purified from milk was purchased from US Biochemicals Corp., Cleveland, OH. Bovine lactoferrin purified from whey was kindly provided by Dr *Hans Burling*, Swedish Dairies Association, Malmö, Sweden. Both protein preparations were homogenous in ion-exchange (Mono-Q column, Pharmacia, Uppsala, Sweden) or molecular-sieve (TSK G4000 SW, LKB produkter AB, Bromma, Sweden) high-performance liquid chromatography. Fibronectin was purified from human plasma according to *Vuento & Vaheri* (65). Laminin and collagen type IV purified from basement membrane of the Engelbreth-Holm-Swarm transplantable tumour were purchased from Collaborative Research Inc., Bedford, MA. Vitrogen 100[TM] collagen (containing 95% type I and 5% type III collagens) was purchased from Collagen Corporation Inc., Palo Alto, CA. Fibrinogen (human plasma), transferrin (from human serum), α-1 acid glycoprotein (human), D(+)galactose, D(+)mannose, L(−)fucose, sialic acid type VI, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine were purchased from Sigma Chemical Co., St. Louis, MO. All chemicals used in the preparation of buffer solutions were of analytical grade.

^{125}I-lactoferrin binding assay

Proteins were labelled with Na(^{125}I) (DuPont Scandinavia AB, Stockholm, Sweden) using Iodobeads (Pierce Chemical Co., Rockford, IL) (32) to a specific activity of 0.15 MBq/μg of HLf, and 0.16 MBq/μg of BLf. The labelled proteins were tested and confirmed for homogeneity by autoradiography.

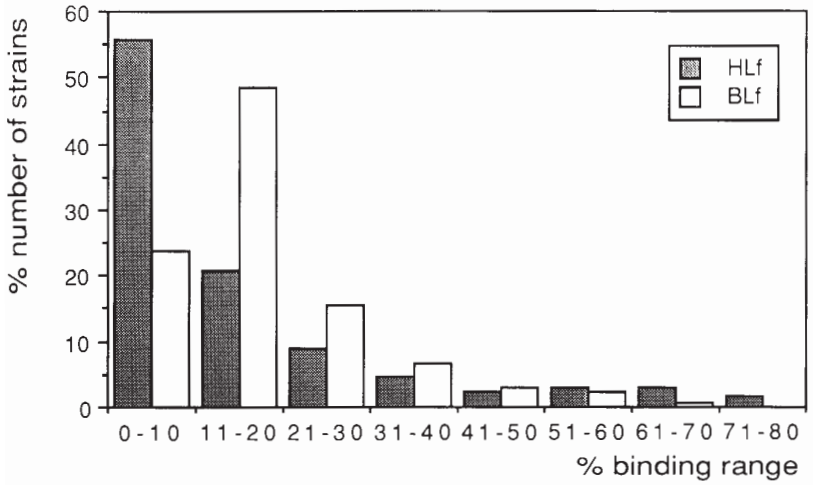

Fig. 1. Frequency distribution of *E. coli* strains according to the degree of binding to HLf or BLf. Binding was expressed as a percentage calculated from the total labelled ligand added to the bacteria. Binding below 10% was considered negative.

Binding assays were performed as described earlier by *Naidu et al.* (40, 41). Briefly, 10^9 bacteria (in 0.1 ml PBS) were incubated with 0.1 ml of ^{125}I-labelled HLf or BLf ($\sim$8 ng) at room temperature for one h. The binding reaction was terminated by adding 2 ml of ice-cold PBS (containing 0.05% Tween 20 to reduce non-specific hydrophobic interactions) and centrifuged at 4,500 $\times g$ 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). Background radioactivity (from incubation mixtures without bacteria) was deducted, and the binding was expressed as a percentage calculated from the total labelled ligand added to the bacteria. Binding below 10% was considered negative. Strains E34663 and HH45 served as positive (60%) and negative (5%) binding controls, respectively. Samples were always tested in triplicate and each experiment was repeated at least twice.

^{125}I-HLf binding displacement with unlabelled HLf or BLf

Strain E34663 (EPEC isolate belonging to serotype O127), a high HLf and BLf binder, was selected for the characterization experiments. For determining the reversible nature of the binding, increasing amounts (0.01–300 µg) of unlabelled HLf or BLf (both iron-saturated and apo forms) in 0.1 ml PBS were mixed with an equal volume of PBS containing $\sim$8 ng of ^{125}I-HLf. Approximately 10^9 bacteria were added to this solution (final volume 0.3 ml) and the mixture was incubated at room temperature for one h. Furthermore, increasing amounts (0.01 to 10 µg) of labelled HLf were incubated with 10^9 bacteria under the above conditions, either in the absence (total binding) or in the presence (non-specific binding) of 50-fold excess (calculated on the basis of the binding-displacement data) of unlabelled HLf. The derived specific binding data were analysed in a Scatchard plot (52).

^{125}I-HLf binding inhibition by various proteins or carbohydrates

The competitive inhibitory effect of different proteins, such as transferrin, fibronectin, fibrinogen, collagen type I, collagen type IV, laminin and α-1 acid glycoprotein, and of different carbohydrates, such as N-acetylneuraminic acid type VI (from *E. coli*), N-acetylglucosamine, N-acetylgalactosamine, D(+) galactose, D(+) mannose and L(−) fucose, was examined on ^{125}I-HLf binding. A 0.1 ml volume of 0.1% protein or carbohydrate solution was mixed with approximately 10^9 bacteria (in a 0.1 ml volume) and kept at room temperature for 30 min. Finally, 0.1 ml ($\sim$8 ng) of ^{125}I-HLf was added and the mixture was incubated at room temperature for one h. ^{125}I-HLf binding measurements were made as described above.

Aerobactin production

A previously described bioassay (19, 45) was used for the qualitative determination of aerobactin production. The bioassay medium contained 2,2′-dipyridyl (160 µM) and nitrilotriacetic acid (20 µg/ml). Approximately 10^6 cells/ml of the aerobactin-requiring *E. coli* LG1522 were uniformly spread on an agar surface and allowed to dry at room temperature for 30 min. Test strains were spot inoculated with sterile toothpicks and incubated at 37 C for 16 h. A test strain was considered an aerobactin producer if growth of strain LG1522 was observed around the inoculation spot. Aerobactin-producing (n = 13) and aerobactin-non-producing (n = 15) strains of *E. coli* were tested for Lf binding.

Statistical analyses

For comparisons of HLf or BLf binding between different clinical groups or between different serotypes of EPEC, the Mann-Whitney U test was used. Significance of differences in HLf or BLf binding between aerobactin-producing and non-producing strains was assessed using Student's t test. P values

TABLE 1. *Per cent binding of ^{125}I-labelled HLf or BLf to* E. coli *strains belonging to different clinical groups and normal flora*

Group	No. of strains	Median (min-max)	
		HLf	BLf
Enteropathogenic *E. coli*	112	9 (4–73)	14 (5–62)
Enterotoxigenic *E. coli*[a]	27	19 (5–70)	18 (6–55)
Enteroinvasive *E. coli*[b]	25	7 (4–15)	14 (7–29)
Enterohaemorrhagic *E. coli*[c]	5	6 (6–9)	10 (9–18)
E. coli (normal flora)[d]	68	9 (3–35)	10 (2–33)

[a] ETEC strains were positive for heat-labile or heat-stable enterotoxin
[b] EIEC strains were positive for Shigella keratoconjunctivitis (Sereny test)
[c] EHEC strains were positive for verotoxin production
[d] Isolated from healthy individuals with no symptoms of diarrhoea.

less than or equal to 0.05 were considered indicative of significant differences.

RESULTS

The distribution of 169 *E. coli* strains according to the degree of ^{125}I-HLf and ^{125}I-BLf binding is shown in Fig. 1. The degree of HLf binding among strains ranged from 4 to 73%, and a majority demonstrated binding below 10%. For BLf, the degree of binding ranged from 5 to 62%, and strains were frequently above the 10% binding level.

Among the four clinical groups, ETEC strains demonstrated a high HLf-binding capacity and differed significantly from EPEC ($p<0.01$), EIEC ($p<0.001$), EHEC ($p<0.01$) strains or isolates from faeces of normal individuals ($p<0.001$) (Table 1). Such significant differences in HLf binding were not evident between other groups. Neither did the degree of BLf binding significantly differ among the five groups.

The degree of HLf or BLf binding to seven serotypes of EPEC strains, i.e. O26 ($n = 10$), O44 ($n = 7$), O55 ($n = 17$), O111 ($n = 34$), O119 ($n = 10$), O126 ($n = 10$), and O127 ($n = 8$), is shown in Fig. 2. When binding capacities were compared between different serotypes, O44 and O127 strains demonstrated significantly higher HLf binding than the remaining five serotypes. For BLf, only serotype O44 demonstrated significantly higher binding compared to other serotypes (except O127).

Aerobactin-producing ($n = 13$) strains demonstrated a mean binding of 9% and 16% for HLf and BLf, respectively. The corresponding mean values for aerobactin-non-producing ($n =$

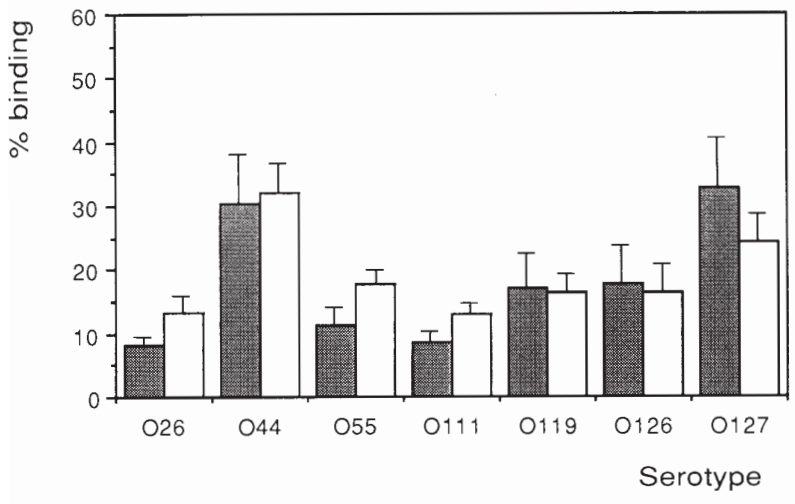

Fig. 2. Mean and SEM of HLf (filled bars) or BLf (open bars) binding to different EPEC serotypes.

15) strains were 10% and 13%. No significant difference in the degree of binding was found between the groups (Fig. 3).

A high HLf- (73.4%) and BLf-binding (55.6%) *E. coli* strain E34663, belonging to EPEC serotype O127, was selected for characterization studies. The binding of ^{125}I-HLf to E34663 was stable (ranging from 70.1 to 72.1%) over the pH range 4.0 to 7.5 and did not dissociate in the presence of 2M NaCl or 2M urea. The kinetics of HLf binding was time-dependent and reached complete saturation within 110 min (Fig. 4). ^{125}I-HLf binding to strain E34663 was reversible, and was effectively displaced by unlabelled HLf or BLf in a dose-dependent manner. The displacement kinetics were similar for both unlabelled ligands (Fig. 5). Apo- and iron-saturated forms of Lf showed similar binding and displacement kinetics. Other proteins and carbohydrates also affected the ^{125}I-HLf binding to a varying extent (Table 2). However, this effect was considerably lower compared to unlabelled HLf or BLf.

The extent of non-specific ^{125}I-HLf interaction with E34663 was about 10%. The magnitude of specific binding was estimated at different concentrations of HLf (Fig. 6a), and the data were further analysed in a Scatchard plot (Fig. 6b). The plot was linear and implied a single type of binding mechanism with an affinity constant (K_a) of 1.4×10^{-7} M. The estimated number of specific HLf binding sites was 5,400 per cell.

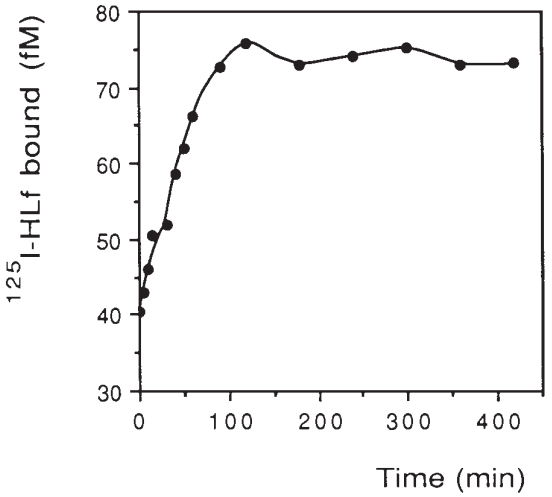

Fig. 4. Time course of ^{125}I-HLf binding to *E. coli* strain E34663. Bacteria (10^9 cells) were incubated with I-HLf at time intervals indicated. Values represent the radioactivity bound to bacteria after deducting the background (radioactivity) measurements.

DISCUSSION

In the present study we showed a binding of both HLf and BLf to diarrhoeagenic *E. coli*. Among different categories, ETEC strains ex-

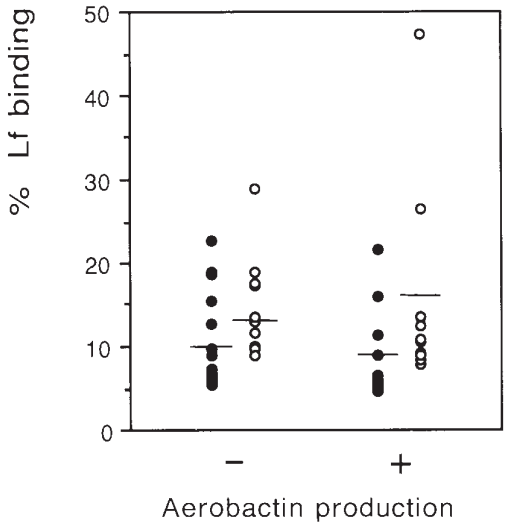

Fig. 3. HLf (●) and Blf (○) binding to aerobactin-producing (+) and non-producing (−) *E. coli* strains.

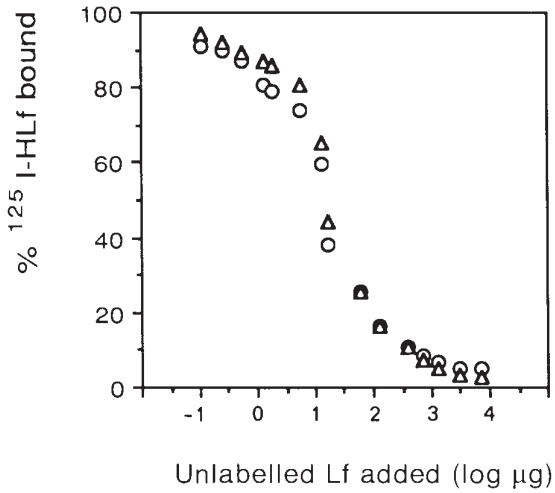

Fig. 5. Displacement of ^{125}I-labelled HLf binding as a function of unlabelled HLf (○) or BLf (△) added.

hibited a significantly higher HLf binding compared to EPEC, EIEC and EHEC strains, and isolates from healthy individuals. The high Lf-binding ETEC strains may generally be considered as less invasive than the other groups (61, 66). We have recently shown that low-invasive (pyoderma or toxic shock syndrome) strains of *Staphylococcus aureus* exhibit a significantly higher HLf binding than the high-invasive (endocarditis or septicaemia) strains (43). Whether a similar correlation exists for *E. coli* is not known, since the invasive property of our strains was not examined. Adsorption to the bacterial surface may damage the outer cell membrane and enhance the antimicrobial effects of Lf (12, 16). Therefore, the low Lf-binding capacity may protect the normal intestinal *E. coli* flora from the antimicrobial action of Lf.

The most frequent classical EPEC (class I, and EAF-positive) serotypes O26, O55, O111, O119 and O126 (28, 29, 62) demonstrated a low magnitude of Lf binding, with the exception of serotype O127. On the other hand, Lf binding was significantly higher in the less frequent EPEC serotype (class II, rarely EAF-positive) O44 (28, 29, 62). Thus, the data suggest that Lf binding in EPEC strains might be serotype-associated.

Iron is acquired in most *E. coli* through the production of enterochelin and/or aerobactin,

TABLE 2. *Effect of various proteins and carbohydrates on* ^{125}*I-HLf (~8 ng) binding to* E. coli *strain E34663*

Inhibitor (0.1 mg/ml)	% Inhibition Mean ± SEM[a]
Lactoferrin (human)	93 ± 2
Lactoferrin (bovine)	94 ± 2
Transferrin	13 ± 3
Fibronectin	37 ± 1
Fibrinogen	32 ± 6
Type-I collagen	−27 ± 9
Type-IV collagen	−11 ± 1
Laminin	16 ± 1
α-1 acid glycoprotein (human)	−11 ± 2
N-acetylneuraminic acid type VI	−4 ± 3
N-acetylglucosamine	−9 ± 4
N-acetylgalactosamine	0 ± 0
D(+) galactose	3 ± 1
D(+) mannose	−9 ± 1
L(−) fucose	13 ± 1

[a] Based on four experiments. Per cent inhibition (positive value) or enhancement (negative value) was calculated from ^{125}I-HLf binding to cells in PBS.

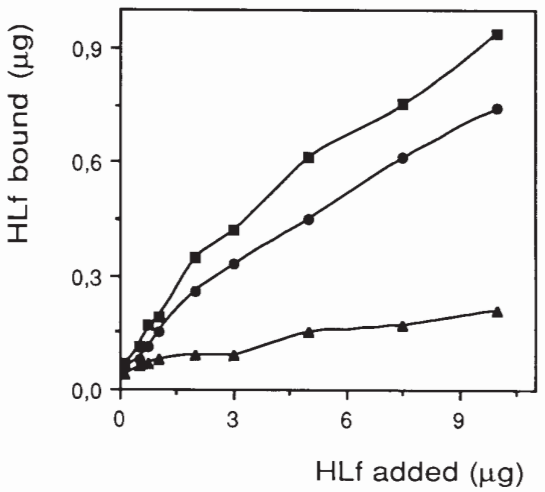

Fig. 6a. Binding of increasing amounts of ^{125}I-HLf to bacteria in the absence (■ – total binding) or presence (▲ – non-specific binding) of 50-fold excess of unlabelled HLf. Specific binding (●) was calculated at each point by subtracting the corresponding non-specific HLf binding.

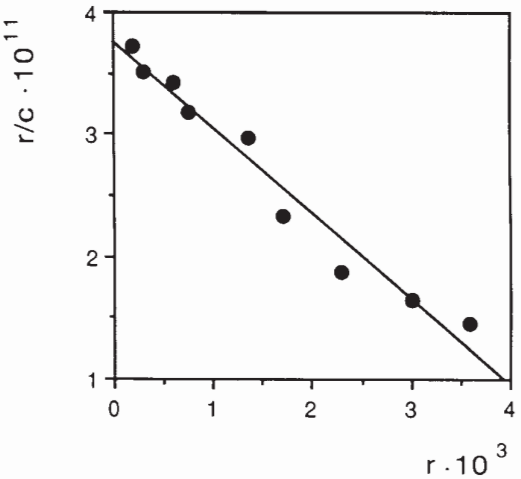

Fig. 6b. Scatchard plot ($r/c = nK_a - rK_a$) analysis (52) of ^{125}I-HLf binding to *E. coli* strain E34663 ($y = 3.7528 - 0.6999x$; $r = 0.98$). The molecules of HLf bound to one bacterial cell (r) and the molecules of HLf free in the medium (c) were calculated assuming a mol.wt. of 76,500 for HLf. The intercept on the X axis represents the number of binding sites per cell (n). The slope of the line represents the effective association constant (K_a) inverted.

siderophores which demonstrate higher affinity for iron than Lf or transferrin (7, 19). Enterochelin is less effective *in vivo*, since it interacts with albumin (25). In contrast, aerobactin is

effective *in vivo* and even in an acidic environment such as inflamed tissues, and has therefore been suggested to contribute to virulence (26, 39). However, no significant differences were evident in the Lf-binding capacity between aerobactin-producing and non-producing strains. Thus, Lf binding seems to be independent of an iron-associated virulence factor of *E. coli*.

Further characterization of the HLf interaction with one *E. coli* strain, E34663, revealed a stable and saturable binding, implying a limited number of binding sites, which was also supported by Scatchard analysis. The binding-displacement data indicated that *E. coli* could identify both labelled and unlabelled forms of HLf. Displacement studies with homologous and heterologous ligands on ^{125}I-HLf binding showed that both human and bovine proteins are recognized. Furthermore, various proteins and carbohydrates caused considerably less binding inhibition than HLf or BLf. Thus, the data suggest a specific nature of the interaction. Similar results were also obtained with an ETEC strain H10407 (data not shown).

E. coli can recognize specific proteins (i.e. fibronectin, laminin, collagen etc.) or carbohydrates occurring in the host mucosal and submucosal milieu (22, 55, 66). These subepithelial matrix components might be exposed at the inflamed sites during enteritis. Furthermore, the inflammatory milieu is heavily infiltrated by Lf-excreting polymorphonuclear leukocytes, and is acidic (15, 24, 63). Our *in vitro* results indicate that even under these conditions Lf may bind to *E. coli*, and can probably exert an antibacterial effect *in vivo*. Specific receptors for fibronectin have been reported on *E. coli* (18), and thus a moderate HLf binding inhibition elicited by this protein may be due to steric hindrance. The basic nature (high pI) of Lf may promote non-specific charge interactions with acidic compounds (21), and therefore an increase in HLf binding in the presence of collagen type I and type IV could be expected.

Several mammalian receptors in the mononuclear-phagocyte system recognize different terminal carbohydrate units, in particular, N-acetylglucosamine, mannose and fucose in the glycoproteins (23). In HLf, the fucose residue at the α1–3 linkage with N-acetylglucosamine may promote binding to eukaryotic cells, and, in particular, to the intestinal brush border epithelia (11, 37). Furthermore, mannose may be involved in the binding of Lf to monocytes and B lymphocytes (5). In our carbohydrate inhibition experiments, no blocking of HLf binding to *E. coli* was obtained with fucose, mannose and other carbohydrates. Thus, the Lf interaction with bacteria seems different from that with eukaryotic cells.

In summary, we have established a specific binding of *E. coli* to Lf, an acute-phase, iron-binding, antimicrobial host component. This interaction is associated with certain clinical groups and serotypes of *E. coli*. The biological significance of this interaction remains to be elucidated.

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Correlation between Human Lactoferrin Binding and Colicin Susceptibility in *Escherichia coli*

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***Escherichia coli* H10407 demonstrated low ^{125}I-human lactoferrin (HLf) binding (7%) and was insusceptible to group A (A, E1, E2, E3, E6, and K) and group B (B, D, Ia, Ib, and V) colicins. Conversely, a spontaneous HLf high-binding (44%) variant, H10407(Lf), demonstrated an increased susceptibility to both colicin groups. Colicin-insusceptible *E. coli* wild-type strains 75ColT, 84ColT, and 981ColT showed a low degree of HLf binding, i.e., 4, 8, and 10%, respectively. The HLf binding capacity was high in the corresponding colicin-susceptible mutants 75ColS (43%), 84ColS (32%), and 981ColS (43%). Furthermore, HLf low- (<5%) and high- (>35%) binding *E. coli* clinical isolates (10 in each category) were tested for susceptibility against 11 colicins. Colicin V susceptibility did not correlate with HLf binding in either categories. However, with the remaining colicins, three distinct HLf-binding, colicin susceptibility patterns were observed; (i) 10 of 10 HLf low-binding strains were colicin insusceptible, (ii) 6 of 10 HLf high-binding strains were also colicin insusceptible, and (iii) the remaining HLf high binders were highly colicin susceptible. Certain proteins in the cell envelope and outer membrane of wild-type H10407 (HLf low binder, colicin insusceptible) showed a lower mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis compared to the corresponding proteins of mutant H10407(Lf) (HLf high binder, colicin susceptible). These mobility differences were also associated with HLf-binding proteins in Western blot (ligand blot) analysis. The wild type showed a smooth form of lipopolysaccharide (LPS) with a distinct ladder of O-chains, compared to the rough LPS of the mutant. Exogenous smooth LPS from wild-type H10407 inhibited ^{125}I-HLf binding to mutant H10407(Lf) in a dose-dependent manner, while rough LPS was ineffective. These data establish a correlation between HLf binding and colicin susceptibility in *E. coli*. The LPS seems to be associated with the HLf binding components and the colicin receptors in a similar manner and interferes with the interaction of these eucaryotic and procaryotic antimicrobial agents with *E. coli*.**

Lactoferrin (Lf) is an antimicrobial, iron-binding, mammalian glycoprotein secreted by polymorphonuclear leukocytes and exocrine glands, in milk and at the mucosal surface (27, 28). This protein is thought to play an important role in nonspecific host defense during early events of inflammatory host response (11, 46). The antimicrobial effect of Lf has been mainly attributed to its capacity to deprive iron from bacteria (4, 37). Lf also mediates bacterial uptake and killing during phagocytosis (5, 24).

Escherichia coli is a predominant member of the normal intestinal flora (12) and also a frequent causative agent of human intestinal infections (22, 23). Lf inhibits the growth of *E. coli* and is more effective with immunoglobulin G1 and immunoglobulin A (6, 39, 40, 43, 44). Lf may damage the outer membrane of *E. coli* by releasing lipopolysaccharides (LPS) and facilitate the entry of certain antibiotics (13, 35). Lf adsorption to *E. coli* seems to enhance antibacterial effects (9). We have recently demonstrated specific binding of Lf to *E. coli*, *Aeromonas hydrophila*, and certain gram-positive bacteria (19, 30–33).

Certain *E. coli* strains produce colicins, a class of bacteriocins (20). Colicins are known to kill susceptible *E. coli* and related bacteria by adsorbing to specific receptors in the outer membrane (18, 20). Some of these receptors perform key functions such as selective uptake of various nutrients (20, 36) and adsorption of bacteriophages (48) and may also demonstrate porin activity (7, 8). Adsorption to the bacterial surface is an essential step for colicin action (38). The O-antigenic chains of LPS in *E. coli* may shield the receptors and inhibit colicin adsorption (45). Accordingly, most colicin-insusceptible strains of *E. coli* become colicin susceptible by partial removal of LPS after EDTA or Lf treatment (13, 15).

We have found that a Lf low-binding *E. coli* strain insusceptible to a wide range of colicins, when modified into a Lf high-binding strain, also showed an increase in colicin susceptibility. Furthermore, three colicin-insusceptible *E. coli* strains that do not bind to human Lf (HLf), when converted to colicin susceptibility, demonstrated a high HLf binding capacity. In this communication, we have elucidated a relation between Lf binding and colicin susceptibility profiles in genetically defined strains and clinical isolates of *E. coli*.

MATERIALS AND METHODS

***E. coli* strains.** Lf high- and low-binding strains (10 in each category) were selected from an earlier study (33) and tested for colicin susceptibility. Strain H10407 was received from A. Thorén, Department of Infectious Diseases, Malmö General Hospital, Malmö, Sweden, and strain H10407(Lf) is an analogous Lf high-binding spontaneous variant. Strains 75ColT, 84ColT, and 981ColT are colicin-insusceptible wild

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types and 75ColS, 84ColS, and 981ColS are corresponding colicin-susceptible mutants described in an earlier study (16). The nine *E. coli* and *Citrobacter freundii* reference strains used in the production of group A (A, E1, E2, E3, and K) and group B (B, D, Ia, and Ib) colicins were kindly provided by G. Lebek, Institut für Hygiene und Medizinische Mikrobiologie, University of Bern, Bern, Switzerland. *Shigella sonnei* 617/89 was used for colicin E6 production. *E. coli* HB101 was used for colicin V production after transformation with colicin V-producing genes originating from *E. coli* ColV.K30, and incorporation into vector pSN1.

^{125}I-labeled lactoferrin binding assay. HLf (lot 63541; U.S. Biochemicals Corp., Cleveland, Ohio) was labeled with Na[^{125}I] (specific activity, 629 GBq/mg) (DuPont Scandinavia AB, Stockholm, Sweden) by using Iodobeads (Pierce Chemicals Co., Rockford, Ill.) (26). The labeled protein was tested and confirmed for homogeneity by autoradiography. A binding assay was performed as described earlier (30–33). Briefly, 10^9 bacteria (in 0.1 ml of phosphate-buffered saline [PBS]) were incubated with 0.1 ml of ^{125}I-labeled HLf (radioactivity was adjusted with cold PBS to 2.5×10^4 to 3.0×10^4 cpm, corresponding to ~8 ng) at room temperature for 1 h. The binding reaction was terminated by adding 2 ml of ice-cold PBS (containing 0.1% Tween 20, to reduce nonspecific interactions) and centrifuged at 4,400 × *g* for 15 min. The supernatant was carefully aspirated, and radioactivity bound to the bacterial pellet was measured in a gamma counter (LKB Wallac Clinigamma 1272, Turku, Finland). Background radioactivity (from incubation mixtures without bacteria) of 2.5% was deducted while interpreting the binding data. Samples were always tested in triplicate, and each experiment was repeated at least twice.

Colicin susceptibility assay. Susceptibility of test strains against 11 colicins belonging to group A (A, E1, E2, E3, E6, and K) and group B (B, D, Ia, Ib, and V) was examined (29). Briefly, the colicin-producing reference strains were grown in nutrient broth (Oxoid no. 2) containing 0.2 μg of mitomycin (Sigma) per ml (0.05 μg of mitomycin per ml for colicin A) at 37°C for 16 h with aeration. For colicin V production, mitomycin was omitted from the growth media. After centrifugation at 4,400 × *g* for 30 min, the supernatant was carefully collected and filtered through a membrane filter (pore size; 0.2 μm) or sterilized with chloroform (chloroform was later removed by an additional centrifugation step). Colicin activity was determined by placing serially diluted (10-fold) supernatant on nutrient agar (Oxoid no. 2) spread with a colicin-susceptible laboratory strain, *E. coli* K-12. The plates were examined for a clear inhibition zone after an overnight incubation at 37°C. The highest inhibitory dilution was considered to contain one "colicin unit." The inhibitory effect of colicins on test strains was performed as described above, and the susceptibility values were scored relative to those for strain K-12.

Preparation of total cell protein, envelope, and outer membrane. Strain H10407 and mutant H10407(Lf) were characterized and compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For total cell proteins, bacteria were grown in nutrient broth (Oxoid no. 2) at 37°C for 16 h. After centrifugation at 4,400 × *g*, cells were harvested, washed, and resuspended in Tris-buffered saline (TBS), pH 7.4, to a density of 2×10^{10} bacteria per ml (25^E [E, extinction] at 600 nm). Three milliliters of bacterial suspension was centrifuged, and the pellet was mixed with 0.1 ml of Laemmli solution (containing 2% SDS, 4% β-mercaptoethanol, 10% [vol/vol] glycerol, and 0.2% bromophenol blue in TBS, pH 6.8) and boiled for 10 min.

For the cell envelope preparation, bacteria were sonicated (200 W, four 2-min sonications) in the presence of phenylmethylsulfonyl fluoride (0.1 mg/ml). The sonicate was centrifuged at 4,400 × *g*, and the supernatant was carefully collected. After centrifugation at 10,000 × *g* for 30 min, the pellet was washed in TBS and solubilized in Laemmli solution as described above.

For the outer membrane preparation, a pellet containing cell envelope (from the above-described procedure) was extracted with sarcosyl solution (2% sarcosyl, 0.01 M EDTA, 0.01 M HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], and 0.05 M Tris, pH 7.4) or SDS solution (2% SDS, 20 mM Tris, pH 7.4) for 1 h at room temperature. The sample was centrifuged at 10,000 × *g* for 30 min, and the pellet was dissolved in Laemmli solution as described above.

Gel electrophoresis of proteins. A volume of 10 to 20 μl of the total cell protein, cell envelope, or outer membrane sample was boiled for 5 min and run in SDS-PAGE in the discontinuous buffer system described by Laemmli (21). The resolving gel contained 12.5% acrylamide and 0.1% SDS, and the electrophoresis was preformed in a Miniprotean II apparatus (Bio-Rad, Richmond, Calif.) at 33 mA of constant current for 80 min.

Western blot (ligand blot) analysis with HRPO-conjugated HLf. After SDS-PAGE, proteins in the gel were transferred to a nitrocellulose membrane (Sartorius, Göttingen, Germany) at 400 mA for 1 h, using a Trans-Blot cell (Bio-Rad). The free sites on the membrane were blocked with 1% Tween 20 for 30 min. HLf was coupled to horseradish peroxidase (HRPO) by the method of Nakamura et al. (34), and the membranes were probed with this conjugate. The color reaction was developed with diaminobenzidine (0.25 mg/ml; Sigma) dissolved in 0.1 M sodium acetate buffer, pH 5.0, containing hydrogen peroxide (3×10^{-4}% [vol/vol]), and the reaction was terminated by adding sodium pyrosulfite (1% [wt/vol]).

LPS analysis by SDS-PAGE. The LPS was isolated from strain H10407 and mutant H10407(Lf) by phenol-water extraction (47). Furthermore, a second preparation was made with the sarcosyl-extracted outer membranes of the two strains, which were boiled for 10 min and digested with proteinase K (30 to 60 U/ml; Sigma) at 37°C for 2 h. Both the preparations were run on SDS-PAGE as described above. The gels were stained with a silver-staining kit (lot 35709; Bio-Rad) according to the manufacturer's instructions.

RESULTS

Binding of ^{125}I-labeled HLf and susceptibility to group A as well as group B colicins was tested and compared in four wild-type *E. coli* strains and their derivatives (Table 1). Wild-type H10407 showed 7% HLf binding, and H10407(Lf) was isolated as a spontaneous HLf high-binding (44%) variant. The former strain was insusceptible to both group A and group B colicins, while the latter demonstrated a susceptibility to all colicins (except moderate insusceptibility to colicins A, B, and Ia). On the other hand, strains 75ColT, 84ColT, and 981ColT, the colicin-insusceptible wild types, bound ^{125}I-HLf to a low degree, i.e., 4, 8, and 10%, respectively. Conversely, the corresponding colicin-susceptible mutants demonstrated a high magnitude of ^{125}I-HLf binding, i.e., 75ColS, 43%; 84ColS, 32%; and 981ColS, 43%.

Ten ^{125}I-HLf low-binding (<5% binding) and ten ^{125}I-HLf high-binding (>35% binding) strains were tested for susceptibility to group A and group B colicins. Colicin V suscepti-

TABLE 1. HLf binding and colicin susceptibility in *E. coli* strains and mutants

Strain	% ^{125}I-HLf bound[a]	MIC of colicin (log concn colicin units/ml)[b]										
		Group A						Group B				
		A	E1	E2	E3	E6	K	B	D	Ia	Ib	V
H10407	7.2	>3	2	3	3	4	>2	>4	4	3	>2	1
H10407(Lf)	43.9	1	0	0	0	0	0	1	0	1	0	0
75ColT	4.2	2	2	2	2	2	>1	4	4	2	>1	2
75ColS	42.5	0	0	0	0	0	0	0	0	0	0	0
84ColT	7.8	>4	3	3	4	4	>2	>3	>4	>2	>2	>1
84ColS	32.3	1	0	0	0	0	0	1	2	1	>1	1
981ColT	10.2	3	3	>4	>3	4	>1	>4	>4	>2	>1	>2
981ColS	42.9	1	1	1	1	1	1	1	1	>2	>1	>2

[a] Percentages are based on the total ^{125}I-labeled protein added.
[b] The minimal quantity of colicin to elicit inhibition of *E. coli* strain K-12 was considered a colicin unit.

bility did not correlate with HLf binding in either categories. Three distinct HLf-binding, colicin susceptibility patterns were observed with the other colicins (Table 2). In pattern 1, all of the HLf low binders (10 of 10 strains) were also highly insusceptible to group A and B colicins. In pattern 2, 6 of the 10 HLf high-binding strains demonstrated a high degree of insusceptibility to colicins. In pattern 3, the remaining four HLf high binders were susceptible to colicins.

The mobilities of proteins from cell envelope and outer membrane of strain H10407 and mutant H10407(Lf) were analyzed and compared in SDS-PAGE (Fig. 1). The total cell protein profiles appeared to be similar for both strains; however, certain proteins in the 20- to 80-kDa range in strain H10407 were less mobile than in the mutant H10407(Lf). Such difference in protein migration was also observed to a lesser extent in cell envelope and sarcosyl-extracted outer membrane preparations. The SDS-extracted outer membrane proteins, however, showed no mobility variations. Finally, Western blot analysis revealed that the outer membrane proteins with HLf binding capacity also demonstrated a mobility difference.

Phenol-water-extracted and proteinase K-treated LPS from strain H10407 and mutant H10407(Lf) were analyzed by silver stain and compared (Fig. 2). The HLf low-binding, colicin-insusceptible (wild-type) strain exhibited a smooth (S) form of LPS with regular ladders of O-chains in both the preparations. On the other hand, in the HLf high-binding, colicin-susceptible (mutant) strain the O-chain was absent.

TABLE 2. Colicin susceptibility in HLf high- and low-binding *E. coli* strains

Strain (serotype)[a]	% ^{125}I-HLf bound[b]	MIC of colicin (log concn colicin units/ml)										
		Group A						Group B				
		A	E1	E2	E3	E6	K	B	D	Ia	Ib	V
Pattern 1												
E227 (O2:H−)	1.0	>3	2	5	>5	>5	>3	>4	>4	>3	3	>1
HH41 (O124:H−)	1.2	>3	2	5	5	5	>3	>4	>4	2	2	0
E206 (O2:H5)	1.3	>3	>3	>4	>4	>4	>3	>3	>3	>2	>2	>1
E221 (O15:H1)	1.9	>3	>2	>5	5	5	>3	>4	>4	>3	>3	1
E229 (O18ac:H−)	2.3	>3	>2	5	5	5	>3	>4	>4	>3	>3	>1
E223 (O18ac:H−)	3.1	>3	>1	5	5	5	>3	>4	>4	>3	>3	>2
HH25 (O86:HNT)	3.2	>3	>2	5	5	5	>3	>4	>4	>3	>3	>1
HH26 (O86:H11)	3.3	>3	1	4	4	4	>3	>4	>4	>3	>3	2
HH45 (O7:HNT)	4.4	>3	>1	>5	5	5	>3	>4	>4	>3	>3	2
E84 (O126:H−)	4.6	>3	3	4	>4	>4	>3	>3	>3	>2	>2	−1
Pattern 2												
E21 (O44:H49)	30.2	>3	>1	>5	5	5	>3	>4	>4	>3	>3	2
E66 (O44:H18)	33.6	>3	1	5	5	5	>3	>4	>4	>3	>3	2
E308 (O167:H−)	34.0	>3	1	4	4	4	>3	>4	>4	>3	>3	>2
E302 (O92:H33)	38.1	>3	1	4	4	4	>3	>4	>4	>3	>3	2
HH2 (O26:H11)	70.0	>3	3	4	>4	>4	>3	>3	>3	>2	>2	1
E1 (O44:H−)	73.4	>3	>2	>5	5	5	>3	>4	>4	>3	>3	1
Pattern 3												
E322 (Sp:H−)	34.6	1	0	−1	−1	−1	0	0	−1	1	0	0
HH19 (O109:H−)	43.9	>3	1	2	2	2	3	2	1	1	1	−1
HH47 (Sp:H−)	46.6	1	1	1	1	0	0	0	0	0	0	−1
F18 (Sp:H5)	47.1	2	1	1	1	2	1	2	1	>3	3	>1

[a] The O and H serotypes were shown; Sp, spontaneous agglutination.
[b] Percentages are based on the total ^{125}I-labeled protein added.

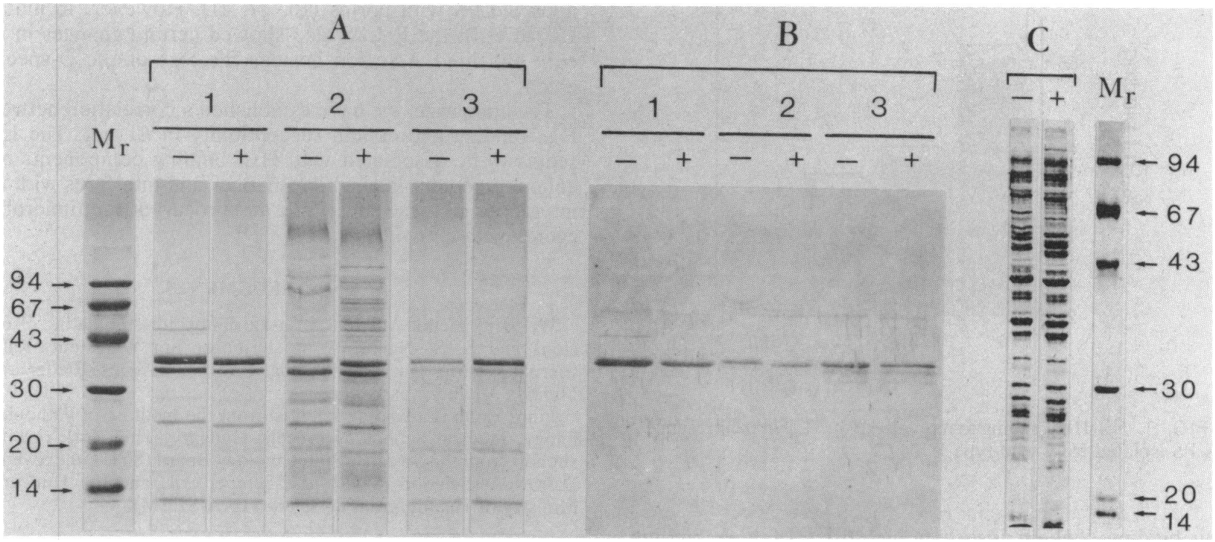

FIG. 1. Analysis of total cell proteins, cell envelope, and outer membranes of *E. coli* in SDS-PAGE. −, wild-type H10407: HLf low binder and colicin insusceptible; +, mutant H10407(Lf): HLf high binder and colicin susceptible. (A) Gels stained with Coomassie brilliant blue. (B) Proteins from section A were transferred to nitrocellulose, and Western blot analysis was performed with HLf-HRPO conjugate. 1, outer membranes (sarcosyl extract); 2, cell envelopes; 3, outer membranes (SDS extract). (C) Total cell protein preparation.

In addition, the LPS from the S strain showed a distinct ladder pattern in the core and lipid A region.

Exogenous S LPS (from *E. coli* serotype O128:B12) decreased the HLf binding in the mutant H10407(Lf), and rough (R) LPS (from *E. coli* serotype O111:B4) was ineffective (Table 3). Furthermore, phenol-water-extracted S LPS from H10407 caused a concentration-dependent decrease in the binding of ^{125}I-labeled HLf to mutant H10407(Lf) (Fig. 3).

DISCUSSION

Lf is a mammalian antimicrobial protein with an important role in nonspecific host defense (1, 4, 37), while colicins are a class of antibacterial proteins produced by certain species of the family *Enterobacteriaceae* (20). Specific receptor-mediated interaction of colicins with *E. coli* is a necessary initial step in bacterial killing (3, 8, 18, 20). In analogy, Lf interaction with *E. coli* surface seems necessary to elicit an antibacterial effect (9). We have recently demonstrated binding of Lf to diarrheagenic *E. coli* (33). Both Lf- and colicin-mediated antimicrobial systems prevail in the mammalian gastrointestinal tract. Therefore, we have examined a possible relation between the two in a common bacterial gut pathogen, *E. coli* (22, 23). On the basis of two lines of experimental evidence, we have demonstrated a correlation between the HLf binding property and colicin susceptibility in *E. coli*.

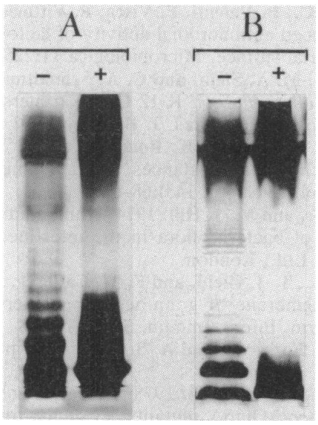

FIG. 2. LPS analysis of *E. coli* by SDS-PAGE and silver staining. −, wild-type H10407; +, mutant H10407(Lf). (A) LPS prepared by phenol-water extraction. (B) LPS preparation digested with proteinase K.

First, from three colicin-insusceptible, wild-type strains, when colicin-susceptible mutants were isolated, a simultaneous change from HLf low- to high-binding capacity was observed. Second, from an HLf low-binding strain H10407, when an HLf high-binding variant H10407(Lf) was isolated, a shift from colicin insusceptibility to colicin susceptibility followed.

To further investigate, different HLf high- and low-binding isolates (10 strains in each category) were tested for colicin susceptibility. Three distinct patterns were noted: (i) HLf

TABLE 3. Effect of exogeneous LPS on ^{125}I-HLf binding to *E. coli*[a]

Addition (0.5 mg/ml)	% ^{125}I-HLf binding	
	Wild-type H10407	Mutant H10407(Lf)
Control (PBS)	5.9	64.7
S LPS (from *E. coli* O128:B12)	3.0	46.5
R LPS (from *E. coli* O111:B4)	4.2	64.6

[a] Bacteria (10^9 cells) were mixed with 0.1 mg of LPS (final volume, 0.1 ml) and incubated at 37°C for 1 h. ^{125}I-HLf (~8 ng in 0.1 ml of PBS) was added to this mixture (final volume, 0.2 ml), and the binding assay was performed as described in Materials and Methods.

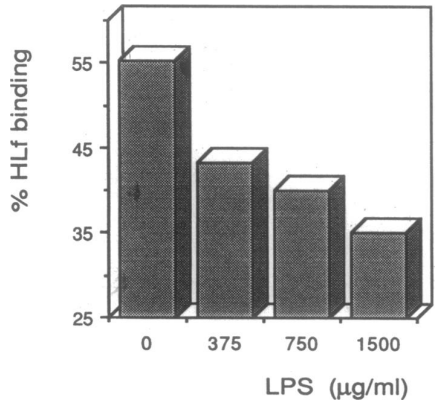

FIG. 3. ^{125}I-HLf binding to mutant H10407(Lf) in the presence of S LPS isolated from wild-type H10407.

low binding, colicin insusceptible; (ii) HLf high binding, colicin insusceptible; and (iii) HLf high binding, colicin susceptible. There was no fourth, HLf low-binding, colicin-susceptible pattern. Thus, an independent degradation process(s) for the expression of these two properties seems unlikely.

Colicins bind to specific protein receptors such as btuB, OmpF, tsx, etc., in the outer membrane (3, 8, 25). These outer membrane proteins are associated with LPS and may be shielded by O-specific chains (10, 45, 48). Thus, rough laboratory strains of *E. coli* are more readily available for colicin interaction (45). Similar laboratory strains also showed a high degree of Lf binding. Eight to nine molecules of LPS are loosely associated, and at least one molecule is tightly bound to each porin trimer (17, 41). The S LPS bind porins with a higher affinity than the R LPS (2). In a recent study, Diedrich et al. (10) have demonstrated a slower mobility of S-LPS-associated OmpF trimers in SDS polyacrylamide gels compared with trimers from R strains.

Accordingly, the SDS-PAGE results indicated that certain proteins were of lower mobility in the wild-type strain H10407 compared with the corresponding proteins in the mutant H10407(Lf). The mobility differences were distinct in total cell protein and cell envelope preparations. The outer membranes extracted with sarcosyl demonstrated less pronounced protein mobility differences, and this phenomenon was absent in samples extracted with SDS. The latter extraction may have reduced LPS association with the outer membrane proteins (42). The HLf binding components in the cell envelope preparation also showed slight mobility differences. Recently, we have demonstrated that the lactoferrin-binding outer membrane proteins in *E. coli* are porins (14).

In the case of the strain pairs described in Table 1, the low HLf binding and colicin insusceptibility may be due to the masking effect of S LPS. The gradual degradation of LPS seems to expose receptors for the two antimicrobial agents. In support of this hypothesis, we have demonstrated two lines of evidence: (i) an S-to-R variation in the LPS coincided with a shift from HLf low binding and colicin insusceptibility (wild-type H10407) to HLf high binding and colicin susceptibility [mutant H10407(Lf)], and (ii) the addition of exogenous S LPS from strain H10407 or from another *E. coli* serotype effectively inhibited the HLf binding in mutant H10407, compared with exogenous R LPS. This inhibitory effect may be due to the reassociation of exogenous S LPS with porins (10, 17, 41). However, in mutant H10407(Lf), the R LPS also showed certain changes in the core and lipid A region besides the loss of the O-specific chain.

To summarize, we have established a correlation between HLf binding and colicin susceptibility in *E. coli*. The LPS seems to be associated with HLf binding components and colicin receptors in a similar manner and interferes with the interaction of these eucaryotic and procaryotic antimicrobial agents with *E. coli*.

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Lactoferrin Binds to Porins OmpF and OmpC in *Escherichia coli*

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Lactoferrin (Lf) is an iron-binding antimicrobial protein present in milk and on mucosal surfaces, with a suggested role in preimmune host defense. Certain strains of *Escherichia coli* (bacterial whole cells) demonstrate specific interaction with ^{125}I-labeled Lf. A band with a mass of ~37 kDa, which was reactive with horseradish peroxidase-labeled Lf, was identified in the boiled cell envelope and outer membrane preparations of an Lf-binding *E. coli* strain, E34663, and a non-Lf-binding strain, HH45, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). Such a band was not detected in the unboiled native cell envelope and outer membrane preparations. The molecular mass and the property of heat modifiability suggested that the Lf-binding proteins were porins. The native trimeric form of porin OmpF isolated from strain B6 and its dissociated monomeric form both reacted with horseradish peroxidase-labeled Lf and with monoclonal antibodies specific for OmpF. Furthermore, by using *E. coli* constructs with defined porin phenotypes, OmpF and OmpC were identified as the Lf-binding proteins by urea-SDS-PAGE and Western blotting and by ^{125}I-Lf binding studies with intact bacteria. These data establish that Lf binds to porins, a class of well-conserved molecules common in *E. coli* and many other gram-negative bacteria. However, in certain strains of *E. coli* these pore-forming proteins are shielded from Lf interaction.

Lactoferrin (Lf) is an iron-binding mammalian glycoprotein secreted by the polymorphonuclear leukocytes and various exocrine glands (2, 21). This protein occurs at high concentrations in milk and also bathes mucosal surfaces (21, 31). A regulatory function for Lf in various physiological pathways (5), including the adhesion of polymorphonuclear leukocytes to the endothelial surface (30), feedback inhibition of the granulocyte-monocyte colony-stimulating factor (6, 7), and the regulation of antibody production (11), has been suggested. Specific interaction of Lf with certain mammalian cells seems to be involved in the above pathways, and, accordingly, specific receptors for Lf were identified on macrophages, monocytes, B lymphocytes, polymorphonuclear leukocytes, activated T lymphocytes, and hepatocytes (3, 10, 20, 32, 41).

Lf inhibits the growth of *Escherichia coli* and certain other microorganisms in vitro (8). Iron chelation and the resulting iron limitation for bacteria have been suggested to form the mechanism of Lf-mediated antimicrobial action (1, 18). This effect may be enhanced when Lf adsorbs to the bacterial surface (9, 26). The specific nature of Lf interaction with *E. coli* has recently been elucidated by our laboratory (25).

However, the mechanism of Lf-mediated antimicrobial action seems to be more complex than simple iron deprivation (36). Lf may alter bacterial membrane permeability by causing a release of lipopolysaccharides (LPS) and by damaging the gram-negative bacterial outer membrane (OM) (12). Porins constitute a permeability barrier against nutrients and antibiotics in the OM of *E. coli* (29). These pore-forming proteins belong to a class of well-conserved molecules in the family *Enterobacteriaceae* and many other gram-negative bacteria (19). Porins also serve as receptors for certain bacteriocins and bacteriophages (4, 19). Furthermore, a strong correlation exists between colicin sensitivity and human Lf-binding ability in *E. coli* (15). The present communication presents evidence that Lf binds to porins OmpF and OmpC in *E. coli*.

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MATERIALS AND METHODS

***E. coli* strains.** Strain E34663 is an enteropathogenic *E. coli* strain belonging to serotype O127 and was previously characterized with regard to Lf interaction (25). The non-Lf-binding strain HH45 is an enterotoxigenic *E. coli* strain belonging to serotype O7:HNT (15). Strain B6, which expresses OmpF as the only porin under normal growth conditions (34), was from our existing strain collection. Strains that phenotypically express different porins, i.e., PC0479 (PhoE$^-$ OmpF$^+$ OmpC$^+$), PC2415 (PhoE$^-$ OmpF$^-$ OmpC$^-$), PC2416 (PhoE$^+$ OmpF$^-$ OmpC$^-$), PC2421 (PhoE$^-$ OmpF$^-$ OmpC$^+$), and PC2407 (PhoE$^-$ OmpF$^+$ OmpC$^-$), were kindly provided by J. Tommassen, Department of Molecular Cell Biology, University of Utrecht, Utrecht, The Netherlands, and were described elsewhere (38, 39). A second set of strains (constructs with defined porins), i.e., JF568 (OmpF$^+$ OmpC$^+$), JF701 (OmpF$^+$ OmpC$^-$), and JF703 (OmpF$^-$ OmpC$^+$), were kindly provided by H. Nikaido, Department of Molecular and Cell Biology, University of California, Berkeley, Calif., and were described elsewhere (14). Bacteria were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) at 37°C for 16 h.

^{125}I-labeled-Lf binding assay. Human Lf (lot 63541) purified from milk was purchased from U.S. Biochemicals Corp., Cleveland, Ohio. Bovine Lf (BLf) purified from whey was kindly provided by H. Burling, Swedish Dairies Association, Malmö, Sweden. Both protein preparations were homogeneous in ion-exchange (Mono-Q column; Pharmacia, Uppsala, Sweden) or molecular-sieve (TSK G4000SW; LKB Produkter AB, Bromma, Sweden) high-performance liquid chromatography. The interaction of Lf with intact bacteria was tested in a ^{125}I-labeled-Lf binding assay (23, 24). Bacteria (10^9 cells in 0.1 ml of phosphate-buffered saline [PBS]) were incubated with

0.1 ml of PBS containing ~8 ng of ^{125}I-Lf (specific activity, 0.16 MBq/μg of Lf) at room temperature for 1 h. The reaction was terminated with 2 ml of ice-cold PBS (containing 0.1% Tween 20 to reduce nonspecific interactions). After centrifugation at 4,400 × *g* for 15 min, the supernatant was aspirated, and the radioactivity retained in the bacterial pellet was measured with a gamma counter (Clinigamma 1272; LKB Wallac, Turku, Finland). Background radioactivity (from incubation mixtures without bacteria) was deducted, and the binding was expressed as a percentage of the total amount of labeled ligand added to the bacteria. Strains E34663 and HH45 served as positive (60%) and negative (5%) binding controls, respectively.

Isolation of *E. coli* CE and OM material (16, 37). Bacteria were harvested during the late exponential phase, washed in 20 mM Tris-buffered saline, pH 7.4, and resuspended in 20 mM Tris-HCl, pH 7.4, containing 2 mM EDTA. After sonic disruption was performed three times for 2 min during cooling, unbroken cells were removed by centrifugation (1,200 × *g*, 20 min). The supernatant was recentrifuged (100,000 × *g*, 1 h), and a pellet containing cell envelope (CE) material was obtained; this pellet was washed in the above-described buffer and resuspended in a buffer containing 1% sarcosyl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM EDTA, and 50 mM Tris-HCl, pH 7.4, at room temperature for 1 h, with gentle agitation. The suspension was centrifuged (100,000 × *g*, 1 h), and a pellet containing OM proteins associated with peptidoglycan fragments (13) was suspended in a sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue).

OmpF isolation and detection. CE material of strain B6 (expressing OmpF as the only porin under normal growth conditions) was prepared as described above. The envelope material was extracted three times with 2% SDS in 10 mM Tris, pH 7.4, at 55°C, to remove membrane proteins other than porins. OmpF was released from the peptidoglycan by end-to-end rotation at room temperature for 1 h with the above-described extraction buffer containing 0.5 M NaCl and 0.7 M 2-mercaptoethanol. The peptidoglycan was removed by high-speed centrifugation, and the OmpF-containing supernatant was dialyzed against 10 mM $NaHCO_3$ containing 0.1% SDS, at 26°C, twice for 16 h. An OmpF-specific mouse monoclonal antibody (catalog no. 0411; Immunotech S.A., Marseille, France) was reconstituted in 1 ml of distilled water and used at a 1:1,000 working dilution in a buffer containing 0.5% ovalbumin, 0.5 M NaCl, 0.05% Tween 20, and 20 mM Tris-HCl, pH 7.4. Horseradish peroxidase (HRPO)-labeled rabbit antibodies to mouse immunoglobulins (Dakopatts A/S, Glostrup, Denmark) were used at a 1:1,000 dilution in the same buffer. Each antibody was incubated with nitrocellulose strips for 60 min during Western blotting (immunoblotting). Strips incubated only with labeled second antibody were included as controls.

Electrophoresis and Western blotting. The samples were either boiled for 10 min or run without boiling. Electrophoresis was carried out at 30 mA of constant current according to Laemmli's procedure (17) in a Miniprotean II apparatus with 1-mm-thick gels. The stacking and separating gels contained 4 and 12.5% acrylamide, respectively. A 20-μl sample containing 10 to 20 μg of proteins was applied to each lane. Molecular weight standards (Pharmacia AB) were used according to the manufacturer's instructions. After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were electrophoretically transferred from the gel to a nitrocellulose membrane (Sartorius, Göttingen, Federal Republic of Germany), at 400 mA for 1 h, by using a Trans-Blot cell (Bio-Rad, Richmond, Calif.) containing blotting buffer with 25 mM Tris base, 192 mM glycine, and 20% (vol/vol) methanol (pH 8.3). Free sites on the membrane were blocked with 1% Tween 20 for 8 min and probed with HRPO-labeled Lf (diluted in PBS containing 0.05% Tween 20) for 1 h. The HRPO was coupled to Lf according to the periodate method (28), and the conjugate was used at a 1:1,000 dilution. A color reaction was developed with diaminobenzidine (0.25 mg/ml) dissolved in 0.1 M sodium acetate buffer, pH 5, containing hydrogen peroxide (3 × 10^{-4}%, vol/vol), and the reaction was terminated by soaking the solution in sodium pyrosulfite (1%, wt/vol) for 2 min. Probes with native HRPO adjusted to the appropriate concentration (2 μg/ml) were included as controls. Also, two preparations of smooth *E. coli* LPS (lots 113F-4007 and 108F-4015; Sigma Chemical Co., St. Louis, Mo.) and a rough LPS extracted from *E. coli* 10407 (Lf) (15) were tested for direct binding to HRPO-labeled human and bovine Lfs by Western blotting.

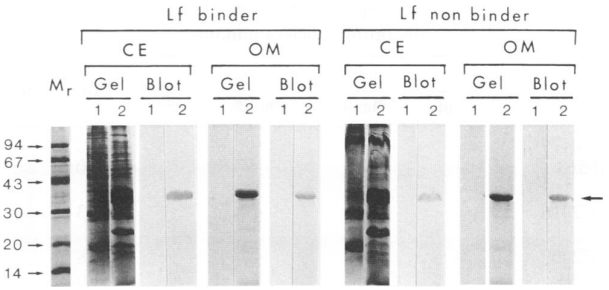

FIG. 1. Analysis of CE and OM (SDS extraction) material of strain E34663 (Lf binder) and strain HH45 (non-Lf-binder) by SDS-PAGE and Western blotting with HRPO-labeled BLf. Lanes 1, unboiled samples; lanes 2, samples boiled for 10 min. Molecular weight standards (in thousands) are indicated at the left. The arrow on the right indicates the Lf-reactive bands.

RESULTS

The CE and OM fractions of the Lf-binding strain E34663 and those of the non-Lf-binding isolate HH45 were examined by SDS-PAGE and Western blotting with HRPO-labeled Lf (Fig. 1). The boiled CE preparation of both strains showed many protein bands in the gel; however, only a single Lf-reactive band with an estimated molecular mass of ~37 kDa was identified in the Western blot. On the other hand, the corresponding protein was clearly absent in the gels and blots of unboiled CE material. The porin-enriched OM fraction of both strains obtained by SDS extraction also demonstrated Lf-reactive bands only in the boiled preparations. A similar band was readily demonstrated at the identical position and intensity in each *E. coli* strain (n = 26) tested, irrespective of the level of Lf binding to intact cells (data not shown). However, no such band was developed with the unboiled CE and OM preparations from any of the strains. Both bovine and human Lfs were used throughout the study, and similar results were obtained with the two.

The binding of ^{125}I-labeled Lf to intact cells of *E. coli* mutants with defined porin phenotypes was examined (Table 1). The lack of either OmpF or OmpC did not considerably alter the Lf-binding capacity compared with those of the parental strains PC0479 and JF568, which contain both of the proteins. The mutant PC2416, which expresses neither OmpF nor OmpC but only PhoE, demonstrated half as much binding as its parent strain. However, the OmpF-, OmpC-, and PhoE-

TABLE 1. Binding of ^{125}I-labeled BLf to the wild-type strain and its porin-deficient mutants

Strain	Porin phenotype			% of ^{125}I-BLf bound[a]
	OmpF	OmpC	PhoE[b]	
PC0479	+	+	−	25.2
JF568	+	+	−	26.0
PC2416	−	−	+	13.3
PC2407	+	−	−	20.8
JF701	+	−	−	31.0
PC2412	−	+	−	25.8
JF703	−	+	−	30.9
PC2415	−	−	−	4.2

[a] Percentage of the total amount of labeled ligand (~8 ng) added to 10^9 bacteria.

[b] In wild-type strain PC0479 PhoE production can be induced by growth under phosphate limitation. In strain PC2416, PhoE is produced constitutively because of a *phoS* mutation (38). Thus, only PC2416 can produce PhoE when grown on normal broth media.

deficient mutant PC2415 demonstrated a negligible level of Lf binding.

The OMs of *E. coli* mutants with defined porin phenotypes were run in urea-SDS-PAGE and analyzed by Western blotting (Fig. 2). Protein bands corresponding to porins OmpF and OmpC demonstrated binding to HRPO-labeled Lf. The OM of the PhoE-expressing mutant PC2416 (lacking OmpF and OmpC) demonstrated a weak Lf-reactive band in the OmpF position. Such Lf-binding proteins were absent in the OM of mutant PC2415. OmpA, one of the major nonporin OM proteins of *E. coli*, showed no reactivity with HRPO-labeled Lf.

Porin OmpF, isolated from *E. coli* B6, was identified as one of the Lf-binding components by simultaneous probing with OmpF-specific monoclonal antibody and HRPO-labeled Lf. It was also shown that the native porin trimers were released from the peptidoglycan, migrated in the gel, and bound Lf just

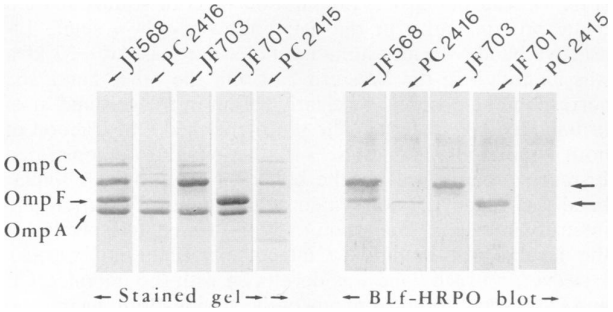

FIG. 2. OM analyses of wild-type and porin-deficient mutants of *E. coli* by urea-SDS-PAGE and Western blotting with HRPO-labeled BLf. The OM of one strain from each porin phenotype (same order as in Table 1) was analyzed. Samples were boiled for 10 min prior to electrophoresis. The figure shows only the relevant parts of the gel and the blot. The arrows on the right indicate OmpC (top) and OmpF (bottom). Electrophoresis and Western blot analyses were performed as described in Materials and Methods, except that the separating gel contained 10% acrylamide, 0.21% bisacrylamide, and 6 M urea, and the electrophoresis was performed at 60 V of constant current. HRPO, horseradish peroxidase.

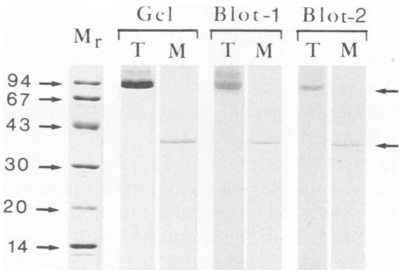

FIG. 3. SDS-PAGE and Western blot analyses of native trimeric (T) and heat-denatured monomeric (M) OmpF isolated from strain B6. Proteins transferred from gel to membrane were probed with an OmpF-specific monoclonal antibody (Blot-1) or with HRPO-labeled BLf (Blot-2). Molecular weight standards (in thousands) are indicated at the left.

as the denatured monomers did (Fig. 3). Neither smooth nor rough LPS of *E. coli* showed reactivity with the HRPO-labeled Lf in Western blot analysis (data not shown).

DISCUSSION

We have previously demonstrated specific binding of Lf to *E. coli* by using a ^{125}I-labeled protein binding assay (25). Neither the degree of iron saturation nor the species origin of Lf affected this interaction. A majority of clinical isolates showed low or negligible levels of interaction with Lf. The nature of Lf-bacterium interaction was characterized in the high-Lf-binding clinical isolate of *E. coli*, strain E34663. Approximately 5,400 specific binding sites per bacterium, having an affinity constant of 1.4×10^{-7} M, were estimated to exist in strain E34663. The OM fractions of this strain and that of the non-Lf-binding isolate HH45 were examined by gel electrophoresis for the presence of Lf-binding components. A band with a mass of ~37 kDa, which reacted with HRPO-labeled Lf, was detected in the boiled OM preparation of strain E34663. Strain HH45 also showed an Lf-reactive band at an identical position. However, such Lf-reactive bands did not appear in the unboiled OM preparations of either strain. A similar Lf-binding protein pattern was observed in the OMs of 26 other *E. coli* strains, irrespective of the level of Lf binding to intact cells.

The molecular weight and heat-modifiable electrophoretic mobility pattern indicated that the Lf-binding component in *E. coli* is a porin. Unheated OM preparations did not reveal Lf-binding proteins in the Western blot, since porin trimers remain attached to the peptidoglycan and failed to enter the gel (27) under the electrophoretic conditions used. The detection of Lf-binding components in the non-Lf-binding strain HH45 seems to be a consequence of protein unmasking. Recently, we have also shown that a phenotypic shift from a low to a high level of Lf binding is connected with variation from smooth to rough LPS in *E. coli* H10407 (15). Furthermore, the O chains of LPS are suggested to cause the shielding of porins from interaction with certain colicins, bacteriophages, and antibiotics (29, 40, 42). Similarly, LPS may also be interfering with the binding of Lf to porins.

Most of the pore-forming proteins tend to form trimers in association with LPS and peptidoglycan, and they protrude to the cell surface during spatial arrangement of the gram-negative bacterial OM (4, 19, 33). Western blot analyses with isolated OmpF (from strain B6) clearly indicated that both the monomeric (heat-denatured) and the native trimeric forms of

porin could bind Lf. Furthermore, monoclonal antibodies specific for OmpF (from strain B6) also recognized both physical forms of OmpF.

Evidence for Lf interaction with porins on intact bacteria was obtained from a ^{125}I-Lf binding assay with *E. coli* genetic constructs with defined porin types. Thus, strain PC2415, lacking porins OmpF, OmpC, and PhoE, demonstrated a negligible level of ^{125}I-Lf binding. The involvement of individual porin types in Lf interaction was examined by Western blot techniques. Since OmpF, OmpC, and PhoE do not resolve in most acrylamide gel systems because of their similar molecular masses (35), the OM preparations were run in urea-containing gels. Lf was avidly bound to OmpF and OmpC, and a weak reaction was also observed with PhoE. Lf has been shown to cause permeability alterations in the gram-negative bacterial OM (12). Porins serve as the major permeability barriers in the OM and protect *E. coli* from a hostile environment such as that of the human intestinal tract. The role of individual porin-Lf interaction in this phenomenon is currently under investigation.

Both binding experiments with isotopic labels (Table 1) and Western blot analysis (Fig. 2) clearly ruled out the involvement of OmpA (the major nonporin OM protein) in Lf binding. The interaction of Lf with LPS (22) is less likely in our system, since LPS was not recognized by HRPO-labeled Lf in the Western blot analysis.

In summary, we have demonstrated that Lf, a mammalian preimmune host defense factor, recognizes porins, conserved molecules common in most members of the *Enterobacteriaceae* and other gram-negative bacteria (4, 19). Though porins occur at a high copy number (up to 10^5 per cell) (34), certain bacteria seem to shield these binding sites from Lf interaction via the polysaccharide core(s) of LPS.

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1993, p. 240–245
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Relationship between Antibacterial Activity and Porin Binding of Lactoferrin in *Escherichia coli* and *Salmonella typhimurium*

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The effect of lactoferrin (Lf) on bacterial growth was tested by measuring conductance changes in the cultivation media by using a Malthus-AT system and was compared with the magnitude of ^{125}I-labeled Lf binding in 15 clinical isolates of *Escherichia coli*. The binding property was inversely related to the change in bacterial metabolic rate ($r = 0.91$) and was directly related to the degree of bacteriostasis ($r = 0.79$). The magnitude of Lf-bacterium interaction showed no correlation with the MIC of Lf. In certain strains, Lf at supraoptimal levels reduced the bacteriostatic effect. Thus, the Lf concentration in the growth media was critical for the antibacterial effect. The cell envelopes of *Salmonella typhimurium* 395MS with smooth lipopolysaccharide (LPS) and its five isogenic rough mutants revealed 38-kDa porin proteins as peroxidase-labeled-Lf-reactive components in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (ligand blot) analysis. However, in the whole cell binding assay, parent strain 395MS demonstrated a very low interaction with ^{125}I-Lf. On the other hand, Lf interaction gradually increased in correspondence with the decrease in LPS polysaccharide moiety in the isogenic rough mutants. Conductance measurement studies revealed that the low-level-Lf-binding (low-Lf-binding) strain 395MS with smooth LPS was relatively insusceptible to Lf, while the high-Lf-binding mutant Rd was more susceptible to Lf. These data suggested a correlation between Lf binding to porins and the Lf-mediated antimicrobial effect. The polysaccharide moiety of LPS shielded porins from the Lf interaction and concomitantly decreased the antibacterial effect.

Lactoferrin (Lf) constitutes one of the major antimicrobial systems in milk and various mammalian exocrine secretions (8, 9, 11, 29, 42). An important role for Lf in nonspecific local secretory immunity at the mucosal surface has been suggested, more effectively in association with immunoglobulin A (45, 46). Lf also occurs in the specific granules and contributes to the microbicidal activity of the polymorphonuclear leukocytes (1, 10, 30). Since Lf could directly have bacteriostatic, bactericidal, and opsonic effects on bacteria, it has been regarded as one of the potential preimmune host defense systems (3, 22, 37, 42).

Lf is an iron-binding protein that reversibly binds two atoms of Fe^{3+} in the presence of bicarbonate (17, 28). This property enables Lf to scavenge iron from the physiological milieu. Eventually, such processes may deprive iron from the microorganisms and inhibit their metabolic activities in vivo. This hypothesis provided an explanation to elucidate the bacteriostasis effect caused by apo-Lf with a variety of bacteria (39, 41, 45, 46). However, other studies have indicated that the mechanism of Lf-mediated antimicrobial action is more complex than simple iron deprivation (4, 47). The bactericidal activity was iron irreversible, and this pathway seems to require an Lf interaction with the target cell surface (2, 3, 5, 13). Furthermore, a peptic fragment from Lf without iron-binding capacity was identified as a potent bactericidal domain (43). The opsonic effect seems to involve specific interaction of Lf with the microbial surface (22); in complement, Lf-binding receptors are present on the phagocytic cell cascade (6, 25, 50).

Our laboratory has previously demonstrated specific binding of Lf in bacteria belonging to the family *Enterobacteriaceae* and has identified porins as the Lf-binding target proteins (15, 16, 21, 35, 48). Lf-binding outer membrane proteins are a class of well-conserved molecules that form ion channels and constitute a permeability barrier against various molecules, including antibiotics, in bacteria (36). Furthermore, these outer membrane proteins serve as receptors for certain colicins and bacteriophages (7, 24). Though porins occur at a high copy number (7), the Lf-binding capacity markedly varied in these bacteria (15, 48). The lipopolysaccharide (LPS) type seemed to affect the interaction of Lf with porins in certain wild-type strains (16, 48).

This study was aimed at elucidating the effect of Lf-porin interaction on the bacterial growth. For this purpose, the Lf susceptibility patterns of different clinical isolates of *Escherichia coli* with various magnitudes of Lf-binding capacity were examined. To illustrate the extent of LPS involvement, various rough (R) mutants of *Salmonella typhimurium* with different lengths of polysaccharide moiety were tested for Lf binding and susceptibility.

MATERIALS AND METHODS

Bacteria. The 15 clinical isolates of *E. coli* included in this study were from our existing strain collection, and the Lf interaction property of these strains was elucidated in an earlier study (35). A previously well-characterized *S. typh-*

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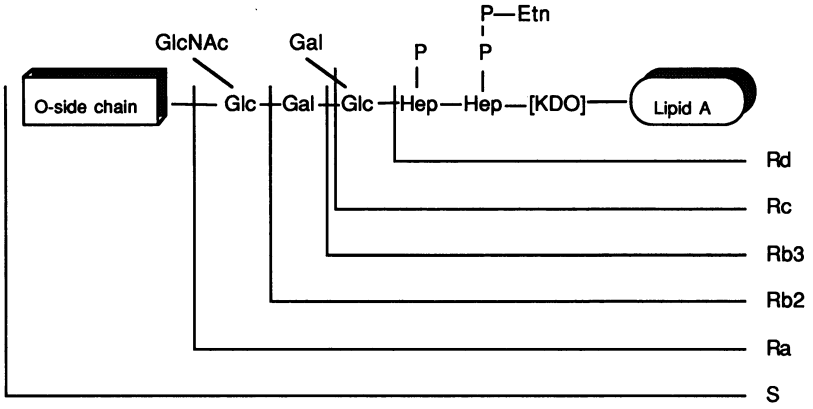

FIG. 1. Schematic representation of the chemical structure of LPS from *S. typhimurium*. Wild-type strain 395MS(S) synthesizes the entire structure, whereas various mutant strains produce the Ra to Rd chemotypes of LPS.

imurium strain, 395MS(S), and its isogenic R mutants with various lengths of LPS polysaccharide moiety (23) (Fig. 1) were kindly provided by O. Stendahl, Department of Medical Microbiology, Linköping University, Linköping, Sweden. Bacterial strains were stored in glycerol at −80°C, revived on special peptone yeast extract (SPYE) agar (Malthus-AT, West Sussex, England), and subsequently cultivated in SPYE broth at 37°C for binding and antibacterial assays.

Lf. Bovine Lf was isolated from milk serum by an industrial-scale process (12). The homogeneity of the protein was determined by ion-exchange (Mono-Q column; Pharmacia AB, Uppsala, Sweden) and molecular-sieve (model TSKG4000SW; LKB Produkter AB, Bromma, Sweden) high-performance liquid chromatography. The iron content was estimated at approximately 250 μg/g of protein by using a Perkin-Elmer (model 560) atomic absorption spectrophotometer. Lf was used in its native form as isolated from the biological reservoir.

Binding of Lf to bacteria and cell envelope (CE) proteins. Lf was labeled with $Na^{125}I$ (DuPont Scandinavia AB, Stockholm, Sweden) to a specific activity of 0.16 MBq/μg of protein by using iodobeads (Pierce Chemical Co., Rockford, Ill.) (26). The binding of ^{125}I-Lf to bacterial whole cells was performed as previously described by Naidu et al. (31, 33, 34).

The interaction of horseradish peroxidase (HRPO)-labeled Lf with CE proteins of *S. typhimurium* was examined according to the method of Kishore et al. (19, 21). The boiled samples of CE were resolved by performing electrophoresis in 1-mm-thick gels at a 33-mA constant current for 70 min in a discontinuous buffer system. The stacking and separating gels contained 4 and 12.5% acrylamide, respectively. After electrophoresis, proteins in the gel were transferred to a nitrocellulose membrane (Sartorius, Göttingen, Germany) at 0.8 mA/cm^2 for 1 h by using Transblot-cell equipment (Bio-Rad, Richmond, Calif.) and Western blotting (ligand blotting) was performed according to the method of Kishore et al. (20, 21). Preincubation of blots with unlabeled Lf abolished the Lf-HRPO interaction and excluded the possibility of direct HRPO binding to CE components.

Antibacterial assay for Lf. The effect of Lf on bacterial growth was estimated by measuring changes in conductance of the cultivation media by using a Malthus-AT system. The assay was performed with special glass tubes (10-ml volume) containing SPYE broth without or with Lf (0.01 to 10 mg/ml). The tubes were inoculated with test strains (~1.4 × 10^4 bacteria per ml) from an overnight SPYE broth culture and sealed with special screw caps fitted with platinum electrodes. The electrodes were immersed in a final volume of 3 ml of growth media, and the tubes were plugged into a Malthus electric adaptor connected to an electronic monitor and were incubated at 37°C. The metabolic activity of the bacteria was continuously measured as the rate of change in conductance (micro-Simmons [μSi] per hour) of the culture media. The conductance measurements were automatically recorded by a built-in computer system equipped with a Malthus-AT laboratory program. The following definitions were used. (i) Detection time (DT) (unit, hours) was the time required to initiate detection of change in conductance, since a minimal metabolic activity was required for a detectable conductance change (i.e., sensitivity of the assay). (ii) The inability of bacteria to cause a change in conductance was considered metabolic inhibition; bacteriostasis (units, hours) was the difference in the DTs obtained with bacteria in the absence and presence of Lf. (iii) The MIC (units, milligrams per milliliter) was the smallest amount of Lf required to elicit a complete inhibition of bacterial growth at the time point when the metabolism of the control (bacteria grown without Lf) reached stationary phase.

Data analysis. Correlations between binding and parameters of antimicrobial activity were made by linear regression analysis.

RESULTS

Fifteen clinical isolates of *E. coli* showing different magnitudes of ^{125}I-labeled Lf binding were examined for growth in SPYE media with or without Lf (Table 1). Lf at a concentration of 1 mg/ml caused bacteriostasis in all strains, with a mean DT increase of 4.1 h (minimum, 0.6 h; maximum, 13.8 h). At an Lf concentration of 2 mg/ml, the DT was increased in nine strains and unaffected or slightly decreased in six strains, compared with the DTs at an Lf concentration of 1 mg/ml. The kinetics of bacterial growth were measured as change in conductance per hour, and in the presence of 1 and 2 mg of Lf per ml the mean rates were 95.3 and 68.9 μSi/h, respectively. Finally, the total change in conductance of growth media during a period of 20 h was also determined. A majority of low-level binders (low binders) (<20% Lf

TABLE 1. Lf binding[a] and growth[b] of 15 clinical isolates of *E. coli* in SPYE media

Strain	% Binding	DT (h) with:			Conductance (μSi)					
					Change/h with:			Change in 20 h with:		
		Lf-0	Lf-1	Lf-2	Lf-0	Lf-1	Lf-2	Lf-0	Lf-1	Lf-2
E450	2 ± 1	3.6	6.7	6.2	377	258	217	619	555	516
E452	2 ± 1	3.9	6.8	8.6	236	171	117	606	632	555
E446	3 ± 1	4.1	5.5	5.9	322	235	202	645	632	606
E453	3 ± 1	6.2	10.0	7.8	264	184	119	632	594	581
E519	9 ± 1	6.2	9.8	11.3	240	110	105	503	413	387
E50	13 ± 2	5.4	6.1	7.4	263	139	97	594	529	516
E520	16 ± 3	6.6	11.5	10.3	126	30	21	555	284	206
E518	18 ± 3	6.8	12.0	13.6	129	35	29	542	284	194
E71	22 ± 2	4.3	6.8	9.0	253	106	104	542	477	400
E355	23 ± 3	6.2	>20	>20	44	0	0	361	0	0
E408	24 ± 1	7.0	7.6	>20	64	31	0	361	374	0
E354	24 ± 2	6.6	8.3	12.3	157	65	12	722	568	103
E412	25 ± 4	6.5	>20	>20	116	0	0	374	0	0
E413	27 ± 3	5.6	7.4	13.2	83	39	10	464	374	64
E386	28 ± 4	7.9	11.5	>20	47	26	0	387	181	0

[a] Bacteria grown in SPYE broth at 37°C for 20 h were harvested and examined for interaction with ^{125}I-labeled Lf as previously described (31, 33, 34) under comparable test conditions. Binding is expressed as the percentage of total labeled protein added (mean ± standard deviation).

[b] A comparable inoculum, i.e., 1.4×10^4 bacteria, was added to SPYE media containing Lf at a final concentration of 1 mg/ml (Lf-1) or 2 mg/ml (Lf-2), and media without Lf (Lf-0) served as a control. Bacterial growth was measured (as change in the conductance of the media) at 37°C during a period of 20 h, using Malthus equipment as described in Materials and Methods.

binding) recovered from the Lf-mediated bacteriostasis, and Lf slightly affected the growth of these bacteria during the 20-h incubation. However, the high binders (except strain E71) failed to recover from the antibacterial effect of Lf during this incubation period.

Data from Table 1 were further analyzed to find a relationship between the magnitude of Lf binding and the antibacterial action of Lf at a 1-mg/ml concentration (Fig. 2). The effect of Lf on the bacterial metabolic rate (expressed as a percentage, considering the rate of change in the conductance of the control to be 100% metabolism) demonstrated an inverse correlation ($r = 0.91$) with the magnitude of Lf binding to bacteria. The three strains that showed total metabolic inhibition with Lf also demonstrated a high level of binding to ^{125}I-labeled Lf (>22%). The Lf-mediated bacteriostasis, i.e., prolongation of DT compared with that of the control, also showed a correlation ($r = 0.79$) with the Lf interaction property of the bacteria. Complete metabolic inhibition could not be achieved, in particular, among low-binding strains. Thus, low-binding strain E446 demonstrated a diminished metabolism and no total inhibition was achieved at an elevated Lf concentration. On the other hand, the metabolism of high-binding strain E386 was totally ceased at an Lf concentration of 0.7 mg/ml (Fig. 3). However, no correlation was found between the MIC determinations and Lf-binding properties of the 15 *E. coli* strains tested (Fig. 2).

The extent of LPS involvement in Lf binding to bacteria was tested by using R mutants of *S. typhimurium*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (using HRPO-labeled Lf) of bacterial CE revealed Lf-binding proteins at approxi-

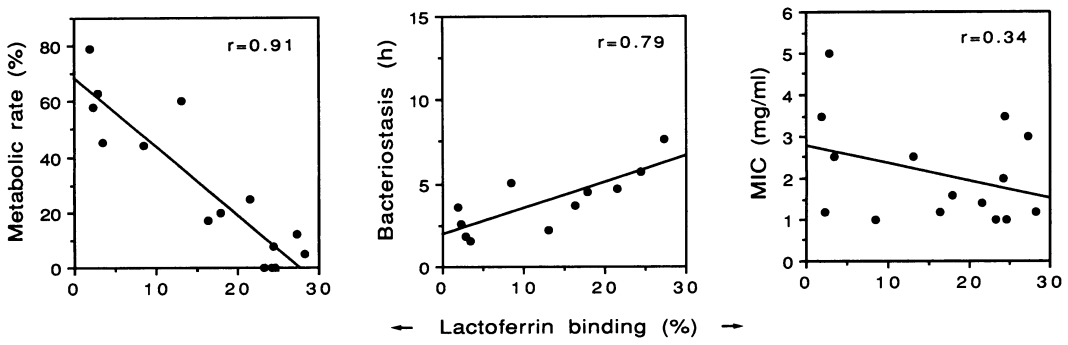

FIG. 2. Relationship between the magnitude of Lf binding and the antibacterial action of Lf in 15 clinical isolates of *E. coli*. Correlations between parameters were made by linear regression analysis. The metabolic rate in the presence of Lf (1 mg/ml) was expressed as a relative percentage, considering the change in the conductance rate of the control (without Lf) to be 100%. Bacteriostasis was estimated as the DT difference between bacterial growth in media with Lf (1 mg/ml) and the control. Three strains that demonstrated complete growth inhibition and one strain that showed growth enhancement at an Lf concentration of 1 mg/ml were excluded during analysis of the bacteriostasis parameter. For MIC determinations, bacteria were grown in the presence of 15 different concentrations of Lf within a range of 0.01 to 10 mg/ml. The smallest amount of Lf that caused complete inhibition of bacterial growth at the time point when the metabolism of the control reached stationary phase was considered to be the MIC.

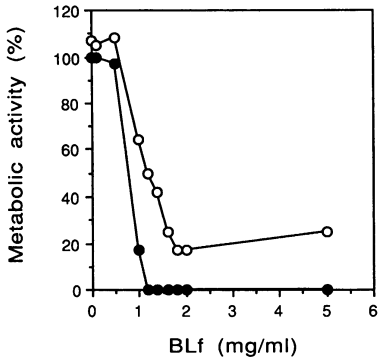

FIG. 3. Inhibition of bacterial growth as a function of Lf concentration in high-binding strain E386 (●) and low-binding strain E446 (○).

mately a 38-kDa position in the smooth parent 395MS(S) and also in the five isogenic R mutants (Fig. 4). The 38-kDa Lf-reactive bands demonstrated a heat-modifiable nature and anti-porin immunoreactivity (data not shown) similar to those of the *S. typhimurium* type strain, ATCC 13311 (21). However, in the whole cell binding assay, the smooth parent strain 395MS(S) demonstrated low binding (10%) and differed from its isogenic R mutants. The Lf interaction capacity gradually increased to a maximum of fourfold in the corresponding R mutants with various lengths of LPS polysaccharide moiety (Table 2). The conductance measurement studies revealed that the low-Lf-binding smooth parent strain was less inhibited by Lf (0.4- and 1.3-h stasis with 1 and 5 mg of Lf per ml, respectively). On the other hand, the high (44%)-Lf-binding mutant Rd was more susceptible to the antibacterial action of Lf (0.8 and 2.0 h with 1 and 5 mg of Lf per ml, respectively) (Fig. 5). The bacterial metabolic rate was slightly affected at 1 mg of Lf per ml and diminished about 50% at 5 mg of Lf per ml in the mutant Rd compared with the smooth parent.

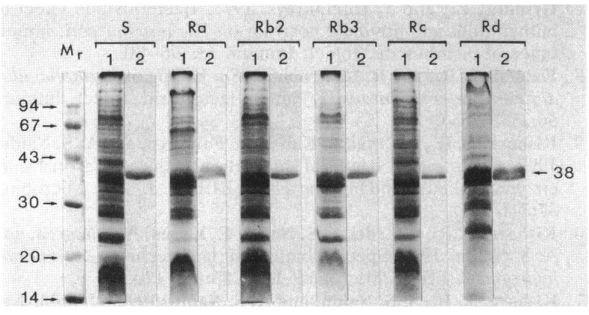

FIG. 4. Demonstration of Lf-binding proteins in the boiled CE preparation of *S. typhimurium* 395MS(S) and its isogenic R mutants with various lengths of LPS polysaccharide moiety (see Fig. 1). The bacterial CE was isolated and analyzed by SDS-PAGE and Western blotting (ligand blotting) as described by Kishore et al. (21). Coomassie-brilliant-blue-R-stained gels (lanes 1) and their corresponding blots probed with HRPO-labeled Lf (lanes 2) are shown. The molecular weight standards are indicated with arrows at the left, and the reactive band with its estimated molecular mass is indicated with an arrow at the right.

TABLE 2. Lf binding and antimicrobial activity in *S. typhimurium* 395MS(S) and its LPS-deficient isogenic mutants[a]

LPS type[b]	Binding[c] (%)	Bacteriostasis[d] (h) with:		Metabolic rate[e] (%) with:	
		Lf-1	Lf-5	Lf-1	Lf-5
S	10 ± 1	0.4 ± 0.2	1.3 ± 0.2	100 ± 8	76 ± 7
Ra	17 ± 2	0.5 ± 0.5	1.3 ± 0.3	96 ± 14	75 ± 13
Rb2	24 ± 3	0.3 ± 0.2	1.0 ± 0.2	91 ± 9	86 ± 11
Rb3	30 ± 2	0.2 ± 0.2	0.9 ± 0.2	101 ± 8	62 ± 7
Rc	34 ± 4	0.6 ± 0.2	0.8 ± 0.3	107 ± 4	82 ± 5
Rd	44 ± 4	0.8 ± 0.1	2.0 ± 0.1	98 ± 5	36 ± 3

[a] Bacterial growth was tested at an Lf concentration of 1 mg/ml (Lf-1) or 5 mg/ml (Lf-5) at 37°C for 24 h; media without Lf served as a control. Mean values and standard deviations are based on four experiments.
[b] Designated according to the scheme shown in Fig. 1.
[c] Binding is expressed as the percentage of total labeled protein added.
[d] DT difference between growth in media with Lf and the control.
[e] Expressed as the relative percentage, considering the change in the conductance rate of the control to be 100%.

DISCUSSION

Studies with 15 clinical isolates of *E. coli* with different Lf-binding capacities revealed that the concentration of Lf in growth media was a critical factor for the antibacterial effect. Lf at a supraoptimal concentration showed either a reduced or no additional effect in certain strains. Excess of free ligand in the media may affect the equilibrium of a receptor-ligand interaction (44). The binding of Lf to porins was reversible and had a low affinity (20, 21, 35, 48). Thus, at a supraoptimal concentration, free Lf in the media could

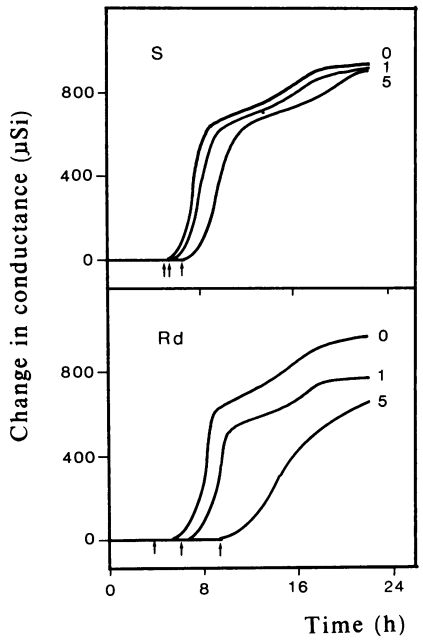

FIG. 5. Growth of *S. typhimurium* 395MS(S) (S; parent with smooth LPS) and its isogenic mutant Rd (with R LPS) in SPYE broth with Lf (1 and 5 mg/ml) or without Lf (0). The bacterial metabolism at 37°C was measured for 24 h as the change in conductance of growth media by using a Malthus-AT system as described in Materials and Methods.

possibly decrease specific interaction and reduce the antimicrobial activity of Lf. Under such conditions, Lf failed to elicit a total inhibitory effect on low binders. Accordingly, the MIC did not correlate with the bacterium binding property of Lf.

The magnitude of the Lf-bacterium interaction was inversely related to the change in the metabolic rate of the bacteria and was directly related to the degree of bacteriostasis. The metabolic suppression did not seem to be iron dependent, since an Lf peptide fragment without iron-chelating capacity showed both porin binding and metabolic inhibitory capacity (32). Interestingly, another peptide, generated by pepsin hydrolysis at acidic pH according to the method of Saito et al. (43), showed antimicrobial activity but failed to inhibit ^{125}I-Lf binding to *E. coli* and 10 other different species of the family *Enterobacteriaceae*. Furthermore, the antibacterial activity of the latter peptide was highly vulnerable to changes in the growth conditions (32). In *Actinobacillus actinomycetemcomitans*, the growth temperature increase of 2°C (37 to 39°C) markedly enhanced the susceptibility of the bacterium to Lf (18). Fatty acid composition and bacterial membrane permeability are highly prone to temperature-induced effects (27). Thus, our findings on Lf-porin interaction in members of the family *Enterobacteriaceae* and Lf-mediated enhancement in antibiotic susceptibility of *S. typhimurium* (15, 21, 48) suggest that Lf could damage the outer membrane of gram-negative bacteria and cause permeability alterations as proposed by Ellison and coworkers (14). It also seems possible that Lf might affect certain cellular pathways in bacteria. Arnold and coworkers (4) demonstrated that Lf could inhibit glucose uptake and metabolism in *Streptococcus mutans*. Peterson and Alderete (38) showed that the specific binding of Lf to *Trichomonas vaginalis* caused a sixfold increase in the pyruvate/ferredoxin oxidoreductase activity, an enzyme involved in energy metabolism.

For certain members of the family *Enterobacteriaceae*, Lf binding to strains with smooth LPS was low or negligible compared with that of their isogenic R mutants with a high Lf-binding capacity (16, 48). Our study with the isogenic R mutants of *S. typhimurium* suggested that the magnitude of Lf binding to whole cells was inversely related to the length of the LPS polysaccharide moiety. Accordingly, the low-Lf-binding smooth parent was less susceptible to Lf, and at corresponding conditions the growth of LPS-deficient mutant Rd was inhibited by 50%. By analogy, such LPS polysaccharide involvement in the blockade of certain colicin and bacteriophage interaction with porin targets was previously reported (49, 51). *S. typhimurium* could also demonstrate an increased susceptibility to magainin 2 (a small cationic antimicrobial peptide) with a gradual decrease in the length of the LPS polysaccharide moiety (40).

In conclusion, our data suggested a correlation between Lf binding to porins and the Lf-mediated antimicrobial effect. Shielding of porins by the polysaccharide moiety of LPS could block the bacterial interaction with Lf and decrease the antibacterial effect.

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Lactoferrin-Binding Proteins in *Shigella flexneri*

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The ability of *Shigella flexneri* to interact with lactoferrin (Lf) was examined with a ^{125}I-labeled protein-binding assay. The percent binding of human lactoferrin (HLf) and bovine lactoferrin (BLf) to 45 *S. flexneri* strains was 19 ± 3 and 21 ± 3 (mean ± standard error of the mean), respectively. ^{125}I-labeled HLf and BLf binding to strain M90T reached an equilibrium within 2 h. Unlabeled HLf and BLf displaced the ^{125}I-HLf–bacteria interaction in a dose-dependent manner. The Lf-bacterium complex was uncoupled by KSCN or urea, but not by NaCl. The interaction was specific, and ~4,800 HLf binding sites (affinity constant [K_a], 690 nM) or ~5,700 BLf binding sites (K_a, 104 nM) per cell were estimated in strain M90T by a Scatchard plot analysis. The native cell envelope (CE) and outer membrane (OM) did not reveal Lf-binding components in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, after being boiled, the CE and OM preparations showed three distinct horseradish peroxidase-Lf reactive bands of about 39, 22, and 16 kDa. The 39-kDa component was also reactive to a monoclonal antibody specific for porin (PoI) proteins of members of the family *Enterobacteriaceae*. The Lf-binding protein pattern was similar with BLf or HLf, for Crb$^+$ and Crb$^-$ strains. The protein-Lf complex was dissociable by KSCN or urea and was stable after treatment with NaCl. Variation (loss) in the O chain of lipopolysaccharide (LPS) markedly enhanced the Lf-binding capacity in the isogenic rough strain SFL1070-15 compared with its smooth parent strain, SFL1070. These data establish that Lf binds to specific components in the bacterial OM; the heat-modifiable, anti-PoI-reactive, and LPS-associated properties suggested that the Lf-binding proteins are porins in *S. flexneri*.

Shigella flexneri is the most common etiological agent of bacillary dysentery, especially that contracted by children in developing countries (6, 37). Bacterial adhesion to colonic epithelia, cell invasion, and multiplication in host tissues are necessary for the onset of shigellosis (19, 27, 45). The formation of ulcerative lesions, as well as the infiltration of erythrocytes and inflammatory cells, mainly polymorphonuclear leukocytes, into the bowel, is an early event in the pathogenesis (20, 27). It has been suggested that intense inflammatory responses prevent further systemic dissemination of *S. flexneri* (37). Various antimicrobial, acute-phase proteins secreted by the activated polymorphonuclear leukocytes may play an important role in the inflammatory host defense (7, 55). However, our knowledge of the interaction of acute-phase proteins with *S. flexneri* is limited.

Lactoferrin (Lf) is an antimicrobial acute-phase protein secreted by polymorphonuclear leukocytes as well as by exocrine glands (3, 7, 8, 33–35) and is found in milk and at the mucosal surface. The mechanism of Lf-mediated antimicrobial action has mainly been attributed to its iron deprivation capacity with bacteria (47). The antimicrobial activity seems to be enhanced when Lf adsorbs to bacteria (2, 12, 16). Furthermore, specific receptors for Lf were reported in the mammalian intestinal brush border (11, 14) and certain peptides from Lf were shown to inhibit the adherence of *S. flexneri* to enterocytes (24).

On the other hand, the ability of bacteria to compete for iron within the host is one of the factors that influence pathogenicity (49, 56). Under iron stress, *S. flexneri* may produce one or more siderophores in vitro, for acquiring the Fe^{3+} ion (48). *S. flexneri* may also utilize various host iron compounds, including Lf, transferrin, hemin, and hematin, in vitro (29). Furthermore, a 101-kDa heme-binding protein in *S. flexneri* has been identified (53). In pathogens such as those belonging to the genus *Neisseria*, Lf-binding proteins were also identified and attributed to iron acquisition (39, 52). Thus, Lf-bacterium interaction may be relevant in bacterial virulence and host defense. However, the interaction of Lf with *S. flexneri* has not been reported.

In this communication, we give evidence for the specific binding of Lf to *S. flexneri*. The nature of the Lf-bacterium interaction has been characterized, and the outer membrane (OM) proteins involved in Lf binding in *S. flexneri* M90T were identified.

MATERIALS AND METHODS

Bacterial strains. A total of 45 *S. flexneri* strains comprising 20 fecal isolates from gastroenteritis patients and 23 type strains (kindly supplied by É. Czirok and H. Milch, National Institute of Hygiene, Budapest, Hungary) were tested. Strains M90T (Crb$^+$) and M90T55 (Crb$^-$) were provided by T. Pál, Institute of Microbiology, University Medical School, Pécs, Hungary. In addition, a wild-type strain, SFL1070, containing smooth lipopolysaccharide (LPS) (Δ*aroD*, serotype 2a) and its isogenic mutant, SFL1070-15, with rough LPS were provided by A. Lindberg, Department of Clinical Bacteriology, Karolinska Institute, Stockholm, Sweden. Bacterial strains were grown in antibiotic (Penassay) medium 3 (Difco Laboratories, Detroit, Mich.) with continuous mechanical shaking (100 rpm) at 37°C for 24 h. Cells were harvested, washed, and resuspended in phosphate-buffered saline (PBS), pH 7.2. The cell density was adjusted photometrically (at 600 nm) to 10^{10} bacteria per ml for binding studies.

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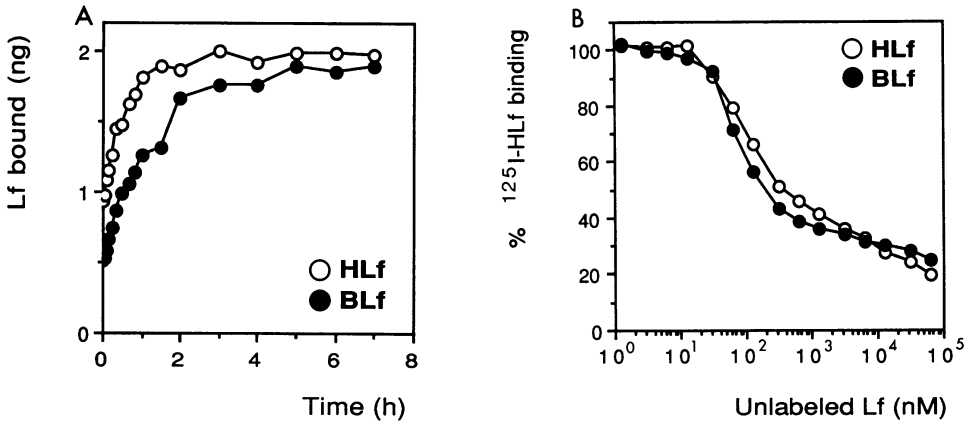

FIG. 1. (A) Kinetics of ^{125}I-HLf and ^{125}I-BLf binding equilibrium with strain M90T. Bacteria (10^9 cells in 0.1 ml) were incubated with ~8 ng of ^{125}I-Lf (~30 kcpm) in 0.1 ml of PBS at 37°C. At different time points (as indicated), the reaction was terminated by adding 2 ml of ice-cold PBS containing 0.1% Tween 20. The supernatant was aspirated after centrifugation (5,000 × *g*), and the radioactivity bound to the bacterial pellet was measured. The values shown represent binding after deducting the background radioactivity measurements. (B) Displacement of ^{125}I-labeled HLf binding by increasing amounts of unlabeled HLf or BLf. Approximately 10^9 cells (in 0.1 ml of PBS) of strain M90T were mixed with ~8 ng of ^{125}I-HLf (in 0.1-ml volumes) and incubated at 37°C for 2 h. Increasing amounts (1 to 10^5 nM) of unlabeled HLf or BLf (in 0.1-ml volumes, diluted in PBS) were added to the HLf-bacterium complex (final volume, 0.3 ml) and further incubated for an additional hour. The ^{125}I-labeled protein-binding measurements were made as described in the text, and the homologous (^{125}I-HLf versus unlabeled HLf, r = 0.98) and heterologous (^{125}I-HLf versus unlabeled BLf, r = 0.97) displacement curves were plotted.

Chemicals. Human lactoferrin (HLf) (lot 63541) purified from milk was purchased from the U.S. Biochemicals Corp., Cleveland, Ohio. Bovine lactoferrin (BLf) purified from milk whey was kindly provided by H. Burling, Swedish Dairies Association, Malmö, Sweden. Both Lf preparations were homogeneous in ion-exchange (Mono-Q column; Pharmacia, Uppsala, Sweden) and in molecular-sieve (TSK G4000 SW; LKB Produkter AB, Bromma, Sweden) high-performance liquid chromatography. Apo-Lf was prepared by removing iron from the protein by exhaustive dialysis against 0.1 M citrate (1). Transferrin (apo form, lot 67F.9457; iron-saturated form, lot 67F.9458), hemin (bovine, type I, lot 104F.0090), mucin type III (porcine stomach, lot 49F.3876), sodium deoxycholate, *N*-acetylglucosamine (lot 87F.0329), *N*-acetylgalactosamine (lot 77F.5053), *N*-acetylneuraminic acid (sialic acid type VIII from sheep submaxillary glands, lot 28F.1046), *N*-acetylmuramic acid (lot 18F.1016), Congo red (lot 118F-3494), and peroxidase type VIA from horseradish (HRPO) (EC 1.11.1.7, lot 69F.9525) were purchased from Sigma Chemical Co., St. Louis, Mo. All chemicals used for the preparation of buffer solutions were of analytical grade.

Congo red binding (Crb) and HeLa cell invasion assay. Congo red (0.01%, wt/vol) was incorporated into Trypticase soy broth and solidified with 1.5% agar. Crb$^+$ strains bound the dye and demonstrated red colonies. The invasive capacity of the strains was examined with HeLa cell monolayers as previously described (46).

^{125}I-Lf binding assay. HLf and BLf were labeled with Na^{125}I (specific activity, 629 GBq/mg; DuPont Scandinavia AB, Stockholm, Sweden) by using Iodobeads (Pierce Chemical Co., Rockford, Ill.) (32). Binding assays were performed as described previously by Naidu et al. (40, 41). Briefly, 10^9 bacteria (in 0.1 ml of PBS) were mixed with 0.1 ml of PBS containing ~8 ng of ^{125}I-Lf (specific activity, 0.16 MBq/μg). After incubation for 2 h at 37°C (according to binding equilibrium kinetics [Fig. 1A]), the binding reaction was terminated by adding 2 ml of ice-cold PBS (containing 0.1% Tween 20). The suspension was centrifuged at 5,300 × *g* for 15 min, and the supernatant was aspirated. Radioactivity bound to the bacterial pellet was measured in a gamma counter (Clinigamma 1272; LKB Wallac, Turku, Finland). The background radioactivity (from incubation mixtures without bacteria) was deducted, and the binding was expressed in percentages calculated from the total labeled ligand added to the bacteria. *Escherichia coli* E34663 and HH45 served as positive (60%) and negative (5%) binding controls, respectively.

A moderately Lf-binding *S. flexneri* laboratory strain, M90T, and a clinical isolate, SH10, were selected for characterization studies. Both strains showed similar binding characteristics; therefore, the data obtained with strain M90T are presented.

Dissociation of Lf-bacterium complex. Binding of ^{125}I-Lf to bacteria was performed as described above, and the following reagents of various molarities, i.e., 1 ml each of sodium chloride (1 to 5 M), potassium thiocyanate (1 to 5 M), and urea (1 to 8 M), were added to the ligand-bacterium complex. Reaction mixtures containing 1 ml of 20 mM potassium phosphate buffer, pH 7.2, served as the control (taken as 100% binding). After incubation at 37°C for 1 h, the mixture was centrifuged (5,300 × *g* for 15 min), the supernatant was aspirated, and the radioactivity bound to the bacterial pellet was measured.

Isolation of bacterial CE and OM. Strains M90T (Crb$^+$) and M90T55 (Crb$^-$) were grown in 400 ml of Penassay medium, harvested (100 × *g* for 15 min), and washed twice with 10 mM Tris HCl, pH 7.4, and the cell density was adjusted photometrically to ~4 × 10^{10} bacteria per ml in the same buffer containing 10 mM $MgCl_2$. Cells were sonicated (200 W, three times for 2 min each) on ice, and the sonicate was centrifuged (3,000 × *g* at 4°C for 15 min). The resulting supernatant was ultracentrifuged (Beckman L7; 100,000 × *g*, at 4°C for 1 h), and a pellet called the "cell envelope" (CE) was obtained. The CE preparation was further treated with 2% (vol/vol) Triton X-100 (Sigma) in 10 mM Tris HCl

containing 10 mM $MgCl_2$ and was kept at 37°C for 1 h with end-to-end rotation. After an additional ultracentrifugation step as described above, a pellet (designated the OM pellet) was obtained. Both CE and OM preparations were dissolved in 0.06 M Tris HCl, pH 6.8, containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and 10% (vol/vol) glycerol and were stored at −20°C.

SDS-PAGE and probing of the CE and OM with HRPO-labeled Lf. The CE and OM preparations were mixed with a sample buffer (0.06 M Tris HCl, pH 6.8; containing 5% β-mercaptoethanol, 2% SDS, and 10% glycerol), and also other CE and OM preparations were boiled in the above buffer at 100°C for 5 min. Both boiled and unboiled samples were tested by performing SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (28) in a Miniprotean II apparatus (Bio-Rad, Richmond, Calif.). The gel consisted of 12% acrylamide and 0.31% bisacrylamide, and the electrophoresis was run at 60 V for ~2 h.

After SDS-PAGE, proteins were electrophoretically transferred from the gel to a nitrocellulose membrane (Sartorius, Göttingen, Germany) at 200 mA for 2 to 3 h, by using semidry blot equipment (Novablot 2117-250; Pharmacia LKB). After being blocked with Tween 20 (1%, vol/vol) in Tris-buffered saline, pH 7.4 (TBS), for 90 min, the membrane was washed three times with TBS. HLf and BLf were coupled to HRPO by a periodate method (43). HLf- or BLf-HRPO (dilution, 1:350 in TBS) was added to the membrane and incubated at room temperature for 2 h. After the membrane was washed three times with TBS containing 0.05% Tween 20, a color reaction was developed with diaminobenzidine (0.25 mg/ml; Sigma) chromophore dissolved in 0.1 M sodium acetate buffer, pH 5.0, containing hydrogen peroxide (0.003%, vol/vol), and the reaction was terminated by adding 5% sodium pyrosulfite (1%, wt/vol). Preincubation of blots with unlabeled HLf abolished the HLf-HRPO interaction and excluded the possibility of direct HRPO binding to bacterial components.

Immunoblotting with an anti-porin MAb probe. Monoclonal antibodies (MAb) against a conserved porin protein domain (PoI) common to 10 different genera of the family *Enterobacteriaceae*, including *S. flexneri*, previously described by Henriksen and Mæland (22) were used. The boiled and unboiled CE preparations were run on SDS-PAGE gels, and the proteins in the gel were transblotted to a nitrocellulose membrane. Free sites on the membrane were blocked with TBS (containing 1% ovalbumin and 1% Tween 20) for 30 min. Anti-PoI antibody (MAb F9-16) was mixed with the blocking solution at a working dilution of 1:5,000, and the solution was incubated at room temperature for 2 h with gentle shaking. After the membrane was washed thoroughly with TBS, it was incubated with HRPO-labeled rabbit antibodies to mouse immunoglobulins (lot 059; Dakopatts A/S, Glostrup, Denmark) at room temperature for 1 h with gentle shaking. After a final washing step, the membrane was developed with diaminobenzidine chromophore. Strips incubated with only labeled second antibody served as controls.

LPS analysis by SDS-PAGE. Strain SFL1070 and its mutant, SFL1070-15, were examined for LPS patterns as previously described (18). The sarcosyl-extracted OM preparations from the bacteria were boiled for 10 min and digested with proteinase K (30 to 60 U/ml; Sigma) at 37°C for 2 h. The preparations were run on SDS-PAGE gels as described above, and the gels were stained with a silver staining kit (Bio-Rad; lot 35709) according to the manufacturer's instructions.

TABLE 1. ^{125}I-labeled HLf and BLf binding to *S. flexneri* strains[a]

Strain	Serotype or subsp.	% Binding (mean ± SEM) to:	
		^{125}I-HLf	^{125}I-BLf
NCTC 9950	Type 1a	13 ± 3	19 ± 5
63-125-700	Type 1a	16 ± 2	19 ± 2
DRL41/53	Type 1b	10 ± 1	14 ± 3
OKI2/68	Type 1b	11 ± 1	11 ± 1
26D	Type 2a	16 ± 1	20 ± 1
Sc592	Type 2a	13 ± 1	17 ± 1
Sc661	Type 2b	14 ± 3	19 ± 3
DRL5/63	Type 3a	14 ± 1	20 ± 2
OKI264	Type 3a	16 ± 1	21 ± 2
1308/68/2	Type 3b	16 ± 1	18 ± 1
PHLS11D	Type 4a	11 ± 2	13 ± 2
Carpenter IV	Type 4a	16 ± 2	20 ± 1
NCTC 8522	Type 4b	10 ± 1	12 ± 2
OKI840	Type 4b	14 ± 1	15 ± 1
M90T	Type 5	17 ± 1	19 ± 2
M213 x$^-$	Type 5	14 ± 2	16 ± 2
OKI273	Type 5	16 ± 1	20 ± 1
67-104-1	Type6	13 ± 1	10 ± 1
NCTC 9729	Type 6	12 ± 1	15 ± 1
Newcastle	6 subsp. newcastle	16 ± 2	22 ± 2
DRL531/61	Subsp. *X*	12 ± 1	14 ± 2
OKI222	Supsp. *X*	14 ± 2	18 ± 2
Ledingham	Subsp. *Y*	12 ± 1	12 ± 1
NCTC 9730	Subsp. *Y*	12 ± 1	15 ± 1

[a] Bacteria grown in Penassay broth were tested for Lf interaction in a ^{125}I-labeled protein-binding assay as described in the text. Values represent the percent Lf bound to 10^9 bacteria incubated with ~8 ng of ^{125}I-protein for 2 h (SEM based on four experiments).

RESULTS

The magnitude of ^{125}I-labeled HLf and BLf binding (expressed as the percent Lf bound from 8 ng of labeled ligand added) to *S. flexneri* type strains (n = 24) is shown in Table 1. The type strains and an additional 20 clinical isolates demonstrated mean percent (mean ± standard error of the mean [SEM]) binding of 19 ± 3 (minimum, 10; maximum, 33) and 21 ± 3 (minimum, 10; maximum, 36) for HLf and BLf, respectively.

The nature of the Lf-*S. flexneri* interaction was characterized in strain M90T. Strain M90T (10^9 bacteria) demonstrated a binding equilibrium within 2 at ~8 ng of ^{125}I-labeled HLf or BLf per ml (Fig. 1A). Of the various unlabeled proteins and carbohydrates tested, only Lf from bovine and human species strongly inhibited (~90%) the ^{125}I-labeled Lf binding to strain M90T, both in homologous and heterologous combinations (Table 2). The iron-saturated Lf preparations were slightly more inhibitory than the apo forms. Iron-binding proteins (i.e., transferrin and hemin) and other commonly occurring substances of the gastrointestinal tract, such as mucin, deoxycholate, and glycoconjugates (i.e., *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, and *N*-acetylmuramic acid), caused less than 30% inhibition of the Lf binding. The displacement of ^{125}I-HLf binding to bacteria by homologous (HLf) or heterologous (BLf) unlabeled Lfs was dose dependent and required ~4 μg of protein per ml to elicit a 50% effect (Fig. 1B). The stability of the ^{125}I-HLf–bacterium complex was examined in a whole-cell binding assay with different chemical agents from 0 to 5 M (Fig. 2). The binding was stable in the presence of 5 M NaCl. However, KSCN and urea caused

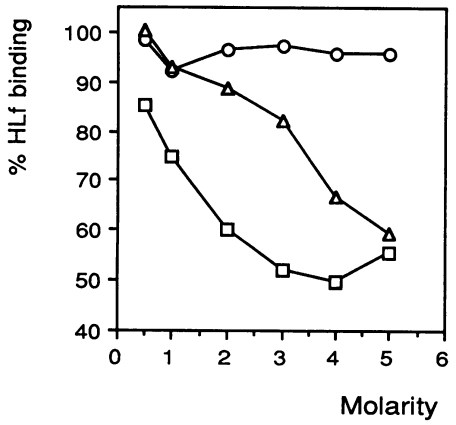

FIG. 2. Stability of ^{125}I-HLf–bacterium interaction in strain M90T. The ^{125}I-HLf binding to bacteria was performed at 37°C for 2 h, as described in the text. The reaction was terminated by adding ice-cold PBS-Tween; after centrifugation, the radioactivity bound to the bacterial pellet was measured. Various molarities (in 1-ml volumes) of NaCl (○), KSCN (□), or urea (▽) were added to tubes containing the ligand-bacterium complex; control tubes received 1 ml of 20 mM phosphate buffer, pH 7.2. The mixtures were allowed to stand for an additional 1 h at 37°C and were then centrifuged (5,300 × *g* for 15 min); the supernatant was aspirated, and the radioactivity retained with the bacterial pellet was measured. Binding was expressed in percentages relative to that of the controls (100%).

a concentration-dependent dissociation of the HLf-bacterium complex.

The concentration-dependent saturability of ^{125}I-HLf and ^{125}I-BLf binding to strain M90T was estimated (Fig. 3). The magnitude of nonspecific binding was higher for the bacterial interaction with HLf than with BLf. In a limited time of 2 h, approximately 5 μg of HLf or 7.5 μg of BLf could saturate Lf binding to ~10^9 bacterial cells. The specific binding values obtained from Fig. 3 were analyzed in a Scatchard plot (50) (Fig. 4). Thus, 4,800 HLf-bacterium binding sites, with an affinity constant (K_a) of 690 nM, and 5,600 BLf-bacterium binding sites (K_a, 104 nM) were estimated for strain M90T.

TABLE 2. ^{125}I-labeled HLf and BLf binding to strain M90T in the presence of various unlabeled proteins and carbohydrates[a]

Substance (0.1 mg/ml)	% Binding (mean ± SEM)	
	^{125}I-HLf	^{125}I-BLf
Control (PBS)	100	100
HLf (apo)	13 ± 1	13 ± 1
HLf (iron saturated)	10 ± 2	12 ± 1
BLf (apo)	11 ± 1	9 ± 1
BLf (iron saturated)	9 ± 1	8 ± 1
Transferrin (apo)	77 ± 1	75 ± 3
Transferrin (iron saturated)	78 ± 1	77 ± 3
Hemin	70 ± 2	71 ± 4
Mucin type III	70 ± 2	75 ± 2
Sodium deoxycholate	95 ± 2	93 ± 1
N-acetylglucosamine	80 ± 6	82 ± 4
N-acetylgalactosamine	82 ± 8	78 ± 5
N-acetylneuraminic acid	81 ± 5	73 ± 1
N-acetylmuramic acid	77 ± 6	76 ± 2

[a] Bacteria (10^9 cells in 0.1 ml) were incubated with unlabeled protein or carbohydrate (10 μg in 0.1 ml) at 37°C for 1 h. A volume of 0.1 ml of ^{125}I-labeled HLf or BLf (~8 ng, corresponding to 30 kcpm) was added to the above mixture and incubated at 37°C for 2 h with gentle shaking. The binding experiment was performed as described in the text. The percent binding was calculated from ^{125}I-Lf binding to cells in PBS (control) (SEM based on four experiments).

The presence of Lf-binding components in the CE and OM of strains M90T (Crb$^+$) and M90T55 (Crb$^-$) was examined by using HRPO-labeled Lf by Western blot (ligand blot) analyses (Fig. 5). Unheated CE and OM preparations did not show any Lf-binding components. However, after boiling, the same preparations revealed three distinct HRPO-Lf reactive bands of approximately 39, 22, and 16 kDa. Of these three heat-modifiable CE components, only the 39-kDa protein was weakly reactive against anti-PoI antibody. The pattern of Lf-binding proteins was similar for BLf and HLf in Crb$^+$ and Crb$^-$ strains. Furthermore, the stability of the receptor-HLf complex in the presence of NaCl, KSCN, and urea was tested by Western blot (Fig. 6). The HRPO-HLf-receptor complex readily dissociated with increasing molarities of KSCN or urea, but not with NaCl, which is similar to the Lf-whole cell dissociation results shown in Fig. 4.

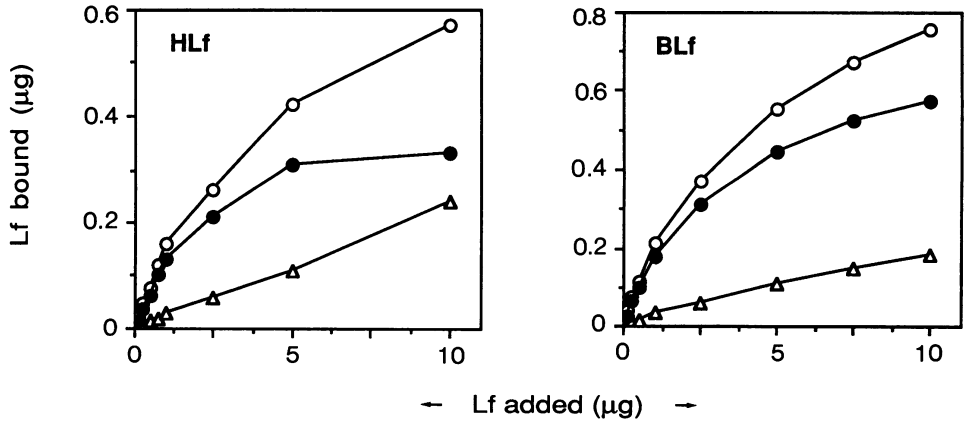

FIG. 3. Saturability of specific binding of ^{125}I-labeled HLf and -BLf to strain M90T. Increasing amounts of ^{125}I-labeled Lf (range, 0.1 to 10 μg) in 0.1-ml volumes were added to bacteria (8 × 10^8 cells per 0.1 ml), and the mixture was kept at 37°C for 2 h. Binding was performed in the absence (○ [total binding]) or presence (▽ [nonspecific binding]) of 50-fold excess unlabeled ligand. Specific binding (●) was calculated by subtracting the nonspecific Lf uptake values from total binding values.

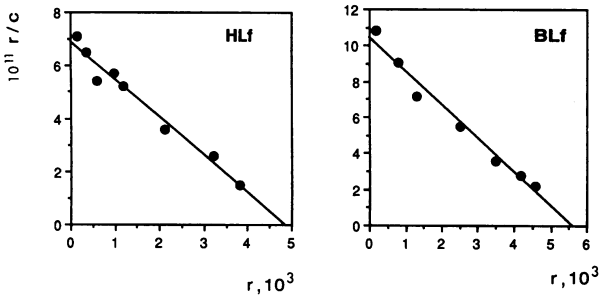

FIG. 4. Scatchard plot analysis ($r/c = nK_a - rK_a$) of ^{125}I-Lf binding to *S. flexneri* M90T. The molecules of Lf bound to one bacterial cell (r) and the molecules of Lf free in the medium (c) were calculated by assuming molecular masses of 82,400 and 92,100 daltons for HLf and BLf, respectively (38, 57). The intercept on the x axis represents the number of receptors per cell (n). The slopes of the lines represent the effective K_a (expressed as the nanomolar concentration).

The ^{125}I-HLf-binding capacity of the parent strain SFL1070 was 15 ± 1% (mean ± SEM), and the corresponding value for the isogenic derivative SFL1070-15 with rough LPS was 42 ± 2% (mean ± SEM) (about threefold higher binding). The boiled OM of the rough strain showed an incomplete O chain in the LPS structure compared with the smooth LPS of the parent strain; however, both strains demonstrated similar Lf-binding protein patterns (Fig. 7).

DISCUSSION

Breast-feeding has been suggested to decrease the frequency of symptomatic shigellosis and also the severity of the illness (10, 36). This protection has been attributed to various components in milk such as immunoglobulins, nonspecific cell receptor analogs, and Lf (8, 9). Lf has been shown to elicit bacteriostatic and also bactericidal effects (2, 34, 47). An enhanced antimicrobial activity could be achieved when Lf adsorbs to the bacteria (12, 16). Recently, we have described specific receptors for Lf in certain bacteria causing intestinal infections, i.e., *E. coli* and *Aeromonas hydrophila* (25, 42). The present study also demonstrates the binding of HLf and BLf to specific outer membrane proteins (OMPs) in *S. flexneri*.

The binding of HLf and BLf to strain M90T reached an equilibrium within a definite time and implied that there are a limited number of interaction sites on the bacterium. Binding inhibition studies indicated that strain M90T interacts with apo- and iron-saturated forms of Lf from human and bovine species. Extraintestinal pathogens, i.e., *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae*, seem to recognize only HLf but not BLf (30, 51, 52). Furthermore, various compounds common in the gastrointestinal tract caused little or no effect on Lf binding to bacteria. The ^{125}I-HLf binding displacement studies suggested that the bacterium recognized both isotope-labeled and unlabeled forms of ligands, preferably the latter form. The binding was reversible, hence the interaction seems to be of a low affinity. The binding characteristics of *S. flexneri* seem to be similar to those reported for *E. coli* and *A. hydrophila* (25, 42).

We have recently demonstrated that Lf recognizes porins OmpC and OmpF in *E. coli* (17). A 40-kDa Lf-binding protein in *A. hydrophila* (25) also seems to be a previously described pore-forming protein (13). Lf also binds to porins in a *Salmonella* sp. and potentiates the action of antibiotics by altering the permeability of the bacterial OM (26). The boiled preparations of CE and OM of *S. flexneri* M90T revealed three Lf-binding proteins of 39, 22, and 16 kDa in an SDS-PAGE and Western blot analysis. The Lf-binding protein pattern was similar for HLf and BLf among Crb$^+$ and Crb$^-$ variants of strain M90T. However, the corresponding protein bands were absent in the native (unboiled) CE and OM preparations. This heat-modifiable property is a characteristic feature for porins (the pore-forming OMPs) (4, 31). Henriksen and Mæland (22) have described an MAb that could specifically recognize a conserved domain in the PoI protein in a majority of bacteria belonging to the family *Enterobacteriaceae*. The 39-kDa heat-modifiable, Lf-binding

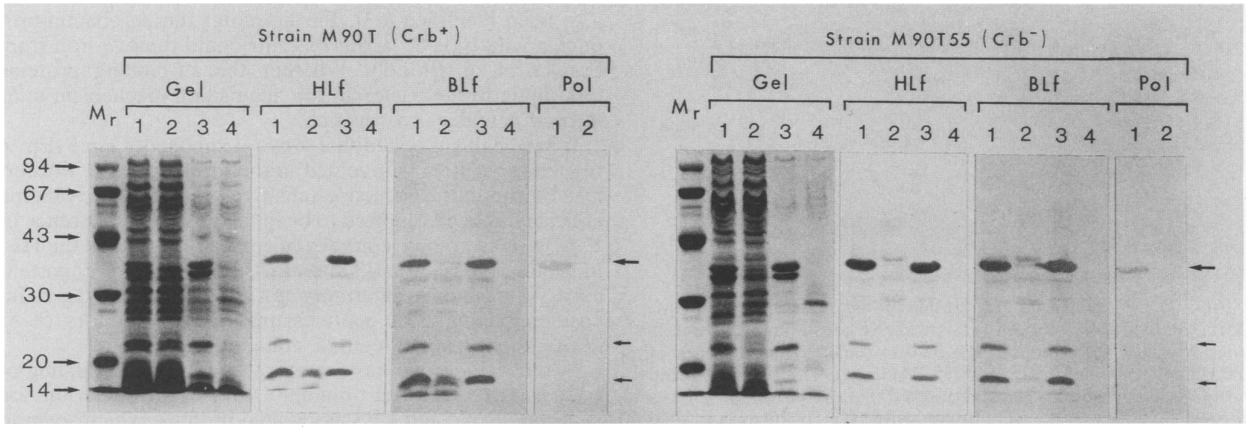

FIG. 5. Demonstration of Lf-binding proteins and porins in Crb$^+$ and Crb$^-$ strains of *S. flexneri* by Western blotting after SDS-PAGE. The CE and OM proteins of Crb$^+$ M90T and its Crb$^-$ variant, M90T55, were analyzed as native and boiled preparations by SDS-PAGE (12% acrylamide, 0.31% bisacrylamide), and the electrophoresis was run at 60 V for ~2 h. Proteins from unstained gels were transblotted to a nitrocellulose membrane, and the free sites on the membrane were blocked. The membranes were probed with HRPO-labeled HLf, HRPO-labeled BLf, or PoI (anti-porin MAb F9-16) as described in the text. Lanes 1, CE boiled at 100°C for 5 min; lanes 2, native CE; lanes 3, OM boiled at 100°C for 5 min; lanes 4, native OM. The gel was stained with Coomassie brilliant blue R. The molecular mass standards (kilodaltons) are indicated by arrows to the left, and the HRPO-Lf-reactive bands are indicated by arrows to the right of each gel.

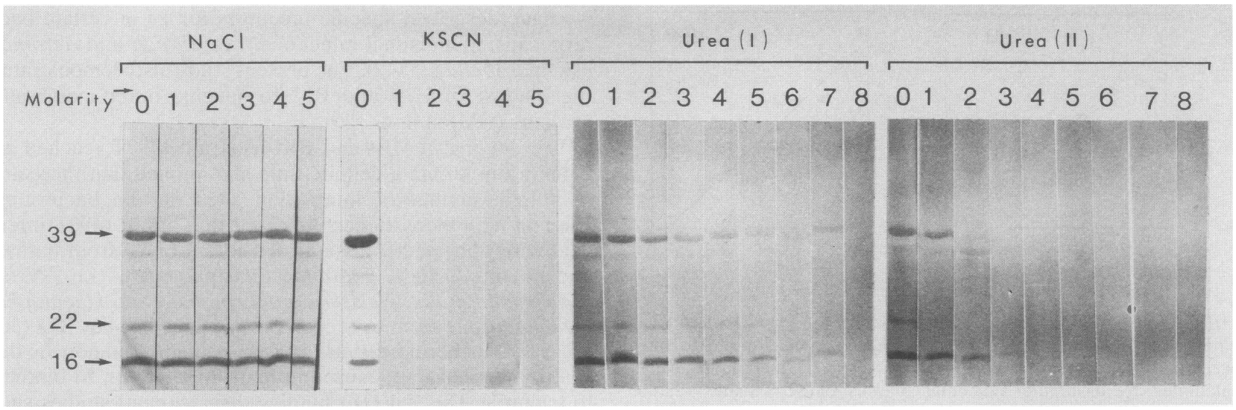

FIG. 6. Effect of various chemical agents on the bacterial OMP-Lf complex. The heat-treated OMPs of strain M90T were run on SDS-PAGE gels and transblotted to a nitrocellulose membrane, and free sites on the membrane were blocked. Each membrane strip was incubated with HLf-HRPO conjugate for 2 h and thoroughly washed with TBS-Tween. The strips were further incubated with various molarities (in 2-ml volumes) of NaCl, KSCN, or urea [Urea(I)] for 1 h. After a thorough wash (three times) with TBS-Tween, the strips were developed with diaminobenzidine as described in the text. An additional set of strips was treated with urea prior to the binding reaction with HLf-HRPO [Urea(II)]. Arrows to the left indicate the molecular masses (kilodaltons) of the HLf-HRPO-reactive bands.

component identified in *S. flexneri* was reactive against this immunoprobe. Porins in their native trimeric form associate with LPS and dissociate into monomers by boiling in SDS (15). Some of these porins perform key functions such as selective uptake of various nutrients and serving as receptors for bacteriophages and colicins (4, 31, 44). The O chain of LPS associated with porins may shield the receptor function (54). In this study, we have also shown a similar LPS O chain-mediated masking of Lf binding in *S. flexneri* by using a smooth strain and its isogenic rough derivative. We have also demonstrated in a previous study that the smooth LPS associated with native porins blocks *E. coli* from Lf interaction (18). Thus, the Lf-binding components in *S. flexneri* also appear to be porins. Though porins occur at a copy number of 10^5 per bacterium (4, 31), an estimated 20-fold less binding sites per cell in strain M90T in Scatchard plot analyses may be due to the availability of a limited number of porins for Lf interaction. The Scatchard plot was linear and implied a single class of receptors; the 22- and 16-kDa Lf-binding components detected in the Western blot are possibly cleaved products of the porin.

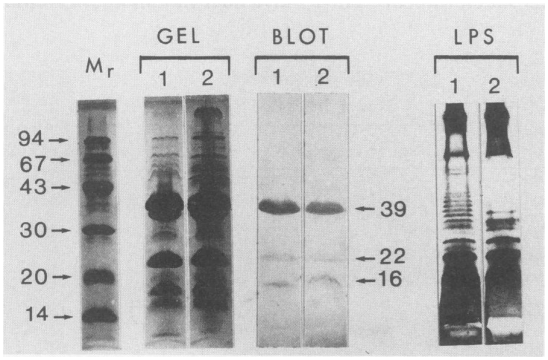

FIG. 7. LPS and Lf-binding protein profile of the *S. flexneri* OM. OMs isolated from a smooth LPS strain, SFL1070 (lanes 1), and its isogenic rough LPS mutant, SFL1070-15 (lanes 2), were boiled at 100°C for 10 min and run on an SDS-PAGE gel. The gel was stained with Coomassie brilliant blue. Proteins transferred from the gel to a nitrocellulose membrane were probed with HLf-HRPO as described in the text. A boiled OM preparation was digested with proteinase K (30 to 60 U/ml; Sigma) at 37°C for 2 h and run on an SDS-PAGE gel; the gel was silver stained to demonstrate LPS. The electrophoresis and blotting conditions were described in the text. Arrows on the left indicate molecular mass standards (kilodaltons), and arrows to the right of the blot indicate the molecular masses (kilodaltons) of the HLf-HRPO-reactive bands.

Lf is a basic protein (pI 8.5) and may demonstrate electrostatic interactions with acidic molecules (21). Such a charge interaction between Lf and bacteria or their OMPs is less likely since NaCl (5 M) failed to dissociate the complex. The binding seems to be a protein conformation-dependent one, since chaotropic agents caused an effective uncoupling of the complex. Nonspecific hydrophobic interactions were reduced by incorporating Tween 20 in the test system.

The ability of bacteria to compete for iron within the host may influence pathogenesis (56). Aerobactin-producing *S. flexneri* strains may utilize host iron sources such as hemin or hematin (29), and a 101-kDa heme-binding protein has also been identified (53). Furthermore, the aerobactin-producing wild-type *S. flexneri* strains could remove iron from transferrin or Lf (29). Whether the Lf-binding proteins contribute to the bacterial iron acquisition mechanism in *S. flexneri* is under investigation.

It has been suggested that a two-component OmpR-EnvZ regulatory system is involved in the virulence of *S. flexneri* (5). Furthermore, a fucose-sensitive, carbohydrate-binding adhesin has been reported to be involved in the adherence of *S. flexneri* to colonic epithelia of guinea pigs (24). In the HLf molecule, fucose at α1-3 and at α1-6 linkages are important eucaryotic binding determinants (23, 38). Interestingly, fucose-containing HLf peptides are strong inhibitors of *S. flexneri* adherence to colonic epithelia (24).

In conclusion, we have demonstrated specific binding of Lf to *S. flexneri*. The Lf-binding OMPs in the bacterium are heat-modifiable and LPS-associated proteins which seem to be porins. The role of specific Lf interaction with *S. flexneri* in the pathogenesis of bacillary dysentery, however, remains to be elucidated.

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Specific binding of lactoferrin to *Aeromonas hydrophila*

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1. SUMMARY

The interaction of lactoferrin (Lf) with *Aeromonas hydrophila* ($n = 28$) was tested in a ^{125}I-labeled protein-binding assay. The mean per cent binding values for human Lf (HLf) and bovine Lf (BLf) were 13.4 ± 2.0 (SEM), and 17.5 ± 2.7 (SEM), respectively. The Lf binding was characterized in type strain *A. hydrophila* subsp. *hydrophila* CCUG 14551. The HLf and BLf binding reached a complete saturation within 2 h. Unlabeled HLf and BLf displaced ^{125}I-HLf binding in a dose-dependent manner, and more effectively by the heterologous (1 μg for 50% inhibition) than the homologous (10 μg for 50% inhibition) ligand. Apo- and holo-forms of HLf and BLf both inhibited more than 80%, while mucin caused approx. 50% inhibition of the HLf binding. Various other proteins (including transferrin) or carbohydrates did not block the binding. Two HLf-binding proteins with an estimated molecular masses of 40 kDa and 30 kDa were identified in a boiled-cell-envelope preparation, while the unboiled cell envelope demonstrated a short-ladder pattern at the top of the separating gel and a second band at approx. 60 kDa position. These data establish a specific interaction of Lf and the Lf-binding proteins seem to be porins in *A. hydrophila*.

2. INTRODUCTION

The habitat of *Aeromonas hydrophila* is fresh water, sewage, unpasteurized milk, etc. and this bacterium has gained acceptance as an aetiological agent of acute diarrhoeal disease, in particular among children [1,2]. The disease transmission is through food and water, with an increasing incidence in developing countries [2]. Breast-feeding is known to protect children against several intestinal infections and this prophylaxis has been attributed to various antimicrobial agents in human milk [3]. Lactoferrin (Lf) is an antimicrobial, iron-binding protein present in milk and in various exocrine secretions at high concentrations

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[4]. However, the interaction of Lf with *A. hydrophila* has not been reported so far.

A. hydrophila is also an increasing cause of nosocomial septicaemia in immunologically compromised hosts [5,6]. A majority of such susceptible individuals, demonstrate both qualitative and quantitative defects in polymorphonuclear leukocytes (PMNLs) [5]. PMNLs are important in the production of various antimicrobial acute phase proteins (APPs) during inflammatory cell-mediated protection [7]. Lf is also an APP produced by PMNLs and its concentration significantly rises during inflammation or endotoxic shock [8,9]. Lf has been suggested to elicit bacteriostatic, bactericidal and opsonic effects on various microbial pathogens [10] and the adsorption of Lf to bacteria may enhance these antimicrobial effects [11].

In this communication we have demonstrated interaction of human and bovine LFs with laboratory strains and clinical isolates of *A. hydrophila*. The nature of Lf-bacteria interaction has been characterized in a type strain *A. hydrophila* subsp. *hydrophila* CCUG 14551 and the proteins involved in the specific binding of Lf were identified in the cell envelope of *A. hydrophila*.

3. MATERIALS AND METHODS

3.1. Bacteria

All of the *A. hydrophila* strains were from the Culture Collection, University of Göteborg (CCUG). The strains originated from divergent clinical specimens and foods, isolated at various geographical regions. The lyophilized cultures were revived on blood agar base No. 2 (Oxoid Limited, Basingstoke, U.K.) supplemented with 10% human blood, at 37 °C, for 18 h. For binding experiments, bacteria were subcultured twice in the above conditions, cells were harvested, washed in phosphate buffered saline, pH 7.2 (PBS), and the density of the suspension was adjusted photometrically (at 600 nm absorbance) to 10^{10} cells/ml. Binding characterization studies were performed with a type strain *A. hydrophila* subsp. hydrophila CCUG 14551 (ATCC 7966; NCTC 8049; NCIB 9240).

3.2. ^{125}I-labeled Lf binding assay

HLf (lot 63541) purified from milk, was purchased from US Biochemicals, Cleveland, OH. BLf purified from milk whey was kindly provided by Dr. H. Burling, Swedish Dairies Association, Malmö, Sweden. Both Lf preparations were homogenous in ion-exchange (Mono-Q column, Pharmacia, Uppsala, Sweden) and in molecular-sieve (TSK G4000 SW, LKB Produkter AB, Bromma, Sweden) high-performance liquid chromatography. Proteins were labeled according to a modified chloramine-T method with Na[^{125}I] (DuPont Scandinavia, Stockholm, Sweden) using Iodobeads (Pierce Chemicals, Rockford, IL) [12] to a specific activity of 0.15 MBq/μg for HLf, and 0.16 MBq/μg for BLf. Binding assays were performed as described earlier by Naidu et al. [13,14]. Briefly, 10^9 bacteria (in 0.1 ml PBS) were mixed with 0.1 ml of ^{125}I-Lf (radioactivity adjusted to $2–2.5 \times 10^4$ cpm, corresponding to approx. 8 ng of the protein). After incubation for 1 h at room temperature, 2 ml of ice-cold PBS (containing 0.1% Tween-20) was added to stop the binding reaction. The suspension was centrifuged at $10\,000 \times g$ for 15 min and the supernate was aspirated. Radioactivity bound to the bacterial pellet was measured in a gammma counter (LKB Wallac Clinigamma 1272, Turku, Finland). Binding was expressed as a per cent value, calculated from the total labeled ligand added to the bacteria. A background radioactivity (from incubation mixtures without bacteria) of 2.5% was subtracted from the values.

3.3. SDS-PAGE analysis of bacterial cell envelope and outer membrane

The cell envelope and the outer membrane were prepared from strain CCUG 14551, according to Schnaitman [15]. Triton X-100 insoluble fractions were recovered by ultra-centrifugation ($100\,000 \times g$) and were used as outer membranes. Both preparations were analysed by SDS-PAGE in discontinuous buffer system described by Laemmli [16]. Separating gels containing 12.5% acrylamide and 0.2% SDS were run in a Miniprotean II apparatus (Bio-Rad, Richmond, VA.) at 60 V for approx. 2 h.

3.4. Western-blot analysis with horseradish peroxidase (HRPO) conjugated HLf

After SDS-PAGE, proteins in the gel were transferred to nitrocellulose membrane (Sartorius, Göttingen, FRG) at 0.8 mA/cm^2 for 2–3 h, using a semi-dry blot equipment (Novablot 2117-250; Pharmacia LKB, Bromma, Sweden). The free sites on the membrane were blocked with 1% Tween-20 for 15 min. HLf was coupled to HRPO according to Nakamura et al. [17] and the membranes were probed with this conjugate. The colour reaction was developed with diaminobenzidine (2.5×10^{-4}% w/v; Sigma) dissolved in 0.05 M sodium acetate buffer, pH 5.0, containing hydrogenperoxide (3×10^{-4}% v/v), and the reaction was terminated by adding 5% sodium pyrosulfite (1% w/v). Preincubation of blots with unlabeled HLf has abolished the HLf-HRPO interaction, excluding the possibility of direct HRPO binding to bacterial components.

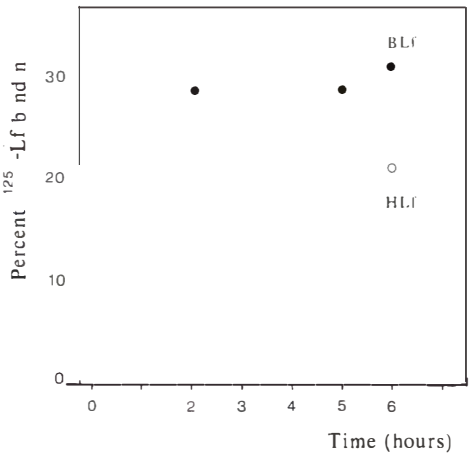

Fig. 1. Time course of ^{125}I-labeled HLf and BLf binding. Cells of strain CCUG 14551 were incubated with ^{125}I-HLf or -BLf at time intervals indicated. Values represent the radioactivity bound to 10^9 bacteria after deducting the background (radioactivity) measurement.

4. RESULTS

A total of 28 *A. hydrophila* strains were tested for HLf- and BLf-binding in a ^{125}I-labeled protein-binding assay. The mean per cent binding values for HLf and BLf were 13.4 ± 2.0 (SEM) (6.0–22.1% range) and 17.5 ± 2.7 (SEM) (6.6–32.6% range) respectively.

The Lf binding to *A. hydrophila* subsp. *hydrophila* strain CCUG 14551 was time-dependent and required approximately 2 h for saturation of both human and bovine proteins (Fig. 1). Unlabeled HLf and BLf displaced ^{125}I-HLf binding in a dose-dependent manner, and more effectively by the heterologous (1 μg to elicit 50% inhibition) than the homologous (10 μg to elicit 50% inhibition) ligand (Fig. 2).

The inhibitory capacity of various unlabeled proteins and carbohydrates (at 0.1 mg/ml concentration) on ^{125}I-HLf binding to strain CCUG 14551 was tested. Iron-free and -saturated forms of HLf and BLf both elicited more than 80%, while mucin caused approx. 50% inhibition of the HLf binding. Other proteins (apo-transferrin, iron-saturated transferrin, fetuin, bovine serum albumin, and ovalbumin) or carbohydrates (D(+)

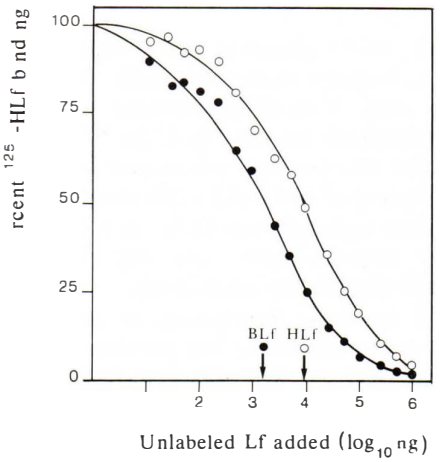

Fig. 2. Displacement of ^{125}I-HLf binding to strain CCUG 14551 by unlabeled lactoferrin. Increasing amounts (as indicated) of unlabeled HLf or BLf (in 0.1 ml volume) were mixed with approx. 8 ng of ^{125}I-labeled HLf (in 0.1 ml volume). Approximately 10^9 bacteria were added to this mixture (final volume, 0.3 ml) and incubated at 37 °C for 2 h. Binding experiment was performed as described. ^{125}I-HLf binding to bacteria in PBS was considered as 100%. Arrows indicate 50% displacement values.

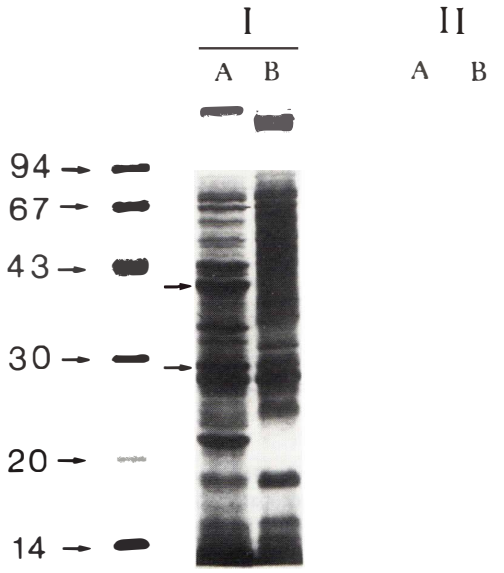

Fig. 3. Cell envelope protein profile in SDS-PAGE and Western blot of HLf-binding proteins in *A. hydrophila*. Boiled (lane **A**) and unboiled (lane **B**) cell envelope preparations I. Gel stained with Coomassie Brilliant Blue. II. Blot with HLf-HRPO conjugate.

glucose, D(+) galactose, D(+) mannose, L(−) fucose, *N*-acetyl neuraminic acid, *N*-acetyl muramin acid, *N*-acetyl glucosamine and *N*-acetyl galactosamine) did not block the ^{125}I-HLf binding. The HLf-bacteria interaction was stable in the presence of 4 M NaCl, while chaotropic agents guanidine hydrochloride (4 M) and KSCN (4 M) caused approximately 50% and 40% ligand-bacteria dissociation respectively.

Cell envelope preparation of strain CCUG 14551 was analysed for the presence of Lf-binding components in SDS-PAGE/Western-blot (Fig. 3). Two HLf-binding proteins with an estimated molecular mass of approx. 40 kDa, and approx. 30 kDa were identified in the boiled sample. The unboiled sample demonstrated two different bands: the first one formed a short ladder pattern at the top of the separating gel and a second one was detected at the approx. 60 kDa position. These bands were demonstrated also in the outer-membrane-protein preparations (data not shown).

5. DISCUSSION

Lf elicits an enhanced antimicrobial effect when adsorbed to the bacterial surface [11,18], a process that may contribute to the host defence. On the other hand, certain microorganisms seem to utilize the iron bound to Lf via specific Lf receptors expressed in the outer membrane [19–21], a property that may contribute to the bacterial virulence. Thus, Lf-bacteria interaction has a pathobiological relevance in either way. In this communication, we have demonstrated evidence for a specific interaction of Lf in *A. hydrophila*.

The HLf and BLf binding to *A. hydrophila* was saturable and implied a limited number of ligand-binding sites on the cell. The ^{125}I-HLf binding to strain CCUG 14551 was displaceable and the bacterium recognized both labeled and native forms of HLf and the BLf with a comparatively higher affinity. In inhibition experiments with excess (100-fold) concentration of different proteins and carbohydates, only HLf and BLf caused a high degree of inhibition and a 50% blocking effect with mucin type-I. Mucin is rich in Lf [22], and Lf fragments contaminating the mucin preparation might account for the inhibition. According to the ligand-bacteria dissociation studies, the interaction does not seem to be of an electrostatic nature since 4 M NaCl failed to dissociate the complex. KSCN and guanidine HCl caused an approx. 50% dissociation of the ligand-bacteria complex, probably by altering the conformation of the proteins involved, by the chaotropic effect.

Western-blot analysis revealed an approx. 40 kDa and an approx. 30 kDa Lf-binding protein in the boiled-cell-envelope preparations of strain CCUG 14551. Binding proteins were less mobile in the unboiled samples, a set of bands formed a ladder at the top of the separating gel, a pattern characteristic to most of the enterobacterial porin oligomers [23,24]. Furthermore, a second band also appeared at the approx. 60 kDa position. Increased mobility by heat treatment in SDS is due to monomer formation and/or higher SDS uptake, also a characteristic of enterobacterial pore-forming proteins. The approx. 40 kDa band detected in the boiled preparation corresponds to

a major protein of *A. hydrophila* that has been identified as a porin in earlier studies [25]. Porins are also resistant to various proteolytic enzymes [23,24,26]. In accordance, our enzyme digestion studies revealed that the HLf binding components are extremely resistant to protease, carbohydrase and lipase digestion (data not shown).

In summary, we have established a specific Lf binding and identified two Lf-binding outer membrane proteins in *A. hydrophila* that are most likely porins.

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