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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.

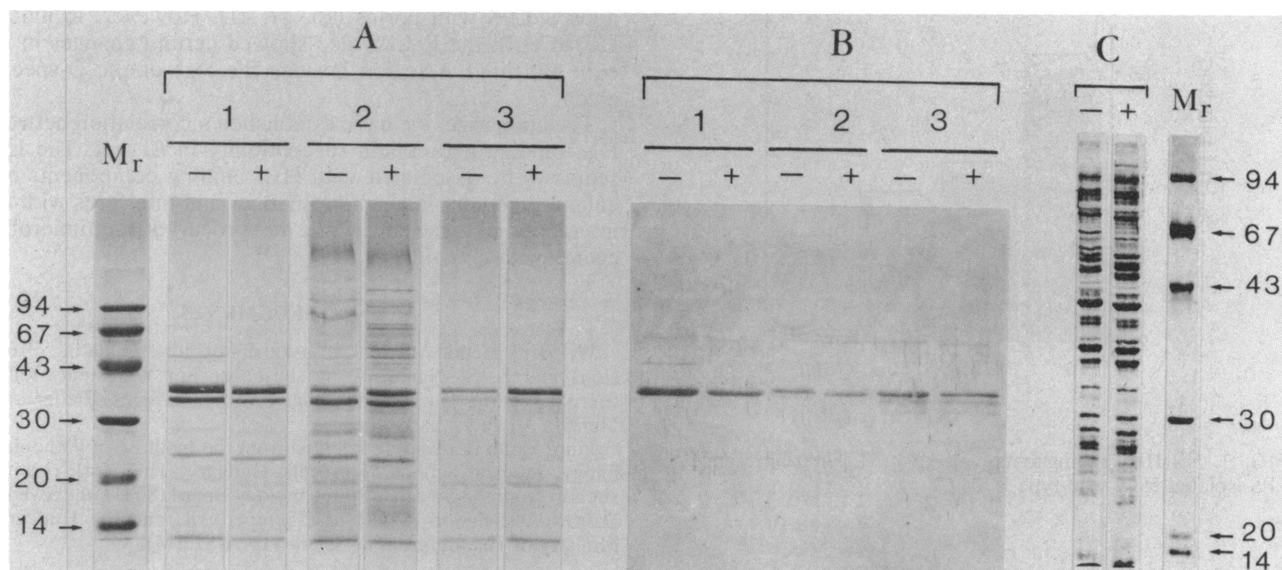

FIG. 1. Analysis of total cell proteins, cell envelope, and outer membranes of *E. coli* in SDS-PAGE. −, wild-type H10407: HLf low binder and colicin insusceptible; +, mutant H10407(Lf): HLf high binder and colicin susceptible. (A) Gels stained with Coomassie brilliant blue. (B) Proteins from section 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*.

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

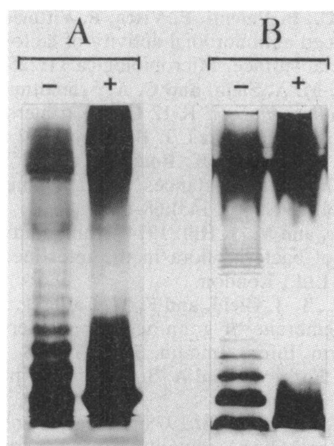

FIG. 2. LPS analysis of *E. coli* by SDS-PAGE and silver staining. −, wild-type H10407; +, mutant H10407(Lf). (A) LPS prepared by phenol-water extraction. (B) LPS preparation digested with proteinase K.

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.

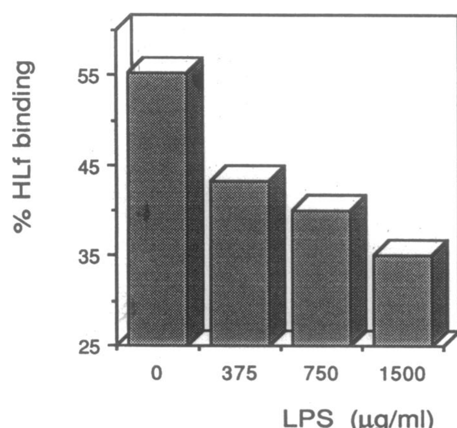

FIG. 3. ^{125}I-HLf binding to mutant H10407(Lf) in the presence of S LPS isolated from wild-type H10407.

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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