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Interaction of lactoferrin with *Escherichia coli* cells and correlation with antibacterial activity

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Abstract. It has been established that the antimicrobial activity of lactoferrin towards *Escherichia coli* is enhanced by a direct contact between the protein and the microbial cell and that, in the case of *E. coli* K-12 strains, an antibacterial activity of lactoferrin unrelated to iron withdrawal is present. Evidence is now reported that lactoferrin binds to surface structures expressed in *E. coli* K-12 strains grown in either an "excess" or "stress" of iron. Under the experimental conditions used, lactoferrin binding both in the apo and in the iron-saturated form yields a maximum of 1.6×10^5 bound molecules/*E. coli* K-12 cell; the amount of lactoferrin bound does not depend on the expression of the iron-regulated outer membrane proteins. In contrast, lactoferrin does not bind to *E. coli* clinical isolates. Apo-lactoferrin (at 500 μg/ml in a chemically defined medium) inhibits the growth of *E. coli* K-12 strains but not of clinical isolates. These findings suggest that the antibacterial activity of the protein could be associated to its binding to the cell surface.

Introduction

The well-known antimicrobial activity of transferrins, such as apo-lactoferrin (Lf), apo-serotransferrin and apo-ovotransferrin, was ascribed originally to their iron-chelating properties (for a review see [11, 13, 30)]. However, experimental evidence obtained in recent years in several species points to the existence of additional antimicrobial mechanisms based on the interference between these proteins and microbial structures. Arnold and co-workers reported a bactericidal effect of human Lf towards a variety of species including *Streptococcus mutans* [2, 4], *Vibrio cholerae* [3] and *Legionella pneumophila* [6] and demonstrated by means of immunofluorescence experiments a direct binding of the protein (both in the apo

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and in the iron-saturated form) to the surface of *E. coli* 0126 [2]. Similarly, Artis et al. [5] and Valenti et al. [28] reported an antimycotic activity of transferrins additional to iron sequestering and showed binding of the fluoresceinated proteins onto the fungal surface. Moreover, Dalmastri et al. [14] showed a binding of apo-Lf coupled to an enhanced antibacterial activity in a variety of microorganisms such as *E. coli, Pseudomonas aeruginosa, Shigella flexneri, Haemophylus influenzae* and *S. mutans.* Ellison et al. [15] demonstrated that the iron-binding proteins damage the Gram-negative outer membrane and alter its permeability.

Other bacterial pathogens, like *Neisseria* spp. and *Bortedella pertussis,* do not secrete siderophores under iron-limiting conditions and appear to obtain iron directly from the host via iron-chelating proteins that bind to specific receptors present on the cell surface in a limited number (approximately 10^3/cell) [23, 24].

Several other observations on the antimicrobial activity of transferrins towards *E. coli* strains with well-characterized iron-transport systems, i.e., enterochelin, aerobactin and their respective receptors, can likewise be ascribed to the interaction between this protein and the bacterial cell. Thus, in the *E. coli* K-12 strains, which possess only the enterochelin-mediated iron-transport system, it has been observed that the antibacterial activity of Lf and ovotransferrin is markedly reduced when they are separated from *E. coli* cells by a dialysis membrane or are used in other experimental set-ups to scavenge iron from the growth medium without any contact with the bacterial cells [25, 26, 29]. Moreover, Lf and ovotransferrin have a strong antibacterial activity when complexed with Zn(II) and, thus, leave iron available in the medium [26, 27, 29]. In contrast, some invasive clinical isolates of *E. coli*, which may possess an additional aerobactin-mediated iron-transport system, are generally much more resistant to Lf in both the apo- and Zn(II)-saturated form [27, 29].

Taken together, all these data indicate that two factors are required to achieve the maximal growth inhibiting effect of Lf: iron chelation and a direct interaction with the microbial surface.

On this basis, the interaction occurring between Lf and *E. coli* K-12 strains and its role in determining the antibacterial activity was investigated. The results obtained indicate that Lf binds to the microbial surface and that Lf binding does not involve iron-regulated outer membrane proteins and does not alter the accessibility to the ferrienterochelin receptor.

Materials and methods

Bacterial strains

The *E. coli* strains used in this study are listed in Table 1.

Media

The defined growth medium was the M9 minimal medium [16] supplemented with glucose (0.2% wt/vol), amino acids (60 μg/ml) or Casamino Acids (0.2% wt/vol) and thiamine hydrocholoride (1 μg/ml). The synthetis of iron-transport systems and iron-regulated outer membrane proteins (IROMPs) was induced by addition of 150 μM 2-2′ dipyridyl to chelate any available iron and repressed by addition of ferric chloride at a concentration of 50 μM. The enterochelin-defective *E. coli* strain AN 263 grew very poorly in minimal medium unless $FeCl_3$ (50 μM) was added. Where required media were solidified by addition of Oxoid agar L11 (0.5% to 1.0% wt/vol).

Table 1. *Escherichia coli* K-12 and reference strains used

Strain	Relevant genotype or characteristics	Reference or source
E. coli 803	*met gal lac hsd*S	[12]
E. coli CSH26	*ara* (*lac pro*) *thi*	[12]
E. coli C600	*thi thr leu*B *lac*Y *ton*A	[1]
E. coli AN263	*ara fep*A *lac leu mtl pro*C *rps*L *sup*E *thi ton*A *trp*E *xyl*	[31]
E. coli HN13	Enteroinvasive (0135:K^-:H^-) aerobactin negative	[20]
E. coli K311	Aerobactin positive	[8]
E. coli 20R966	Colicinogenic for B	P.H.L.S.[a]

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Proteins

Purified apo-Lf from human colostrum was purchased from United States Biochemical Corp. (Cleveland, Ohio, USA). Bovine serum albumin (BSA) and horse heart cytochrome *c* were supplied by Sigma Chemical Co. (St. Louis, Mo., USA). A mouse monoclonal antibody II2C against human apo- and iron-saturated Lf was supplied by H. E. Broxmeyer [10]. When required, proteins were conjugated with fluorescein isothiocyanate (isomer I; Sigma) as described by Wood [32].

Cultivation under different conditions of iron availability

Bacteria collected from stationary growth phases in Luria Broth were washed three times in phosphate-buffered saline (PBS; NaCl, 8 g/l; KCl, 0.2 g/l; Na_2HPO_4, 1.15 g/l; KH_2PO_4, 0.2 g/l; pH7.2) supplemented with 1 mM $CaCl_2$ and 0.50 mM $MgCl_2$, and subcultured twice, for 24 h each time, at 37°C in M9 minimal medium. In the second passage the M9 minimal medium was supplemented either with 50 μM ferric chloride or 150 μM 2-2′ dipyridyl. Cells were harvested by centrifugation, washed twice in PBS and subsequently used for adsorption tests or for assessing the antibacterial activity of Lf. The bacterial growth was monitored by measuring absorbance at 620 nm and by counting the colony-forming units (CFU). Preliminary control experiments performed with several strains proved that no significant differences occur in the cell volumes of bacteria cultivated under iron-poor or -rich conditions. This was determined by adjusting to 1.0 the A_{620} of the cultures under iron stress or excess and by counting the number of CFU and the number of bacterial cells in a Pertoff-Hauser counting chamber. For each strain, we found that iron-rich and -poor cultures with the same absorbance contained about the same number of bacteria as determined by plate and microscopic counts.

Lf adsorption test

Purified Lf (from 25 to 500 μg) dissolved in PBS was added to *E. coli* cells (from 0.125×10^{10} to 2.5×10^{10}) and, unless otherwise stated, the incubation was carried out for 30 min at 37°C either in 1 ml of PBS or in 1 ml of PBS supplemented with 0.50 mM sodium bicarbonate (pH 7.2). After this period the bacterial cells were removed by centrifugation (2,500 *g*, 20 min, 4°C and the Lf

concentration in the supernatants was tested by radial immunodiffusion (LC-Partigen-Lactoferrin kit, Behring, Marburg, FRG). This method for Lf determination allows to quantify with high reproducibility 0.6 μg Lf/ml as the lower limit, but requires a high cell density to detect Lf adsorption. Appropriate controls were performed to exclude the occurrence of nonspecific adsorption of Lf to the test tubes.

Preparation and titration of colicin B

Colicin B was prepared from cultures of *E. coli* 20R966 according to Pugsley and Reeves [22]. Colicin B activity [17] was expressed in units/ml $= 2^n$ where n is the highest number of dilutions giving clear killing in the colicin B adsorption test.

Colicin B adsorption test

Adsorption of colicin B to whole cells was measured according to the method proposed by Guterman [16].

Competition experiments

The competition experiments between Lf and colicin B, BSA or beef heart cytochrome *c* – which, like Lf, is positively charged under the experimental conditions used [9, 18] – were performed on the basis of a modification of the procedure proposed by Guterman [16] for enterochelin. A fixed amount of Lf (50 μg) and a variable amount of the putative competitor were mixed with 1.25×10^{10} viable cells of *E. coli* 803 in 1 ml PBS. Aliquots were collected after incubation of the mixture for 30 min at 37°C. The concentration of Lf was determined in the supernatants after low-speed centrifugation and removal of the bacteria (2,500 *g*, 20 min, 4°C). In these experiments 256 and 512U of colicin B were used, corresponding approximately to the miminum amounts giving 100% adsorption in the absence of inhibitor; BSA and horse heart cytochrome *c* were used in the ranges of 5–500 and 10–100 μg, respectively.

Fluorescence staining of E. coli

The binding of Lf on the *E. coli* surface was checked by direct fluorescence and immunofluorescence using either fluorescein-conjugated Lf or the fluorescein-conjugated monoclonal antibody II2C according to the sandwich technique described by Artis et al. [5].

Results

Adsorption of Lf onto E. coli 803

Figure 1 shows the data obtained upon addition of increasing amounts of Lf (25–500 μg) to 1.25×10^{10} cells of *E. coli* 803 suspended in 1 ml PBS. The cells were derived from cultures grown under conditions of excess or limiting iron, i. e., under conditions that, respectively, repress or induce the synthesis of siderophores and IROMP [12, 19]. Lf is adsorbed to the cells grown under either condition and the amount of Lf bound is slightly higher in the case of cells grown in iron excess (maximal amount 245 μg Lf/1.25×10^{10} iron-rich cells, corresponding to 1.6×10^5 Lf molecules/cell on the basis of the Lf molecular mass, 75,000 [9]).

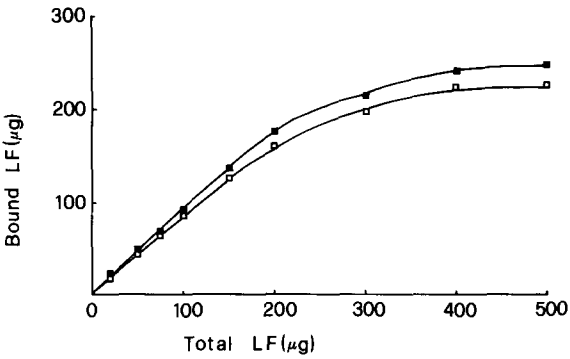

Fig. 1. Binding of lactoferrin onto *E. coli* 803 grown under excess or stress of iron. Lf was added to 1 ml of PBS containing 1.25×10^{10} cells yielding final concentrations between 25 and 500 μg/ml. Cells were derived from cultures in M9 minimal medium supplemented with 150 μM 2–2′ dipyridyl (□) or 50 μM $FeCl_3$ (■)

Table 2. Effect of bacterial concentration on lactoferrin (Lf) binding onto *E. coli* 803 cells; 50 μg of Lf were added to increasing amounts of *E. coli* cells cultivated in M9 minimal medium supplemented with 150 μM 2–2′ dipyridyl or 50 μM $FeCl_3$ and suspended in 1 ml PBS

Cells	Lf adsorbed (μg) on *E. coli* cells grown in:	
	Iron stress	Iron excess
0.125×10^{10}	23.2 (± 0.2)	27.1 (± 0.3)
0.250×10^{10}	32.5 (± 0.5)	33.4 (± 0.3)
1.250×10^{10}	44.3 (± 0.4)	47.8 (± 0.3)
2.500×10^{10}	44.9 (± 0.3)	47.6 (± 0.5)

The data represent mean values (± SD) obtained from five independent experiments

In a second series of experiments, performed to evaluate the influence of the bacterial concentration on Lf adsorption, the binding of a fixed amount of Lf (50 μg) on *E. coli* 803 cells ranging from 0.125 to 2.5×10^{10} was tested. Also in these experiments cells were cultivated either under iron-limiting conditions or in excess of iron. The results shown in Table 2 are consistent with those obtained when using a constant number of bacterial cells in terms of the amount of Lf adsorbed per bacterial cell.

The adsorption of Lf (50 μg) on *E. coli* 803 (1.25×10^{10} cells) was tested also as a function of pH in the range 4–9 (in PBS adjusted with HCl or NaOH) and of temperature (in PBS at pH 7.2). The results reported in Table 3 display a slight pH and temperature dependence with the maximal adsorption occurring at 37°C and at pH values 7–8.

Lastly, the effect of the saturation of Lf with iron and the influence of buffer composition were tested. The saturation of the Lf molecule with iron slightly improves binding; Ca(II) and Mg(II) at millimolar concentrations do not affect significantly Lf adsorption; likewise substitution or supplementation of PBS either with 50 mM bicarbonate, pH 7.2, or with M9 medium does not influence Lf adsorption (data not shown).

Table 3. Influence of pH and temperature on the adsorption of Lf (50 μg) onto iron-starved *E. coli* 803 (1.25×10^{10} cells) in 1 ml PBS.

Experimental variable	μg Lf bound/1.25×10^{10} cells
pH at 37°C	
4	37.4 (± 0.3)
5	39.2 (± 0.2)
6	40.0 (± 0.4)
7	44.3 (± 0.3)
8	44.5 (± 0.4)
9	40.4 (± 0.3)
Temperature at pH 7.2	
4°C	38.2 (± 0.2)
18°C	42.1 (± 0.4)
37°C	44.4 (± 0.5)

The reported data represent mean values (± SD) obtained from three independent experiments

Table 4. Competitive adsorption of Lf, colicin B, bovine serum albumin (BSA) and cytochrome *c* (Cyt *c*) onto *E. coli* strains 803 and AN263

Strain	Incubation conditions	Cultural conditions			
		Iron stress		Iron excess	
		Adsorbed Lf (μg)	Adsorbed colicin B	Adsorbed Lf (μg)	Adsorbed colicin B
803	Lf	44.3 (± 0.4)	–	47.9 (± 0.9)	–
	Colicin B (256 U)	–	256 (± N.D.)	–	111 (± 5.3)
	Colicin B (512 U)	–	452 (± 8.1)	–	117 (± 6.8)
	Lf-Colicin B (256 U)	44.2 (± 0.9)	256 (± N.D.)	48.1 (± 1.2)	113 (± 7.5)
	Lf-Colicin B (512 U)	43.9 (± 1.1)	445 (± 9.8)	48.0 (± 0.8)	117 (± 5.7)
	Lf-BSA	44.3 (± 0.7)	–	48.2 (± 1.1)	–
	Lf-Cyt *c*	43.7 (± 0.9)	–	47.8 (± 0.8)	–
AN263	Lf	46.9 (± 0.5)	–	47.1 (± 1.0)	–
	Colicin B (512 U)	–	N.D.	–	N.D.

The experiments were performed using 50 μg Lf, 256 or 512 U colicin B, 500 μg BSA, 100 μg cytochrome *c* and 1.25×10^{10} *E. coli* cells cultivated in M9 minimal medium supplemented with either 150 μM 2–2′ dipyridyl (iron stress) or 50 μM $FeCl_3$ (iron excess). The reported data represent mean values (± SD) obtained from five experiments. N.D.: not detectable

Influence of Lf on the binding of colicin B to E. coli 803

In view of the well-known interference of Lf with iron uptake in *E. coli*, the effect of Lf adsorption on the accessibility of the ferrienterochelin receptor was tested in *E. coli* 803 which possesses only the enterochelin-mediated iron-uptake system.

Advantage has been taken of the fact that the ferrienterochelin receptor, coded by the *fep*A gene, is also a specific receptor for colicin B and that binding of this bacteriocin causes a rapid killing of sensitive *E. coli* strains. On this basis, the influence of Lf on colicin B binding onto *E. coli* 803 was measured; the *fep*A defective, colicin B-resistant *E. coli* AN263 strain was used as a control.

Competition experiments were performed between Lf (50 μg) and colicin B (256 and 512U), using 1.25×10^{10} *E. coli* 803 cells grown either in the presence of 50 μM $FeCl_3$ or 150 μM 2-2′ dipyridyl, i. e., under conditions of excess or limiting iron. The results (Table 4) show that, as expected, colicin B binds to a higher extent on cells grown in iron-limiting conditions with respect to those grown under iron excess, while no binding occurs on the *fep*A-negative *E. coli* strain AN263. In contrast, Lf binds not only onto *E. coli* 803, but also on *E. coli* AN263. Moreover, the binding of Lf and colicin B is not altered, as compared to the controls, when they are both present in the incubation mixture.

In another series of experiments BSA or horse heart cytochrome *c* were added as unspecific protein competitors. The results obtained with the higher concentration of the competitors are reported in Table 4. Neither protein interferes with the binding of Lf, although the *E. coli* cells bind a small amount (3 μg/1.25×10^{10} cells) of cytochrome *c* as revealed by the absorption spectra of the supernatant in the Soret region [18].

Antibacterial activity and binding of Lf to different E. coli strains

It has been established that the ability of bacteria to multiply in the presence of transferrins can be correlated to their potential virulence; in fact, invasive microorganisms generally display a high resistance to the in vitro bacteriostatic action of transferrins [11, 30]. Therefore, the antibacterial activity of Lf and the binding of this protein on some *E. coli* strains including K-12 derivatives and clinical isolates was assayed. Table 5 shows that 500 μg/ml Lf significantly inhibits the growth of *E. coli* K-12 strains, while all the clinical isolates are unaffected. In addition, all the sensitive *E. coli* K12 strains bind Lf, while no binding could be demonstrated on the clinical isolates of *E.coli*. Moreover, while binding is not significantly affected by saturation of Lf with iron (not shown), the antibacterial activity towards the sensitive *E. coli* K-12 is completely annulled.

Binding measurements were also confirmed by direct fluorescence and immunofluorescence studies performed with fluorescein-conjugated Lf and anti-Lf monoclonal antibody II2C. In both types of experiment all the *E. coli* K-12 strains show a positive fluorescence while clinical isolates appear negative.

Discussion

The experimental data reported in this paper demonstrate that the *E. coli* K-12 strains studied have the ability to bind Lf. Lf binding is slightly pH and temperature dependent (with an optimum at pH 7–8 and 37°C), is not affected by saturation with iron, by millimolar concentrations of calcium and magnesium ions or by the presence of BSA or cytochrome *c* as unspecific protein competitors. Similar amounts of Lf (up to 1.6×10^5 molecules/cell) are bound by *E. coli* cells grown under conditions of excess or limiting iron (the slight differences observed

Table 5. Antibacterial activity and adsorption of Lf onto different *E. coli* strains

	K-12 Strains				Clinical Isolates				
	803	CSH26	C600	AN263	HN13	K311	EC20[a]	EC30[a]	EC33[a]
CFU/ml after 24 h incubation in M9 plus 500 μg/ml apo-Lf	3×10^6	8×10^6	5×10^6	8×10^5	6×10^8	4×10^8	4×10^8	5×10^8	3×10^8
CFU/ml after 24 h incubation in M9 plus 500 μg/ml iron-saturated Lf	3×10^8	7×10^8	5×10^8	9×10^7	5×10^8	4×10^8	6×10^8	4×10^8	6×10^8
Direct immunofluorescence	+	+	+	+	−	−	−	−	−
μg Lf bound/1.25×10^{10} cells (± SD)	44.3 (± 0.4)	26.9 (± 0.3)	42.6 (± 0.7)	46.9 (±0.5)	ND	ND	ND	ND	ND

The antibacterial activity was measured by counting the number of colony forming units (CFU) after 24 h incubation at 37°C in M9 minimal medium supplemented with 500 μg/ml apo- or iron-saturated Lf; the inoculum consisted of approx. 5×10^5 CFU/ml. The binding of Lf was detected by means of direct immunofluorescence and quantitatively determined by adsorption experiments as previously described. The experiments were performed with 50 μg Lf in 1 ml PBS and 1.25×10^{10} *E. coli* cells cultivated in M9 minimal medium supplemented with 150 μM 2–2′ dipyridyl and the reported data represent the mean values (± SD) derived from five experiments. ND; not detectable

[a] The *E. coli* strains EC20 and EC30 are aerobactin-producing clinical isolates (06:K2:H1 and 075:K5:H^-, respectively). *E. coli* EC3 3 is the acapsulated variant of EC20 (06:K^-:H1). These strains have been kindly provided by Dr. A. Caprioli, Laboratorio di Ultrastrutture, Instituto Superiore di Sanità, Roma

may be ascribed to undetectable variations in cell size). These findings indicate that Lf adsorption sites are constitutive rather than regulated by iron, a conclusion which is corroborated by the observation that colicin B does not interfere with Lf binding onto *E. coli* 803 cells. Moreover, the number of Lf molecules bound per bacterial cell is much higher than expected for binding to specific outer membrane protein receptors [7, 19, 23, 24], and is compatible with adsorption onto highly repeated structures on the *E. coli* surface. The results obtained by Ellison and co-workers [15] suggest *E. coli* lipopolysaccharides (LPS) as possible candidates for Lf adsorption. Indeed the bacterial surface is negatively charged due to the presence of acidic LPS and of acidic outer membrane proteins [15, 21] whereas at neutral pH Lf displays a marked positive charge [9].

In contrast to K-12 strains, clinical *E. coli* isolates do not adsorb detectable amounts of Lf as indicated by all the experimental procedures used. Given the possible quantitative and qualitative differences in several cell surface components of the clinical isolates with respect to the *E. coli* K-12 strains, the lack of Lf binding to the former ones could be ascribed either to a decreased accessibility of the target sites or to their modification. In contrast to *E. coli* K-12 strains, all the *E. coli* clinical isolates showed marked resistance to the antibacterial activity of Lf.

On the basis of the data just discussed it is tempting to correlate, in *E. coli,* sensitivity of Lf with the binding of this protein to the microbial cells and, conversely, resistance to Lf with the lack of binding. However, the number of strains tested to date is limited and this correlation cannot be generalized. In any case, the adsorption of Lf on sensitive *E. coli* K-12 strains and, by contrast, the lack of binding to resistant clinical isolates, may provide an explanation for the bacteriostatic activity which requires a direct contact of the apo-protein with the bacterial cell. As expected, iron saturated Lf, although adsorbed by *E. coli* K-12 strains approximately in the same amounts as apo-Lf, does not exert any bacteriostatic effect.

In conclusion, the present data demonstrate that Lf binds to some *E. coli* strains and that the binding could be related with the bacteriostatic activity of the protein while it is independent of the expression of IROMPs. The nature of the Lf adsorption site is being investigated.

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