The effect of iron on the invasiveness of *Escherichia coli* carrying the *inv* gene of *Yersinia pseudotuberculosis*

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Summary. The effect of growth in iron-excess or iron-limitation conditions on the invasiveness for HeLa cells of *Escherichia coli* HB101 carrying plasmid pRI203 which bears the invasion gene of *Yersinia pseudotuberculosis* was examined. Iron-limitation reduced adhesion and the number of organisms internalised by HeLa cells by about 100-fold. The reduced adhesion of iron-starved bacteria correlated with reduced hydrophobicity and the reduced invasiveness appeared to depend on the plasmid copy number, which was 3.5-fold less than in bacteria grown in iron excess.

Introduction

The first step in the interaction between a pathogen and its host is attachment to the host cell surface but, while some bacteria remain on the surface, others proceed to penetrate the host cell.¹

Tests of attachment and invasion *in vitro* are usually performed under standard nutritional conditions² but it is possible that the nutrient status of the bacteria, with special reference to inorganic nutrients, may play an important role in the interaction. Little is known about the effect of ions on these processes. Thus, iron, as Fe³⁺, is an essential bacterial nutrient but, because it is insoluble at physiological pH, its availability *in vivo* is poor (10⁻¹⁸ M).^{3,4} The iron-binding proteins, transferrin and lactoferrin, play an important role in antibacterial defence by maintaining this low iron concentration.⁵

Bacteria isolated directly from infections have surface properties similar to those of iron-starved bacteria^{6,7} and several studies have suggested that iron and iron-containing compounds enhance susceptibility to infection.⁸ However, little is known about the effect of iron overload on the pathogenicity of bacteria for mucosal cells. Thus, mucin, an iron carrier, increases survival of the vaccine strain of *Vibrio cholerae* during intestinal colonisation but the reason is not known.⁹

In *Yersinia pseudotuberculosis*, a 3.2-kb chromosomal region containing the *inv* gene is responsible for the ability of this organism to enter a wide variety of cell lines.² The product of the *inv* gene is a 978-amino acid outer-membrane protein, invasin, which binds to

certain transmembrane proteins of mammalian cells (integrins).^{10,11} The *inv* gene has been cloned into plasmid pBR325 and the resulting plasmid, pRI203, has been introduced into the avirulent *Escherichia coli* strain HB101.¹² The resulting strain, *E. coli* HB101 (pRI203), was able to invade HeLa cells but did not multiply intracellularly.

We have used *E. coli* HB101 (pRI203), grown *in vitro* under conditions of iron availability and iron restriction, to determine whether these conditions affect the ability of this organism to adhere to and invade HeLa cells.

Materials and methods

Micro-organisms and media

E. coli strain HB101 (pRI203) was kindly provided by Dr S. Falkow, Stanford Medical School, CA, USA. It carries a recombinant plasmid pRI203 into which a *Y. pseudotuberculosis* 3.2-kb chromosomal DNA fragment has been cloned and which renders *E. coli* HB101 invasive for cultured animal cells.¹²

E. coli HB101 and *E. coli* HB101 (pRI203) were grown in Trypticase Soy Broth (TSB; BBL) containing 20–50 µM Fe³⁺ as determined by atomic absorption spectrophotometry (Perkin-Elmer 360). Bacteria were also grown in minimal medium M9 plus thiamine 1 mg/L, proline 40 mg/L, threonine 40 mg/L, leucine 40 mg/L and either 150 µM 2,2'-dipyridyl as iron chelator for iron-limiting conditions or 50 µM FeCl₃ for conditions of iron excess. In all experiments, ampicillin was added to a final concentration of 50 µg/ml to ensure maintenance of the recombinant plasmid.

Host cells

HeLa S₃ cells were grown at 37°C as monolayers in Eagle's Minimal Essential Medium (MEM; Gibco Laboratories) plus NaHCO₃ 1.2 g/L, 2 mM glutamine, penicillin 100 U/ml, streptomycin 0.2 mg/ml and heat-inactivated fetal calf serum 10% in a CO₂ 5% atmosphere. Confluent monolayers were trypsinised and 24-well tissue culture plates (Costar) were loaded with 0.5 ml/well of a suspension containing 1.5×10^5 cells/ml and incubated in growth medium without antibiotics for 24 h.

Invasion and adherence assays

Invasion of HeLa cells was assayed as described by Conte *et al.*¹³ Briefly, HeLa cell monolayers (3×10^5 cells) were inoculated with 3×10^7 bacteria in basal Eagle's medium (BME) for 90 min at 37°C. The monolayers were then washed and fresh BME, containing gentamicin 50 µg/ml, was added to kill extracellular bacteria. After incubation for 2 h at 37°C, the cells were lysed to determine the number of viable internalised bacteria by colony counts.

In other experiments, duplicate assays were performed in which infection was synchronised by a temperature shift. Bacteria (3×10^7) were added to pre-cooled cells (3×10^5) and incubated for 90 min at 4°C. After infection, the cells were washed carefully to remove unattached bacteria and then lysed to determine the number of bound bacteria by colony counts. In another experiment the temperature was raised to 37°C after infection to allow bacteria bound at 4°C to penetrate HeLa cells. After incubation for 60 min, gentamicin-containing medium was added for 2 h. After lysis, the number of internalised bacteria was determined by colony counts.

The results of adherence and invasion assays from five independent experiments performed in triplicate were collected. The mean value and SD were determined for each assay.

Adherence of fluorescein-labelled bacteria

Fluorescein isothiocyanate (FITC; Sigma), dissolved in phosphate-buffered saline (PBS), pH 8.0, at a concentration of 2 mg/ml, was incubated with 2×10^7 bacteria/ml at room temperature for 15 min. The bacteria were then washed twice in PBS by centrifugation at 5000 *g*. A suspension of 1×10^7 FITC-labelled bacteria plus Evans blue 0.001% was added to semi-confluent monolayers and left in contact for 2 h. The monolayers were washed five times, fixed in formaldehyde 1% and adherent organisms were observed by microscopy.

Hydrophobic interaction chromatography

Bacteria were loaded on to octyl-sepharose columns (Pharmacia Fine Chemicals) according to a minor modification of the procedure described by Ohman *et*

*al.*¹⁴ Briefly, pasteur pipettes were filled with 1 ml of octyl-sepharose and equilibrated with 0.1 M (NH₄)₂SO₄ buffer (pH 7.0). The bacterial suspensions (10^8 cells/100 µl), washed three times with the above buffer, were applied on to the gel and eluted with 0.1 M (NH₄)₂SO₄. The retention of the cells reflected a hydrophobic interaction and was expressed as the percentage of bacteria retained. In control experiments octyl-sepharose columns were equilibrated and eluted with distilled water.

Analysis of outer-membrane proteins (OMP)

Bacteria (100 mg dry weight) harvested in late exponential phase were suspended in 1 ml of 30 mM Tris-HCl, pH 8.0, and disrupted by three cycles of 1 min each of sonication (18 MHz at Soniprep 150) on ice. Cell debris was removed by centrifugation (15 min at 5000 *g*), the inner membranes were solubilised with sodium-laurylsarcosinate 1% and the OMPs were collected by centrifugation at 51000 *g* for 1 h at 4°C. SDS-PAGE was performed under denaturation conditions in a resolving 8% gel with a stacking 5% gel.¹⁵

Extraction and electrophoresis of total nucleic acids

Briefly, bacteria from 5 ml of bacterial culture, in late exponential phase, were harvested by centrifugation at 5000 *g* for 10 min at 4°C and washed in 1 × TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0). The cell pellet was suspended in 260 µl of sucrose 25%-50 mM Tris-HCl, pH 7.5, containing lysozyme 1 mg/ml and kept for 5 min at 0°C. Then 300 µl of Triton X100 1%, 50 mM Tris-HCl, pH 8.0, 20 mM ribonucleoside vanadyl complexes (Sigma) were added and the sample was held for 5–10 min at 0°C. After centrifugation at 42000 *g* for 20 min at 4°C, the cleared lysate was extracted four times with an equal volume of PCI (phenol 50% saturated with 50 mM Tris-HCl, pH 7.5; chloroform 48%; iso-amyl alcohol 2%) and precipitated twice for 30 min at –70°C with 0.1 vol of 3.3 M sodium acetate, pH 5.2, and 3 vols of absolute ethanol.

Nucleic acids were dissolved in double-distilled water containing diethyl pyrocarbonate 0.5% and stored at –70°C. Nucleic acids were dissolved in formaldehyde 7.5%, formamide 20%, 1 × MOPS (20 mM 3[N-morpholino]propone-sulphonic acid, 5 mM sodium acetate, pH 7.0, 1 mM EDTA, pH 8.0) and incubated for 10 min at 65°C. Electrophoresis was performed in agarose 1%-1 × MOPS gel containing formaldehyde 6%.

Hybridisation procedures

After electrophoresis, nucleic acids were transferred by capillary blotting on to Hybond-C nitrocellulose membranes (Amersham) and filters were treated according to the recommendations of the manufacturer. The invasins gene, kindly provided by Dr A. Crisanti

Fig. 1. Binding of FITC-labelled *E. coli* HB101 (pRI203) to HeLa cells after 90 min at 4°C; **a**, grown in iron excess; **b**, grown in iron-limiting conditions.

(Institute of Parasitology, University of Rome), was labelled with ^{32}P -dATP with a Random Primed DNA Labelling Kit (Boehringer Mannheim) and unincorporated nucleotides were removed on Quick Spin[®] columns G-50 Sephadex (Boehringer Mannheim). After hybridisation, filters were washed to remove non-specific binding of DNA according to the method of Sambrook *et al.*¹⁶

Table I. Invasion of HeLa cells by *E. coli* HB101 (pRI203) grown under different conditions of iron availability

Growth conditions	Mean (SD) number of viable internalised bacteria* (cfu)
TSB (20–50 μM Fe^{3+})	$2.0 (0.37) \times 10^5$
M9 plus 50 μM Fe^{3+}	$1.1 (0.45) \times 10^5$
M9 plus 150 μM 2,2'-dipyridyl	$1.2 (0.70) \times 10^3$

*Mean values of intracellular bacteria / 3×10^5 HeLa cells derived from five independent experiments.

Results

The adherence of FITC-labelled organisms placed in contact with cell monolayers at 4°C for 120 min is illustrated in fig. 1. Bacteria grown in iron excess adhered better (1.2×10^3 /100 HeLa cells; fig. 1a) than those grown under iron-limiting conditions (50/100 HeLa cells; fig. 1b).

In the standard penetration assay, a mean of 2×10^5 *E. coli* HB101 (pRI203) bacterial cells were internalised by 3×10^5 HeLa cells. The efficiency of penetration was increased when the bacteria were centrifuged on to the cell monolayers or when the period of infection was increased. The results obtained with organisms after six serial passages in medium M9 supplemented with 50 μM FeCl_3 or with 150 μM 2,2'-dipyridyl are shown in table I. This shows that organisms grown under iron limitation had a reduced ability to penetrate HeLa cells compared with those grown in iron excess.

Adherence and cell invasion were also studied by determination of viable counts in a temperature shift

Table II. Adherence and penetration into HeLa cells of *E. coli* HB101 (pRI203) grown under different conditions of iron availability

Growth conditions	Mean (SD) number of bound bacteria/ 3×10^5 HeLa cells (cfu)	Mean (SD) number of internalised bacteria (cfu)
TSB (20–50 μM Fe^{3+})	$3.0 (1.12) \times 10^6$	$5.4 (0.45) \times 10^5$
M9 plus 50 μM Fe^{3+}	$1.4 (0.95) \times 10^6$	$2.5 (0.64) \times 10^5$
M9 plus 150 μM 2,2'-dipyridyl	$1.3 (0.78) \times 10^4$	$2.6 (0.55) \times 10^3$

Fig. 2. Percentage of bacteria retained in an octyl-sepharose gel. *E. coli* HB101 (pRI203) grown in iron excess—A, TSB with 20–50 μM Fe^{3+} ■, B, M9 plus 50 μM Fe^{3+} ▨—or, C, in iron-limiting conditions—M9 plus 150 μM 2,2'-dipyridyl ■.

experiment. The results are given in table II. Both adherence and cell invasion were greater with organisms grown in TSB or in iron excess.

The effect of iron availability during growth on the surface hydrophobicity of *E. coli* HB101 (pRI203) is shown in fig. 2. Some 60% of organisms grown in iron excess were retained on the octyl-sepharose column, in contrast to 25% of those grown under iron restriction. The results obtained with the non-invasive *E. coli* HB101 were similar.

The OMP profiles of the invasive and non-invasive strains grown under iron-limiting and iron-excess conditions are shown in fig. 3. Under iron-limiting conditions, both strains produced OMPs of 74, 76 and 81 kDa. The function of the 76-kDa protein is unknown but the 74- and 81-kDa proteins are known to be receptors for colicin I and ferric enterochelin, respectively.^{6,17,18} The 103-kDa protein band, corresponding to the invasins of *E. coli* HB101 (pRI203), was not observed in these experiments.

Determinations of the plasmid copy number of

organisms grown under conditions of iron restriction and excess with the same amount of DNA from each type of organism subjected to electrophoresis and blotted on to nitrocellulose and hybridised with ^{32}P -labelled invasins gene, showed that the signal from iron replete organisms was 3.5-fold greater than that for organisms grown under iron restriction.

Discussion

Infection induces a state of hypoferraemia and bacteria isolated from sites of infection are similar to those grown in culture under iron restriction.⁸ Therefore, it was of interest to determine the effect of iron deprivation *in vitro* on the expression of bacterial virulence factors. Several studies have shown that iron-stressed pathogenic bacteria have evolved mechanisms, such as siderophore synthesis, for the acquisition of iron.^{8,17} However, little information is available about the modulation by iron of the factors involved in the attachment and penetration of cells by enteropathogens. In particular, it is still unclear how iron overload, which increases susceptibility to bacterial infections,³ interferes with host defence mechanisms or with bacterial virulence factors, or both.

The results reported here show that *E. coli* HB101 (pRI203) grown in iron-deficient medium has a reduced ability to bind to and penetrate HeLa cells. In a manner similar to that of other iron-starved bacteria, *E. coli* HB101 and HB101 (pRI203) showed OMP changes corresponding to the synthesis of OMPs of 74, 76 and 81 kDa. Although the function of the 76-kDa protein is unknown, the 74- and 81-kDa proteins are known to be the receptors for colicin I and enterochelin.^{6,17,18} As these OMPs are expressed

Fig. 3. SDS-PAGE of OMPs of *E. coli* HB101 (pRI203) (lanes 1 and 2) and of the non-invasive strain *E. coli* HB101 (lanes 3 and 4), grown in iron-excess (lanes 2 and 4) or iron-limiting conditions (lanes 1 and 3). Lane 5: mol. wt markers (BioRad): myosin (200 kDa), *E. coli* β -galactosidase (116.25 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (42.7 kDa) and bovine carbonic anhydrase (31.0 kDa). The arrows indicate the three proteins expressed in iron-limiting conditions.

equally by invasive and non-invasive *E. coli* strains, they are probably not involved in the early phases of invasion.

It was observed that the hydrophobicity of organisms was markedly reduced by iron restriction. This may be due to the induction of exopolysaccharide production by iron restriction, as has been suggested by others.^{19, 20} This lower hydrophobicity seems to contribute to a decrease of the invasion efficiency by acting on the adhesion step.

As *E. coli* HB101, which lacks the *inv* gene, is incapable of penetrating HeLa cells, it may be that the

most important factor in the invasion process under different iron-availability conditions is the significant change of the copy number of plasmid pRI203. This is consistent with our observation that iron-stressed bacteria had a lower plasmid copy number than bacteria grown in iron excess, and also a decreased expression of invasin.

We thank Dr S. Falkow for providing the *E. coli* HB101 (pRI203) strain, Dr A. Crisanti for producing the *inv* probe and Professor C. Romano Caratelli for her helpful contribution to hydrophobic chromatography assays. This research has been carried out with Ente Nazionale Energie Alternative (ENEA), Ministero Pubblica Istruzione (MPI) and CNR grants.

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