Iron Regulated Outer Membrane Proteins of *Escherichia coli*: Variations in Expression Due to the Chelator Used to Restrict the Availability of Iron

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Iron restriction was induced in *Escherichia coli* O111, *E. coli* O164 and *E. coli* C by growing the organisms in trypticase soy broth containing ovotransferrin, desferal, EDDA (ethylenediamine-dihydroxyphenylacetic acid) or α,α' -dipyridyl. There were marked qualitative and quantitative differences in the iron regulated outer membrane proteins expressed in the presence of the various iron chelators. Differences in the kinetics of growth were also noted. *E. coli* C was devoid of a ferric enterobactin iron uptake system.

INTRODUCTION

Escherichia coli produce iron chelating compounds (siderophores) which sequester iron and also derepress the synthesis of several outer membrane proteins when grown in iron restricted environments (Neilands, 1982). Some of these proteins (molecular mass range 70–83 kDa) act as receptors for ferric siderophores and are essential for transporting iron into the bacterial cell; they are not synthesized in significant amounts by organisms grown in iron replete conditions. Such mechanisms for acquiring iron are of particular interest in the study of bacterial pathogenicity since the availability of iron in the tissues of the mammalian host is severely restricted. Most of the iron is found intracellularly as ferritin, haemosiderin or haem, and that which is extracellular in body fluids is tightly bound to the iron binding glycoproteins transferrin and lactoferrin. As these proteins have high association constants for iron and are usually only partially saturated, they ensure that little free iron is available to invading bacteria. Recently, it has been shown that bacteria obtained without subculture, from infected animals or from patients, express iron regulated membrane proteins (Griffiths *et al.*, 1983; 1985a; Brown *et al.*, 1984; Anwar *et al.*, 1984; Lam *et al.*, 1984). The expression of these iron regulated outer membrane proteins *in vivo* during infection makes the proteins potential candidates for vaccine production and/or possible targets for inhibitors of siderophore mediated iron uptake, especially since these proteins have been shown to be surface exposed (Griffiths *et al.*, 1983, 1985a).

One of the iron regulated outer membrane proteins of *E. coli*, the 81 kDa product of the *fep* gene, is the receptor for ferric enterobactin (also called enterochelin; Neilands, 1982). As part of a study to determine whether antibodies directed against this receptor interfere with enterobactin mediated iron transport and inhibit bacterial growth, we recently investigated the degree of antigenic homology of the 81 kDa protein in 17 strains of *E. coli*. Both the molecular mass and some antigenic properties of this protein were conserved within the strains tested (Chart & Griffiths, 1985). Interestingly, two of the strains examined, the laboratory strain *E. coli* C and an

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Abbreviations: EDDA, ethylenediaminedihydroxyphenylacetic acid; TSB, trypticase soy broth.

enteroinvasive strain *E. coli* O164, were unusual in that neither produced an 81 kDa protein when grown in media containing the iron chelator α, α' -dipyridyl. This chelator was used to induce iron starvation in these studies instead of a natural iron-binding glycoprotein such as ovotransferrin merely because it was more economical and convenient to use. However, when *E. coli* O164 was grown in the presence of ovotransferrin, an 81 kDa protein was expressed (Griffiths *et al.*, 1985*b*). In the present study we have examined further the iron uptake systems of *E. coli* O164 and *E. coli* C using a range of chelating agents and have compared the iron uptake mechanisms of these strains with those of *E. coli* O111.

METHODS

Bacteria. *E. coli* O164 H⁻ was obtained from the Public Health Laboratory, Colindale, London. An isolate of *E. coli* C was obtained from the American Type Culture Collection (ATCC 23461) and *E. coli* O111 K58 H2 was from this laboratory.

Media and culture conditions. Strains were stored at -70°C in brain-heart infusion broth containing 10% (v/v) glycerol. Iron restricted growth was achieved by growing bacteria in trypticase soy broth (TSB, BBL) supplemented with 6.25 μM -ovotransferrin (Sigma) (Griffiths & Humphreys, 1978), 200 μM - α, α' -dipyridyl (Sigma), 0.5 mM-desferal (Ciba-Geigy), or 0.28 mM-ethylenediaminedihydroxyphenylacetic acid (EDDA, Sigma). EDDA was made iron free by the method of Rogers (1973). Bacteria were incubated for 6 h at 37°C but growth of *E. coli* in TSB containing α, α' -dipyridyl resulted in a small yield of cells. Consequently, in order to obtain sufficient cells for the preparation of outer membranes, *E. coli* was grown in 200 ml TSB for 3 h (37°C), collected by centrifugation (6000 g, 15 min, 37°C), resuspended in TSB containing 200 μM - α, α' -dipyridyl and incubated for a further 3 h. The outer membrane protein profiles of *E. coli* grown with α, α' -dipyridyl by the two above methods appeared to be identical, and consequently the latter culturing procedure was used in this work. Iron replete bacteria were grown in TSB alone.

Isolation of outer membranes. Outer membranes were prepared for SDS-PAGE and the proteins detected as outlined by Chart & Griffiths (1985). Immunoblotting was done as described by Stevenson & Griffiths (1985). A rabbit antiserum raised to the ferric enterobactin receptor protein of *E. coli* O111 (Chart & Griffiths, 1985) was used in the present work. Antibodies bound to antigens on nitrocellulose sheets were detected using an ^{125}I -labelled goat anti-rabbit antiserum (Stevenson & Griffiths, 1985). Unlabelled goat anti-rabbit antiserum was obtained from Miles Laboratories.

Growth kinetics of *E. coli* in TSB containing iron chelating agents. Portions (20 ml) of TSB, with or without ovotransferrin (6.25 μM), EDDA (0.28 mM) or α, α' -dipyridyl (200 μM) were gently stirred at 37°C . Broths containing ovotransferrin were gassed with sterile air containing 5% (v/v) CO_2 (100 ml min^{-1}). Each medium was inoculated with sufficient bacteria to give an initial bacterial density of 10^3 viable organisms ml^{-1} and growth was monitored by viable counts. Samples of each broth (0.5 ml) were diluted appropriately in broth saline (10%, v/v, brain-heart infusion broth in 8 g NaCl l^{-1}) and plated on to MacConkey agar (Oxoid).

RESULTS

Expression of iron regulated outer membrane proteins

When growing in TSB containing ovotransferrin, *E. coli* O111 synthesized three new outer membrane proteins with molecular masses of 81, 78 and 74 kDa as determined by SDS-PAGE (Griffiths, 1985). These proteins were used to determine the relative mobilities of the iron regulated outer membrane proteins of *E. coli* O164 and *E. coli* C. The three outer membrane proteins of *E. coli* O111 were expressed regardless of which of the four chelators was used to produce iron restriction (Fig. 1*a*). However, the apparent quantity of a given protein clearly depended upon which chelator was used to induce the restriction. Cultures of *E. coli* O111 grown in TSB containing either ovotransferrin or desferal (Fig. 1*a*, lanes 2 and 5) synthesized similar amounts of the 81 kDa protein. In both cases, the amounts synthesized were similar in quantity to the major outer membrane proteins (Fig. 1*a*). Furthermore, *E. coli* O111 grown in TSB containing ovotransferrin produced comparable amounts of the 81 kDa and 74 kDa proteins (Fig. 1*a*, lane 2); *E. coli* O111 grown in TSB containing desferal, however, synthesized slightly more of the 81 kDa protein (Fig. 1*a*, lane 5) than of the 74 kDa protein. However, in the presence of EDDA or α, α' -dipyridyl considerably less of the 81 kDa and 74 kDa proteins were produced as compared to the major outer membrane proteins (Fig. 1*a*, lanes 3 and 4).

Fig. 1. (a) SDS-PAGE profiles of outer membrane proteins (30 μ g per lane) isolated from *E. coli* O111 (lanes 1–5) and *E. coli* O164 (lanes 6–10). Bacteria were grown in iron replete medium (lanes 1 and 6), or in TSB containing ovotransferrin (lanes 2 and 7), EDDA (lanes 3 and 8), α,α' -dipyridyl (lanes 4 and 9) or desferal (lanes 5 and 10). The variation in the amounts of the iron regulated outer membrane proteins expressed (81, 78 and 74 kDa) can be observed when these bands are compared to the major outer membrane protein bands (ompC, ompA). (b) SDS-PAGE profiles of outer membrane proteins (30 μ g per lane) isolated from *E. coli* O164, showing the improved resolution achieved by extending the time of electrophoresis. *E. coli* O164 was grown in the presence of EDDA (lane 1), α,α' -dipyridyl (lane 2) or ovotransferrin (lane 3). Apparent molecular masses (kDa) are indicated.

As mentioned previously, *E. coli* O164 and *E. coli* C failed to synthesize an 81 kDa protein when grown in TSB containing α,α' -dipyridyl (Chart & Griffiths, 1985); this was confirmed in the present work (Fig. 1a, lane 9). However, surprisingly, *E. coli* O164 expressed this protein when grown in TSB containing ovotransferrin, EDDA or desferal (Fig. 1a, lanes 7, 8 and 10). When the time of electrophoresis was extended as described previously (Griffiths *et al.*, 1985a), resolution of the iron regulated outer membrane proteins of *E. coli* O164 was considerably improved. Using this method it was shown that the nature of the iron chelators used to induce iron restriction was responsible for considerable variation in the quantity of proteins expressed (Fig. 1b), as was found with *E. coli* O111. In the presence of EDDA (Fig. 1b, lane 1) or desferal (not shown), *E. coli* O164 expressed 83 kDa, 81 kDa, 78 kDa, 76 kDa and 74 kDa outer membrane proteins. The 74 kDa protein almost comigrated with the 76 kDa protein. *E. coli* O164 growing in TSB containing ovotransferrin (Fig. 1b, lane 3) also synthesized proteins of 83 kDa and 81 kDa and a protein of 76 kDa, as well as a trace of the 74 kDa protein. In contrast, *E. coli* O164 grown in TSB containing α,α' -dipyridyl (Fig. 1b, lane 2) produced a single protein of 76 kDa. *E. coli* C did not produce an 81 kDa outer membrane protein in the presence of any of the chelators and only trace amounts of the 74 kDa and 78 kDa proteins were seen (data not shown).

Detection of the 81 kDa iron regulated outer membrane protein by immunoblotting

Because of the variation observed in the expression of the iron regulated outer membrane proteins, and because of the possibility of failing to detect small amounts of protein by

Fig. 2. Autoradiogram of an immunoblot showing reaction of antibodies raised to purified enterobactin receptor protein of *E. coli* O111 with the 81 kDa protein (arrowed) in the outer membrane protein profiles of *E. coli* O111 (lanes 1-5) and *E. coli* O164 (lanes 6-10) (the growth media are designated by the same lane numbers as in Fig. 1a). The antibodies did not detect an 81 kDa protein in the protein profiles of iron replete bacteria (lanes 1 and 6) or in *E. coli* O164 grown in TSB containing α, α' -dipyridyl (lane 9); (15 μ g protein per lane; 20 μ l antiserum per lane).

Fig. 3. Growth of *E. coli* O111 (a) and *E. coli* O164 (b) in TSB containing iron chelating agents. The growth of both strains was retarded by ovotransferrin (■---■) and EDDA (▲---▲); however, growth in TSB containing α, α' -dipyridyl (◆---◆) resulted in considerable loss of viability, as compared to growth in TSB alone (●—●). Data shown represent one of four experiments.

Coomassie blue staining, a polyclonal rabbit antiserum raised to the 81 kDa enterobactin receptor protein of *E. coli* O111 was used as a sensitive probe. Using the procedures outlined previously (Chart & Griffiths, 1985) outer membrane proteins that had been subjected to SDS-PAGE were transferred on to nitrocellulose paper and reacted with the antiserum. This antiserum detected the 81 kDa protein in the outer membrane of *E. coli* O111 regardless of which chelator was used to produce iron restriction (Fig. 2, lanes 2-5). Furthermore, *E. coli* O164 synthesized an 81 kDa outer membrane protein when grown in TSB containing ovotransferrin, EDDA or desferal (Fig. 2, lanes 7, 8 and 10) but no 81 kDa protein was produced by the organism growing in TSB containing α, α' -dipyridyl (Fig. 2, lane 9). No 81 kDa outer membrane protein was detected in *E. coli* C regardless of which chelating agent was used to produce iron restriction (data not shown). Iron replete cells of *E. coli* O111 and *E. coli* O164 did not express the 81 kDa protein (Fig. 2, lanes 1 and 6).

Effect of iron chelators on the growth kinetics of E. coli

The type of chelator used to cause iron restriction influenced greatly the growth kinetics of both *E. coli* O111 and *E. coli* O164 (Fig. 3). However, although the growth of the two strains was noticeably retarded in TSB containing ovotransferrin or EDDA, the addition of α, α' -dipyridyl to

the growth medium resulted in a considerable loss of viability by 4 h. Bacteria which had been grown in the presence of α,α' -dipyridyl had a distinctive pink colour.

Ferric iron uptake by E. coli C

E. coli strain C was unusual in not producing a ferric enterobactin receptor protein with any of the iron chelators used; all other *E. coli* isolates examined to date have been found to synthesize this protein (Chart & Griffiths, 1985, and unpublished data). Possible synthesis of enterobactin by *E. coli* C was examined by growing this strain in the Tris-succinate medium of Simon & Tessman (1983) and analysing the culture supernatants for phenolic compounds using the method of Arnow (1937). No phenolic siderophores were produced by *E. coli* C. In an attempt to determine how *E. coli* C obtains iron, the bacteria were grown on Vogel Bonner Glucose Citrate medium (VBGC). This medium supports the growth of *E. coli ent* mutants by providing iron in the form of ferric citrate. However, *E. coli* C did not grow on this medium, indicating a defect in ferric citrate mediated iron transport. On VBGC plates, growth of *E. coli* C was stimulated by the addition of ferrous sulphate (5 mM), ascorbate (10 mM) or ferrichrome (50 μ M) and to a moderate extent by 2,3-dihydroxybenzoic acid (50 μ M). No stimulation of growth was observed with enterobactin (43 μ M).

DISCUSSION

The qualitative and quantitative differences in the expression of iron regulated outer membrane proteins of *E. coli* O111 and *E. coli* O164 described in this paper demonstrate that the nature of the chelator used to restrict iron supply influences the synthesis of iron regulated proteins. It has been reported that the synthesis of the 81 kDa FepA protein and the 74 kDa Cir protein is coordinately regulated (McIntosh & Earhart, 1977), and this would explain why both these proteins are incorporated into the cell envelope of *E. coli* during growth in TSB containing ovotransferrin. However, the presence of α,α' -dipyridyl, EDDA or desferal in the medium resulted in disproportionate expression of these proteins. This suggests either that the genes encoding these proteins may under certain conditions be expressed differently or that the synthesis of these proteins could be sensitive to inhibition by the various iron chelators used. The iron chelator α,α' -dipyridyl is of particular interest as *E. coli* O164 growing in TSB containing this compound synthesized only one iron regulated outer membrane protein (76 kDa) although this strain can clearly produce an 81 kDa and a 74 kDa protein and does so when grown in the presence of ovotransferrin. Strain O164 has been shown to produce aerobactin and is similar to *Shigella flexneri*, which also produces aerobactin as well as a 76 kDa iron regulated outer membrane protein (Griffiths *et al.*, 1985*b*). Indeed, when strain O164 is grown in TSB containing ovotransferrin the SDS-PAGE profile of the iron regulated outer membrane proteins is virtually identical to that of *E. coli* K12-*Shigella* hybrid strains which have acquired the *arg-mtl* region of the *S. flexneri* chromosome.

The effects observed when the iron chelators EDDA, α,α' -dipyridyl or desferal are used might possibly be explained by the molecular masses of these compounds. These three chelators have molecular masses sufficiently small to allow their entry into the bacterial cell and consequently to interfere with cellular metabolism. The fact that α,α' -dipyridyl causes *E. coli* to become pink suggests that this chelator does enter the bacterial cell. Furthermore, it has been reported that α,α' -dipyridyl can lyse bacterial cells (Neilands, 1982); this may explain the bactericidal effects observed here.

The chelator dependent variation in the expression of iron regulated outer membrane proteins in *E. coli* suggests that some caution should be exercised when extrapolating results obtained from *in vitro* studies on bacterial iron uptake to the events occurring in the host during infection. It is likely that data obtained from bacteria growing in media containing transferrins most closely describe conditions *in vivo*. However, our observations that some strains of *E. coli* express outer membrane proteins *in vivo* which are not synthesized during growth *in vitro* in the presence of ovotransferrin indicate that even by using naturally occurring iron binding glycoproteins it is not always possible to mimic entirely *in vitro* the conditions encountered within the host

(Griffiths *et al.*, 1985*a*). It is to be expected, of course, that invading pathogens will encounter other environmental conditions *in vivo* in addition to iron restriction (Griffiths, 1983). Interestingly, the 68 kDa outer membrane protein produced by *E. coli* O18 growing *in vivo* is also expressed during growth in TSB containing α, α' -dipyridyl (Griffiths *et al.*, 1985*a*).

Our data concerning *E. coli* C suggest that this strain has a defect in the iron transport systems for both iron citrate and ferric enterobactin, but not for ferrichrome or ferrous iron; the idea that *E. coli* C can use ferrous iron is deduced from the fact that growth was stimulated by ascorbate. It was reported by G. Bertani that *E. coli* C grew poorly in minimal media and has a prolonged lag when subcultured from nutrient broth to minimal media [notes from p. 1 of Bertani's 1964 strain list kindly provided by Barbara Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., USA]. This may reflect the defect in iron assimilation analysed by the present work. A faster growing derivative of *E. coli* C exists (*E. coli* C-1a), indicating a possible reversion of the mutation(s) responsible for the phenotype of *E. coli* C described here.

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