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Identification of the ferrioxamine B receptor, FoxB, in *Escherichia coli* K12

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The photoreactive *p*-azidobenzoyl analog of ferrioxamine B was used to show that ferrioxamine-B-mediated iron transport is separate and distinct from coprogen-mediated iron transport in *Escherichia coli*. Photolysis of this analog inhibited uptake of [^{59}Fe]ferrioxamine B but not [^{59}Fe]coprogen or [^{59}Fe]ferrichrome. Conversely, photolysis of the *p*-azidobenzoyl analog of coprogen B inhibited uptake of [^{59}Fe]coprogen but not [^{59}Fe]ferrioxamine B or [^{59}Fe]ferrichrome. Photolabeling of outer membranes with *p*-azidobenzoyl-[^{59}Fe]ferrioxamine B resulted in the labeling of two iron-regulated peptides with molecular masses of about 66 and 26 kDa. Expression of these peptides was increased when ferrioxamine B was the sole iron source. Both peptides were present in outer membrane preparations of the *fhuF* mutant H1717, but the 66 kDa peptide was not inducible. These results are evidence for an outer membrane receptor in *E. coli* unique for linear ferrioxamines.

Keywords: *Escherichia coli*, ferrioxamine receptor, iron transport, photoaffinity label, siderophores

Introduction

Six iron-regulated proteins have been identified in the outer membrane of *Escherichia coli* K12. Three proteins, FepA (81 kDa), Fiu (83 kDa) and Cir (74 kDa), have been linked with catecholate-mediated iron transport (Pierce *et al.* 1983, Curtis *et al.* 1988, Nikaido & Rosenberg 1990), FepA being the outer membrane receptor for the native iron-siderophore complex, ferrienterobactin. A fourth iron-regulated protein, FecA (80 kDa), serves as the receptor for ferric dicitrate (Wagegg & Braun 1981). The last two proteins, FhuA (78 kDa) and FhuE (76 kDa), are the receptors for ferrichrome and coprogen respectively (Kadner *et al.* 1980, Hantke 1983). Strains carrying the ColV plasmid also have a seventh iron-regulated protein, Iut (74 kDa), which is the receptor for ferriaerobactin (Grewal *et al.* 1982). In addition, a mutant phenotype, *fhuF*, has been reported which has diminished ability to use ferrioxamine B (FOB) as a source of iron for growth (Hantke 1987). This suggests the presence of still another, as yet unreported, receptor protein that functions in FOB-mediated iron transport, although FhuE has been reported to be the receptor for FOB (Hantke 1983, Braun & Hantke 1991).

A tool commonly used to identify receptors in higher eucaryotes is the photoaffinity label. Among the eucaryotic receptors so identified are those for insulin (Yip *et al.* 1980), thrombin (Carney *et al.* 1979), serotonin and histamine (Wouters *et al.* 1985). The photoaffinity labels are ligand derivatives that retain binding affinity for the receptor and which are coupled with a photoreactive group capable of covalently linking the ligand to the receptor (Linsley *et al.* 1981, Ruoho *et al.* 1984). The covalent binding of the ligand to a receptor often prevents transport via that receptor. Thus, photoaffinity labels can be used in receptor–ligand interaction and competition studies, such as the use of the photoaffinity label *p*-azidobenzoylcoprogen B (ABCB) to block siderophore-mediated iron transport in *Neurospora crassa* (Bailey *et al.* 1986). In addition, a photoaffinity label containing a radionuclide can be used as a probe to identify the receptor protein following polyacrylamide gel elec-

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trophoresis (Yip *et al.* 1985, Shanahan *et al.* 1985). We have used the binding affinity of receptor for photoaffinity label to identify and characterize the FOB receptor in *E. coli*, which we have called FoxB using the Fox nomenclature of Berner & Winkelmann (1990).

Materials and methods

Bacterial strains and media

The *E. coli* strains used in this study are listed in Table 1 and were maintained on Luria Bertani agar (Maniatis *et al.* 1982). Inocula for growth response assays and iron-uptake studies were grown in the iron-replete (8 μM) medium, AAM, which contains 2 g K_2HPO_4, 0.5 g $(NH_4)_2HPO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 1 g yeast extract (Difco Laboratories), 1 g vitamin assay casamino acids (Difco) and 10 g sucrose/l distilled deionized water—ddH_2O (Powell *et al.* 1983). For iron-starved cells, the medium EAM was prepared by chelating the free iron in AAM with 20 μM ethylenediamine-di(*o*-hydroxyphenylacetic acid)—EDDHA (Nelson *et al.* 1988). Alternatively, AAM was deferrated by removing the iron from the yeast extract, casamino acids and sucrose with Chelex 100 (BioRad) prior to mixing the organic and inorganic components of the medium. This medium, ECAAM, was supplemented with 0.1 mM $CaCl_2$ and the pH was adjusted to 7 with concentrated HCl. As a precaution against the presence of free iron in the medium when siderophores were used as sole iron sources, 20 μM EDDHA was also added. Adventitious iron was removed from impure EDDHA (Sigma) using 8-hydroxyquinoline (Rogers 1973).

Preparation of siderophores and photoaffinity labels

Desferal, purchased from Ciba Pharmaceutical Co. was ferrated to obtain FOB by dissolving Desferal and an equimolar amount of ferrous ammonium sulfate in water, neutralizing the solution with NaOH, and then purifying the FOB by adsorption onto a column of Amberlite XAD_2 (Sigma). The FOB was subsequently eluted from the column with methanol. After a small amount of water was added to the eluate, the methanol was evaporated by vacuum distillation. The FOB was then freeze-dried. Coprogen, desferricoprogen and coprogen B were either gifts from Professor Günther Winkelmann (Universität Tübingen, Germany) or extracted and purified from culture filtrates of *N. crassa* (Wong *et al.* 1983) and *Fusarium dimerum* (Diekmann 1970), respectively. Ferrioxamine (FOD_1) was a gift from Dr Walter Keller-Schierlein (Eidgenössische Technische Hochschule, Zürich, Switzerland). Ferrichrome and ferrienterobactin were extracted and purified from culture filtrates: ferrichrome from cultures of *Ustilago sphaerogena* (Emery & Neilands 1960), and ferrienterobactin from *E. coli* AN102 (Pierce *et al.* 1983).

For ^{59}Fe-uptake studies, ferrichrome was deferrated with KOH (Emery & Neilands 1960) and coprogen was deferrated with 8-hydroxyquinoline (Wiebe & Winkelmann 1975). The desferriferrichrome and desferricoprogen were referrated with 2 μCi/ml $^{59}FeCl_3$ (New England Nuclear). Ferrous ammonium sulfate was added to bring the ferrated siderophore concentration to 1 mM. [^{59}Fe]FOB was prepared by dissolving Desferal in 10 mM HEPES pH 7.4 and adding $^{59}FeCl_3$ and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ as before. All [^{59}Fe]siderophores were prepared with deferrated siderophore in excess of iron by at least 5%.

Table 1. *E. coli* strains used

Strain	Genotype	Origin or reference
RW193	*F⁻. thi proC leu trp lacY rpsL galK ara mtl entA403 xyl azi tsx*	Wayne *et al.* (1976)
5AN	*F⁻. thi proC leu trp lacY rpsL tsx fhuA entA403 fepA*	This study
22H1	*araD139 lac169 aroB rpsL thi fepA fhuE::Mud1(Ap lac)*	Nelson *et al.* (1988)
BN3306	*RW193 leu⁺ supE44 fhu B panB*	Prody & Neilands (1984)
14BN	*BN3306 fepA*	Nelson *et al.* (1988)
MFT5	*fur::Tn5 rpsL thi ade*	Schmitt & Payne (1988)
H1717	*araD (argF-lac) U169 rpsL relA flbB deoC aroB fhu::lplacMu*	Hantke (1987)

Photoreactive siderophores were synthesized by reacting coprogen B or FOB with *N*-hydroxysuccinimidyl-4-azidobenzoate (Sigma) in dimethylformamide with a small amount of triethylamine to form ABCB or *p*-azidobenzoylferrioxamine B (ABFOB) respectively. Coprogen B or FOB was dissolved first in <50 μl methanol, to which was added the dimethylformamide and triethylamine. If necessary, the reaction mixture was gently heated in a warm water bath to aid dissolution. The *N*-hydroxysuccinimidyl-4-azidobenzoate was then dissolved in the solution and the reaction was allowed to proceed for 24 h at 25 °C. The photoreactive siderophores were purified using preparative thin-layer chromatography (TLC) on 1000-μm silica gel GF plates (Analatech) using a chloroform:methanol:water (65:2:4) solvent system. The R_f for ABCB is 0.57 (Carrano *et al.* 1986); the R_f for ABFOB was 1.0. Methanol was used to elute the photoreactive siderophore from the silica gel. [$^{55/59}$Fe]ABFOB was prepared by deferrating ABFOB with an equal volume of 1 M KOH. The resulting ferric hydroxide precipitate was removed by centrifugation. The supernatant containing the deferrated ABFOB (ABDFOB) was quickly neutralized by adding 0.5 volumes of 0.5 M Tris/HCl pH 8 and concentrated HCl. The compound was referrated by adding 100 μCi (0.1 μmol) $^{55}FeCl_3$ and 2.7 μCi (4.5 nmol) $^{59}FeCl_3$ to 100 μl (0.225 μmol) of ABDFOB. Since the latter flocculated over time, chelation of the radionuclides was allowed to proceed for 24 h until neither a white flocculant (ABDFOB) nor an orange precipitate (ferric hydroxide) was visible. The [$^{55/59}$Fe]ABFOB was checked for purity via TLC with the $CHCl_3/CH_3OH/H_2O$ solvent. All preparations involving photoreactive compounds were done under very low light conditions.

Effect of ABFOB photolysis on growth

Assays for the growth response of *E. coli* to different siderophores have been described previously (Powell *et al.* 1983, Nelson *et al.* 1988). This methodology was used to test the ability of ABFOB to be the sole iron source for *E. coli*. Concomitantly, the ability of ABFOB to inhibit growth of *E. coli* upon photoactivation was tested. The inocula for these experiments were grown overnight in AAM, transferred (1:100) to EAM supplemented with 0.4 μM FOB and grown for 6 h at 37 °C in shake culture. After 6 h, the cells were harvested by centrifugation and washed three times with ddH_2O to an absorbance of 0.1 (50 Klett units) using a Klett–Summerson colorimeter with a blue-400 filter. Samples of the ABFOB dilution series were inoculated with RW193 and then photolyzed using a mercury vapor lamp (American Electric, 175 W) for 2 min prior to incubation. Inhibition due to ABFOB-receptor binding was differentiated from death caused by ultraviolet irradiation or nitrene generation of free radicals by adding 0.1 μM ferrichrome or ferrienterobactin following photolysis.

To determine the binding affinity of ABFOB, 1 ml cells (A_{400} = 0.1) was diluted 1:10 in EAM supplemented with 10 μM ABFOB. The cells were photolyzed with duplicate samples withdrawn after 0, 0.5, 1 and 2 min of ultraviolet exposure. The samples were diluted 1:10 in EAM or EAM supplemented with 0.1 μM ferrichrome, 0.1 μM coprogen, or 2 μM FOB, and incubated at 37 °C for 14 h.

Inhibition of iron uptake following ABFOB or ABCB photolysis

Inocula for uptake experiments were grown in 100 ml AAM at 37 °C in shake culture for 3 h (midlog phase). A quarter of this culture was harvested by centrifugation (6800 × *g*, 4 °C, 5 min) and washed with 10 ml ddH_2O. The washed pellet was resuspended in 100 ml EAM and grown in shake culture at 37 °C for 3 h. The resulting starved cells were harvested by centrifugation and the pellet resuspended in EAM and incubated at 37 °C for 30 min. For the ^{59}Fe uptake experiments, 2 μM ABFOB or ABCB was added to starved cells, followed by addition of 10 μM [^{59}Fe]FOB, [^{59}Fe]ferrichrome or [^{59}Fe]coprogen. Duplicate samples were taken 3, 10, 20 and 30 min after addition of ^{59}Fe. Photolyzed samples were irradiated with the ultraviolet lamp for 2 min after the 10-min sample was taken. All samples were centrifuged through a mixture of dibutylphthalate/diisononylphthalate (6:4, by volume; Sigma; Sivaprasadarao & Findlay 1987). The supernatant was then decanted and radioactivity in the cell pellets was measured in a Beckman 7000 gamma counter.

Preparation of outer membrane

Iron-sufficient cells were grown in AAM supplemented with $FeCl_3$ to 100 μM total iron concentration. Iron-starved cells were first grown in AAM overnight, harvested by centrifugation (6800 × *g*, 5 min, 4 °C), washed with ddH_2O and then starved for 4 h in EAM. For induction of FoxB, cells were grown in ECAAM supplemented with 10 μM FOB. To obtain outer membranes, the cells were pelleted, resuspended in 1 ml 10 mM HEPES pH 7.4 per 100 ml culture and disrupted by passage three times through a French pressure cell at 138 MPa. Cell lysates were diluted with 17 ml HEPES buffer and centrifuged (2000 × *g*, 5 min, 4 °C) to remove unbroken cells and debris. The supernatants were ultracentrifuged (100 000 × *g*, 45 min, 4 °C) to pellet the membranes. The membrane pellets were resuspended in HEPES buffer with 2% Triton X-100 (Sigma) to solubilize the cytoplasmic membrane (Schnaitman 1973, Mizushima & Yamada 1975). The outer membranes where then pelleted by ultracentrifugation, resuspended in HEPES buffer and stored frozen at −70 °C. Total protein concentration in each sample of outer membrane suspension was determined spectrophotometrically at 280 nm using a Beckman 25 spectrophotometer and the samples were diluted with HEPES buffer to equalize the total protein per sample.

Photolabeling of the FOB receptor protein

Outer membranes were diluted with HEPES buffer to an A_{280} of 0.29 and 20 μl of each sample was mixed with 5 μM

[$^{55/59}$Fe]ABFOB. The mixture was chilled on ice for 10 min and then photolyzed for 2 min. The outer membrane proteins were precipitated with an equal volume of acetone. The precipitate was pelleted by centrifugation, washed with acetone, dried and resuspended in HEPES buffer.

Photolabeling of whole cells and of the periplasmic fraction were also done to determine if FhuD was photolabeled. For labeling of whole cells, either RW193 or BN3306 were starved for iron in ECAAM as previously described. The cells were harvested by centrifugation as above and resuspended in 10 ml aliquots of ECAAM. [$^{55/59}$Fe]ABFOB (10 μM) was added to the cells, which were then incubated at 37 °C for 30 min, then photolyzed for 2 min. After the cells were osmotically shocked (Koshland & Botstein 1980), periplasmic proteins were precipitated with an equal volume of $CHCl_3$ and 1.19 volumes of methanol and resuspended in 10 μl 10 mM HEPES pH 7.4. For labeling of the periplasmic fraction, RW193 was grown in ECAAM plus 10 μM FOB and then osmotically shocked. The periplasmic proteins were precipitated with $CHCl_3$ and methanol and resuspended in 10 mM HEPES pH 7.4, and photolabeled.

Electrophoresis

Outer membrane proteins were solubilized by adding an equal volume of 2X electrophoresis sample buffer consisting of 60 mM Tris/HCl pH 6.8, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate and 10% glycerol (Guellaen *et al.* 1984), and boiling for 2 min in a 100 °C water bath. After boiling, 0.1% bromophenol blue was added. Proteins were separated electrophoretically using 4–20% polyacrylamide gradient gels (Daiichi Pure Chemicals) in Seprabuff (Integrated Separation Systems) on a Mighty Small gel apparatus (Hoefer Scientific Instruments) with a BioRad 3000 Xi electrophoresis power supply. Non-radioactive gels were stained with Coomassie blue G250, which has the sensitivity of silver staining (Neuhoff *et al.* 1988). Gels for the [$^{55/59}$Fe]ABFOB labeling were washed in 0.1 M Tris/HCl pH 8 with 20% methanol, dried without staining, and subjected to autoradiography using Kodak X-Omar film and Lightning Plus intensifying screens (DuPont Cronex). Following autoradiography these gels were stained with Coomassie blue G250 and photographed.

Results and Discussion

Photoaffinity labels

In the non-aqueous polar solvent, dimethylformamide, the nucleophilic free amino group of FOB reacted with the carbonyl group of *N*-hydroxysuccinimidyl-4-azidobenzoate causing the ester to cleave forming *N*-hydroxysuccinimide and the azidobenzoyl analog of the siderophore (Figure 1). The absorption spectrum of ABFOB showed three peaks in the visible to ultraviolet range: a broad peak at about 430–440 nm typical of ferric trihydroxamate

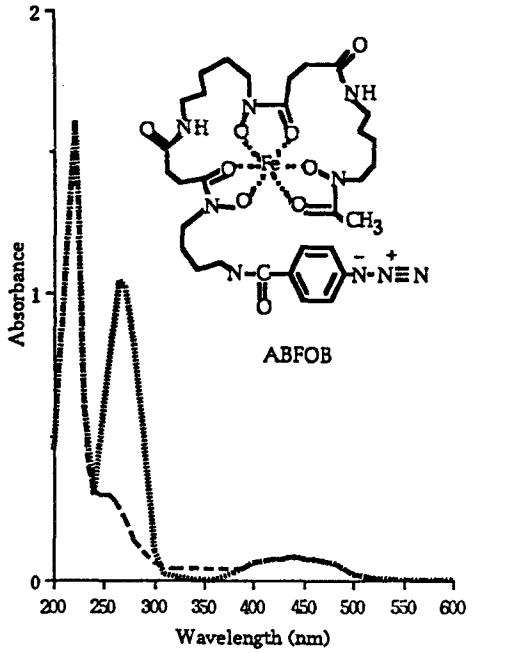

Figure 1. The structure and the absorption spectra of ABFOB before (——) and after (- - -) photolysis.

siderophores (Wong *et al.* 1983), a sharp peak at 265 nm typical of aryl azides (Bayley & Knowles 1977) but not of FOB, and a sharp peak at 220 nm typical FOB. The peak at 265 nm totally disappeared after exposure to ultraviolet light for 1 min in a quartz cuvette. This phenomenon was also reported for ABCB (Bailey *et al.* 1986).

Growth response of E. coli *to various siderophores*

The maximum growth response of *E. coli* RW193 to ferrichrome, coprogen and ferrienterobactin is achieved with addition of 1 μM siderophore (Nelson 1988). In contrast, 10 μM FOB and more than 40 μM non-photolyzed ABFOB were required to achieve about the same growth response (Figure 2). Likewise, 5AN, 22H1 and BN3306 achieved a maximum growth response from 1 μM coprogen, ferrichrome and ferrienterobactin, respectively (Nelson *et al.* 1988), but 5AN and 22H1 required 20 μM FOB to achieve the maximum growth response. The FhuA and FepA mutations in 5AN and the FhuE and FepA mutations in 22H1 did not significantly ($F_{0.05} = 0.96$, d.f. = 2/8) modify their growth response to FOB (Table 2). However, in the *fhuF* background of H1717, the growth response to FOB was decreased 65%, and in the *fhuB* (no hydroxamate-mediated iron transport) background of BN3306 and 14BN was eliminated to all intents and purposes. These growth responses contradict published reports that *E. coli* is not able to use FOB as

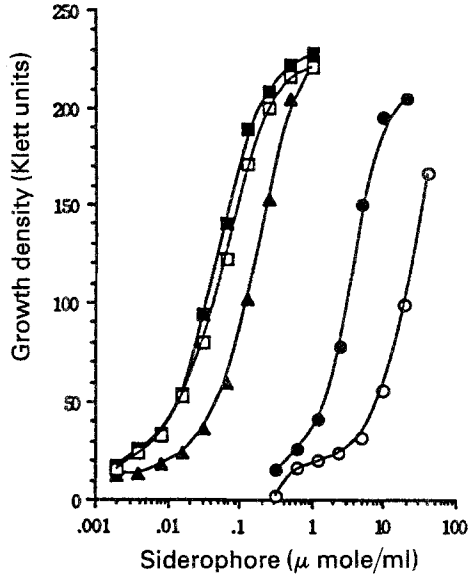

Figure 2. The growth of *E. coli* RW193 in response to increasing concentrations of ferrichrome (■), coprogen (□), ferrienterobactin (▲), FOB (●) and ABFOB (○). 50 Klett units ≡ A_{400} of 0.1.

Table 2. The growth response of different *E. coli* strains in EAM with and without FOB

Strain	Mutation in siderophore uptake	Mean growth response (A_{400}) with FOB at		
		10 μM	20 μM	0 μM
RW193		0.440	—	0.020
5AN	*fhuA fepA*	0.448	—	0.012
22H1	*fhuE fepA*	0.380	—	0.014
H1717	*fhuF*	0.132	—	0.055
BN3306	*fhuB*	—	0.018	0.030
14BN	*fhuB fepA*	—	0.040	0.020

an iron source (Rabsch & Reissbrodt 1988) or that FhuE is the receptor for FOB (Hantke 1983, Braun & Hantke 1991).

Effect of ABFOB photolysis on growth

ABFOB proved to be very suitable for working with biological systems. First, it was activated by longer ultraviolet wavelengths. Since most of the hazards to biological systems stemming from ultraviolet radiation are caused by wavelengths shorter than 300 nm, using longer wavelengths to activate the photoaffinity probe no doubt reduced cell damage. In addition, photoactivation by wavelengths longer than 360 nm allowed the use of Pyrex glass tubes in photoaffinity reactions. The glass added protection to cells, since it blocks shorter, more dangerous, ultraviolet rays. Second, both the photoreactive and the light-inactivated compounds functioned as suitable sources of iron for *E. coli*, although they were less effective in supplying iron for growth than other hydroxamates or ferrienterobactin. When ABFOB was light-inactivated prior to addition to *E. coli*, it was only 79% as effective an iron source as the photoreactive compound (data not shown).

Photolysis of ABFOB in the presence of RW193 resulted in almost total growth inhibition (Figure 3). In contrast, photolysis of FOB in the presence of *E. coli* resulted in only 9% growth inhibition (data not shown). The growth inhibition of *E. coli* by ABFOB photolysis was overcome by the addition of 0.1 μM ferrichrome, ferrienterobactin or coprogen but not by 2 μM FOB (Figures 3 & 4).

Inhibition of iron uptake following ABFOB or ABCB photolysis

Confirmation that neither FhuE nor FhuA is the receptor for the ferrioxamines was provided by experiments using ABFOB to block ferrioxamine-mediated iron uptake (Figure 5; Table 3). Both FOB- and FOD_1-mediated ^{59}Fe uptake by RW193 were blocked after the cells were photolyzed in the presence of ABFOB. Although the presence of ABCB reduced the uptake rate of [^{59}Fe]FOB, especially compared to the uptake rate of [^{59}Fe]FOB in the presence of ABFOB, there was essentially no

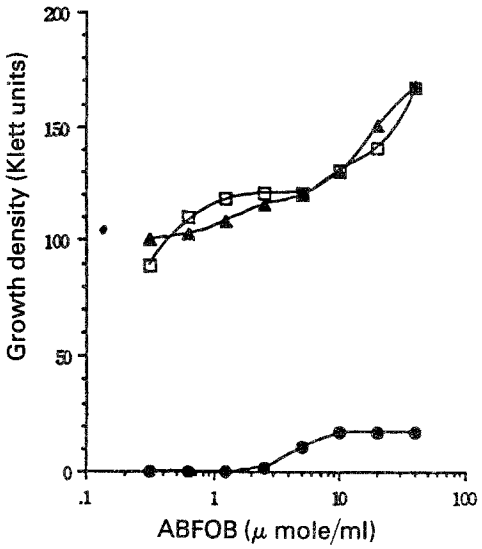

Figure 3. Photolytic inhibition of the growth of RW193 in the presence of increasing concentrations of ABFOB (●). Ferrichrome (□) and ferrienterobactin (▲) (0.1 μM) were added following photolysis. 50 Klett units ≡ A_{400} of 0.1.

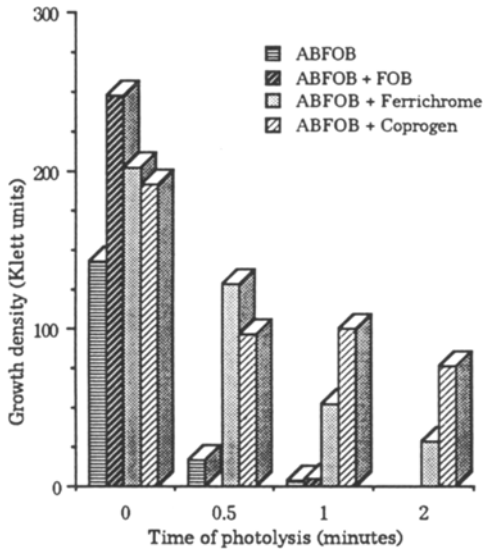

Figure 4. Growth of RW193 in the presence of 10 μM ABFOB following increasing exposure to ultraviolet light. FOB (2 μM), ferrichrome (0.1 μM) or coprogen (0.1 μM) were added following photolysis. 50 Klett units $\equiv A_{400}$ of 0.1.

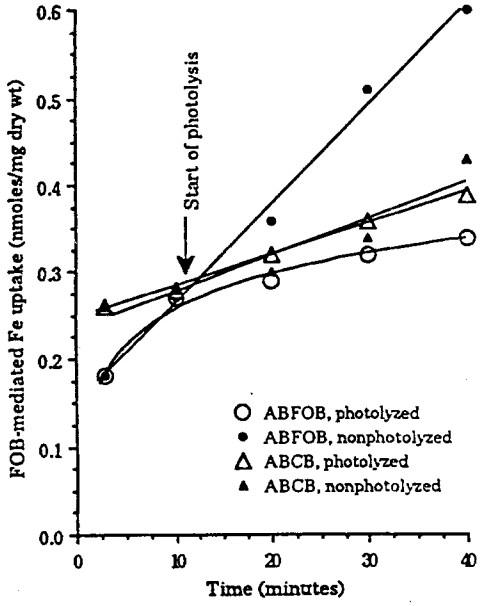

Figure 5. Uptake of [^{59}Fe]FOB by RW193 in the presence of ABFOB and ABCB. The concentration of [^{59}Fe]FOB was 10 μM; the concentration of ABFOB or ABCB was 2 μM.

difference in the uptake rate of [^{59}Fe]FOB caused by photolysis in the presence of ABCB (Figure 5). In contrast, photolysis in the presence of ABCB but not ABFOB inhibited coprogen-mediated ^{59}Fe uptake (Table 3). Also, photolysis of RW193 in the presence of either ABFOB or ABCB had no effect on ferrichrome-mediated ^{59}Fe uptake.

Identification of the FOB receptor

Expression of proteins involved in high-affinity iron transport is regulated by the availability of iron from the medium (McIntosh & Earhart 1977, Hantke 1981). Accordingly, the outer membrane protein profiles of *E. coli* strains grown under different levels of iron stress were compared. A comparison of outer membrane profiles of RW193 grown in AAM, ECAAM and ECAAM plus FOB revealed a number of other bands that were expressed only under iron limitation or when FOB was the iron source. The major iron-regulated proteins, Fiu, FepA and Cir, were expressed more when FOB was present (Figure 6). In addition to these bands, two other bands showed increased expression in the presence of FOB. These corresponded to proteins with apparent molecular masses of approximately 66 and 26 kDa. These proteins were not seen in the outer membranes of cells grown with coprogen as the sole iron source. A comparison of 22H1 and 5AN outer membrane protein profiles with those of RW193 showed that these strains also had both the 66 kDa and the 26 kDa proteins when they were starved for iron or grown with FOB as the sole iron source.

Photoaffinity labeling with [$^{55/59}$Fe]ABFOB of the outer membranes of RW193 grown in ECAAM with 10 μM FOB and of RW193 growin in EAM labeled three loci on the autoradiogram corresponding to apparent molecular masses of about 27, 69 and 171 kDa and one at the dye front (Figure 7). This labeling was more intense in the lane containing the outer membrane proteins of cells grown in FOB. The labeling was diminished by addition of 100 μM FOB to the membranes prior to photolysis. This suggests that the native siderophore protected the receptor from photolabeling. With outer membranes of RW193 grown in AAM only a signal at the dye front was seen. Therefore, we interpret the label at the dye front to represent nonspecific labeling. The radioactive photolabeled peptides at 69 kDa and 27 kDa appeared to correspond to the 66 kDa and 26 kDa bands seen on the Coomassie-stained gels. The discrepancies in the molecular masses may be due to a change in migration caused by the bound ABFOB, or they may stem from the inherent inaccuracy of determining the values from the diffuse loci given off by the radioactive iron. No bands were seen on stained gels in the region

Table 3. The effect of photolysis of ABFOB and ABCB on uptake of ^{59}Fe-labeled siderphores by RW193

^{59}Fe-labeled siderphore	Uptake (nmol/mg dry mass) of							
	ABFOB				ABCB			
	Non-photolyzed		Photolyzed		Non-photolyzed		Photolyzed	
	10 min	40 min	10 min	40 min	10 min	40 min	10 min	40 min
FOD_1	0.22	0.59	0.22	0.21	—	—	—	—
Coprogen	3.40	5.90	3.40	5.60	2.50	5.00	2.50	3.60
Ferrichrome	0.73	1.29	0.73	1.30	0.86	1.11	0.86	1.01

The uptake was measured after incubation for 10 and 40 min with ^{59}Fe-labeled siderphores. Photolysis was for 2 min after the 10-min samples were taken, as described in Methods and Materials.

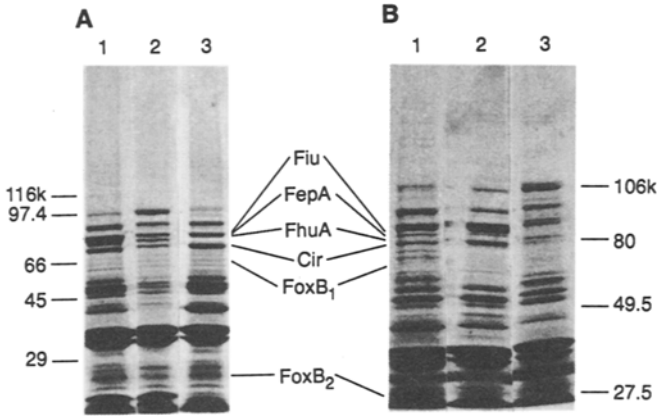

Figure 6. Outer membrane protein profiles of *E. coli* grown with various iron sources. (A) The proteins from RW193 (lane 1), 22H1 (lane 2) and 5AN (lane 3) grown in ECAAM + 10 μM FOB. (B) The proteins from RW193 grown in ECAAM + 10 μM coprogen (lane 1), ECAAM (lane 2) and AAMFe (lane 3). Numbers on the side are molecular masses of standard proteins in kDa.

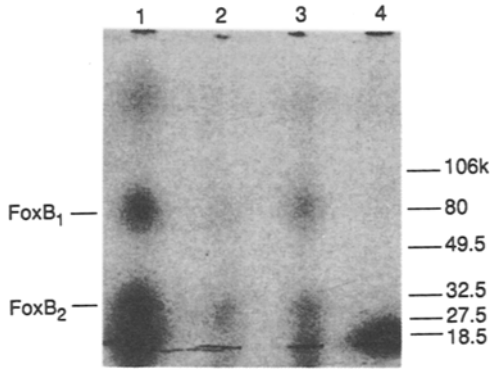

Figure 7. Photolabeling of the outer membrane proteins from RW193. The proteins were from cells grown in ECAAM + 10 μM FOB (lanes 1 and 2), EAM (lane 3) and AAM (lane 4). FOB (100 μM) was added to the proteins in lane 2 prior to photolabeling. Numbers on the right are molecular masses of standard proteins in kDa.

corresponding to 171 kDa. This particular signal, which is weakest on the autoradiograms may be a complex of two of the 69 kDa peptides and one 27 kDa peptide held together by ABFOB.

The 66 and 26 kDa peptides were expressed in outer membrane of the *fur* mutant MFT5 under iron-sufficient conditions and when grown with FOB as the sole iron source (Figure 8). As with RW193, expression of the iron-regulated proteins was greater with FOB as the iron source. MFT5 (*fur*) proteins were also radiolabeled with [$^{55/59}$Fe]ABFOB at 171, 69 and 27 kDa when grown in AAM and when grown in ECAAM plus 10 μM FOB. Once again, labeling of the proteins from cells grown with FOB was more intense than the labeling of proteins not

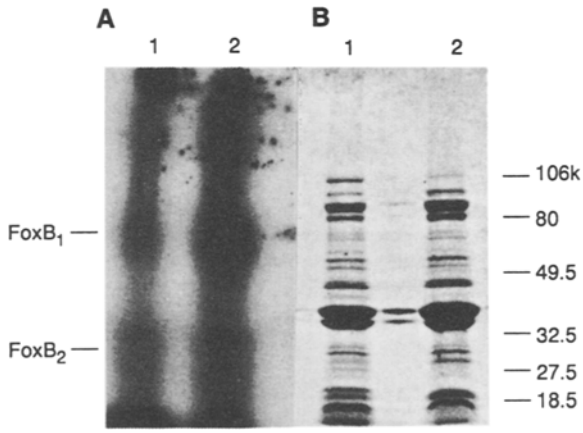

Figure 8. Photolabeling of the outer membrane proteins from MFT5 grown in AAM (lane 1) and ECAAM + 10 μM FOB. (A) The autoradiogram; (B) the Coomassie blue stain. Number on the right are molecular masses of standard proteins in kDa.

grown with FOB. The expression of the 66 and 26 kDa peptides under iron-sufficient conditions in the *fur* mutant indicates that these peptides are regulated by Fur. The increase in the amount of these peptides when FOB was the iron source implies that FOB plays a role in regulation of their production.

The 66 and 26 kDa proteins were also present in membranes of H1717 grown in ECAAM plus 10 μM FOB (Figure 9). However, expression of the 66 kDa protein was at the same level seen in RW193 grown in ECAAM rather than that of ECAAM plus 10 μM FOB. Like RW193 and MFT5, outer membranes of the *fhuF* mutant H1717 were labeled at 27, 69 and 171 kDa when it was grown in ECAAM plus 10 μM FOB. In H1717, labeling at 69 kDa was much weaker than that seen at 69 kDa in RW193 or MFT5. We suggest that the *fhuF* mutation described by Hantke (1987) may be involved in induction of the 66 kDa peptide by FOB. The inability of the *fhuF* mutant to increase production of the 66 kDa part of the ferrioxamine receptor in response to FOB would explain the lower growth response seen with H1717.

The 26 kDa peptide has about the same molecular mass as that reported for FhuD (Fecker and Braun 1983). Although FhuD is a hydrophilic protein located in the periplasm, we attempted to photolabel a periplasmic protein to see if it corresponded with the 26 kDa peptide. No photolabeling was seen in any of the periplasmic preparations leading us to conclude that the 26 kDA peptide is indeed part of the outer membrane receptor and not FhuD.

Although FhuE has been identified (Hantke 1983, Braun & Hantke 1991) as the receptor for both FOB and FOD_1 as well as coprogen and rhodotorulic acid, both FOB and FOD_1 share a receptor that is distinct from FhuE, FhuA, or any other heretofore identified siderophore receptor in *E. coli*. This receptor is unique to FOB and its linear analogs. Because *fhuF* appears to be regulatory in nature and not the outer membrane receptor, we have called the ferrioxamine receptor in *E. coli* FoxB, using the Fox nomenclature that Berner & Winkelmann (1990) used for the ferrioxamine receptor in *Erwinia herbicola*. Photoaffinity labeling of *E. coli* outer membrane proteins suggest that FoxB, unlike other siderophore receptors, is comprised of two peptides ($FoxB_1$ 66 kDa and $FoxB_2$ 26 kDa), both of which contribute to the binding site. FoxB is also unique in that use of FOB as the sole iron souce stimulates greater production of the receptor.

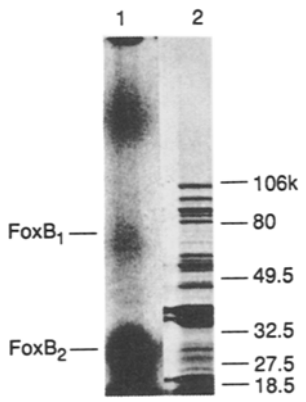

Figure 9. Photolabeling of the outer membrane proteins from H1717 grown in ECAAM + 10 μM FOB. (1) The autoradiogram; (2) the Coomassie blue stain. Numbers on the right are molecular masses of standard proteins in kDa.

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Note added in proof

We have recently isolated a number of ferrimycin A_1 resistant mutants. These mutants showed six different hydroxamate siderophore phenotypes. Most (54%) resistant strains showed no change in their ability to grow using FOB, coprogen B or ferrichrome as iron sources. However, a number (35%) were unable to grow using any of the three hydroxamates; a few (4%) had the phenotype FOB^-, coprogen B^+, ferrichrome$^+$; and another few (4%) had the phenotype FOB^-, coprogen B^-, ferrichrome$^+$. The remaining phenotypes (1% each) were FOB^-, coprogen B^+, ferrichrome$^-$, or FOB^+, coprogen B^-, ferrichrome$^+$. These results suggest that the linear ferrioxamine antibiotic ferrimycin A_1 is also transported by *E. coli* K12. Very possibly this transport is also mediated by the FoxB receptor.