Functions of the siderophore esterases IroD and IroE in iron-salmochelin utilization

Mingang Zhu,1 Marianne Valdebenito,2 Günther Winkelmann2 and Klaus Hantke1

The siderophore salmochelin is produced under iron-poor conditions by Salmonella and many uropathogenic Escherichia coli strains. The production of salmochelin, a C-glucosylated enterobactin, is dependent on the synthesis of enterobactin and the iroBCDEN gene cluster. An E. coli IroD protein with an N-terminal His-tag cleaved cyclic salmochelin S4 to the linear trimer salmochelin S2, the dimer salmochelin S1, and the monomers dihydroxybenzoylsalicylic acid and C-glucosylated dihydroxybenzoylsalicylic acid (salmochelin SX, pacifarinic acid). The periplasmic IroE protein was purified as a MalE–IroE fusion protein. This enzyme degraded salmochelin S4 and ferric-salmochelin S4 to salmochelin S2 and ferric-salmochelin S2, respectively. In E. coli, uptake of ferric-salmochelin S4 was dependent on the cleavage by IroE, and independent of the FepBDGC ABC transporter in the cytoplasmic membrane. IroC, which has similarities to ABC-multidrug-resistance proteins, was necessary for the uptake of salmochelin S2 from the periplasm into the cytoplasm. IroE did not function as a classical binding protein since salmochelin S2 was taken up in the absence of a functional IroE protein. IroC mediated the uptake of iron via enterobactin in a fepB mutant. IroE was also necessary in this case for the uptake of ferric-enterobactin, which indicated that only the linear degradation products of enterobactin were taken up via IroC. PfeE, the Pseudomonas aeruginosa IroE homologue, was cloned, and its enzymic activity was shown to be very similar to that of IroE. It is suggested that homologues in other bacteria are also periplasmic IroE-type esterases of siderophores.

INTRODUCTION

In an iron-poor environment, many bacteria secrete iron-complexing agents called siderophores. The iron-loaded siderophores are utilized by high-affinity uptake systems to allow growth under iron-limiting conditions. Escherichia coli strains often produce the catecholate siderophores enterochelin (O’Brien & Gibson, 1970). The same siderophore isolated from Salmonella enterica strains has been called enterobactin (Pollack & Neilands, 1970). However, analysis of the siderophores from S. enterica and uropathogenic E. coli strains has revealed that the major siderophores in these strains are often salmochelins, and not enterobactin and its degradation products (Hantke et al., 2003; Bister et al., 2004). The key siderophore is salmochelin S4, which is a doubly C-glucosylated enterobactin derivative (Fig. 1). In addition, several hydrolysis products, linear forms of salmochelin S4, analogous to enterobactin and its degradation products, have been observed. These degradation products are best characterized by HPLC or HPLC/MS (Hantke et al., 2003; Bister et al., 2004, Valdebenito et al., 2005).

The iroBCDEN gene cluster is responsible for the glucosylation of enterobactin to salmochelin S4, and for the uptake of iron-loaded salmochelins (Hantke et al., 2003). The iroB gene has been demonstrated in vivo (Hantke et al., 2003) and the IroB protein in vitro (Fischbach et al., 2004) to be responsible for the C-glucosylation of enterobactin. The IroB homologues MceC and McmL, encoded in the microcin gene clusters for the microcins E492 (Lagos et al., 2001), M and H47 (Patzer et al., 2003), seem to have a similar C-glucosylating activity on enterobactin, as recently revealed by the elucidation of the microcin E492 structure (Thomas et al., 2004). The genes iroD and iroE are predicted to code for proteins with hydrolytic activity. IroE is a periplasmic enzyme, and IroD is a cytoplasmic protein with similarity to Fes, the enzyme that preferentially cleaves iron-free enterobactin (Brickman & McIntosh, 1992). To find out why the iro gene cluster encodes two hydrolases, iroD and iroE were cloned, and the functions of the proteins they encode were analysed.
METHODS

Strains and growth conditions. The strains used are described in Table 1. Bacteria were routinely grown in TY medium containing per litre: 8 g tryptone, 5 g yeast extract, 5 g sodium chloride. Growth response to siderophores was tested with bipyridine on nutrient broth plates (NBD) containing per litre: 8 g nutrient broth, 5 g NaCl, 15 g Difco agar, 0.2 mM bipyridine (which was added after autoclaving). Also NTAD plates were used for siderophore-dependent growth tests, especially for strains able to produce enterobactin. NTAD plates contained per litre: 2.5 g NaCl, 4 g tryptone, 15 g Difco agar, 2 g glucose, 0.15 mM bipyridine, 0.15 mM ethylenediameine-bis-(o-hydroxyphenylacetic acid). Minimal medium M63, used for the production of salmochelin and enterobactin, contained per litre: 5.3 g KH2PO4, 13.9 g K2HPO4·3H2O, 2.0 g (NH4)2SO4, 1.3 mg MgSO4·7H2O, 2.6 mg MnSO4·H2O, 0.6% glycerol; no iron was added.

E. coli H1882 was obtained from strain H1559 fepB::MudI (Amp) by selecting for growth at 42 °C, which leads to induction of the temperature-sensitive phage MudI. The development of the defective phage kills the lysogenic cells. Ampicillin-sensitive deletion mutants of E. coli that grew at this temperature were tested for colicin B and D resistance, which indicated a large deletion including the FepA receptor. Some of these mutants, including H1882, were unable to produce 2,3-dihydroxybenzoate, indicating in addition a loss of entC.

Isolation of salmochelin and enterobactin. M63 medium (50 ml) was inoculated with 0.5 ml cells, freshly grown in TY medium, and incubated for 18 h with shaking at 37 °C. After centrifugation, 2 ml FeCl3 was added to the supernatant. The precipitate formed was removed by centrifugation; the clear supernatant was loaded onto a small column containing 0.5-g DE 52 cellulose suspended in water. After washing with five column volumes of water, siderophores were eluted with 2 M ammonium chloride; the coloured fractions were combined and analysed by HPLC.

Salmochelins were prepared as described by Bister et al. (2004). Enterobactin and its degradation products were prepared according to Young & Gibson (1979), and further purified by preparative HPLC as described by Bister et al. (2004).

Construction of plasmids. Plasmid pKHI18 was digested with BlnI and Xhol, and ligated to generate plasmid pKHI20. This treatment removed a 5.3-kb fragment encoding iroN and the C-terminal end of iroE. To generate plasmid pKHI21, plasmid pKHI18 was digested with BstBI and ligated; this removed a 2.9-kb fragment encoding the N-terminal half of iroN. Plasmid pKHI23 was generated by Hpal digestion of pKHI18 and ligation of the two fragments, the first fragment encoding the vector with the promoter region and the first 75 bp of the iroB gene, and the second fragment (7.4 kb) encoding the C-terminal end of iroC (234 bp) and iroDEN.

The primers iroDNdeI (5’-GGATGCTCCATATGCTGACATCGAC-ACACATTCCG-3’) and iroXhol (5’-GGACCTCGAGTCAATCCGACAC-CCCTGTGATGTAACC-3’) were used to amplify iroD from pKHI18. The 1.2-kb PCR fragment and the vector pET19b were digested with NotI/Xhol, ligated, and introduced into E. coli DE3 by transformation. E. coli BL21(DE3) was always transformed freshly with the resulting plasmid pM2038 for isolation of the His-tagged IroD protein (NHIS-IroD).

Primers IroECttaR (5’-GCCAGGATCCCAAGCCGGGATATG-3’) and IroEhind (5’-TGCCAAAGGTGATAGGCGGAGTTAC-3’) were used to amplify the region of iroE encoding residues 33 to the C-terminus in pKHI18. The 0.9-kb fragment and the vector pMAL-p2X were digested with EcoRI/HindIII and ligated. The resulting plasmid, pKHI22, encoded the MalE–IroE fusion protein with a signal sequence that allowed export of the fusion protein into the periplasm.

The iroE mutant plasmid pSP211 iroBCDIN was obtained by religation of pKHI18 after BlnI/Sfi digestion, and treatment with the Klenow fragment to obtain blunt-ended DNA.

Primers PSMalr1 (5’-CGAAGATTCACCGCCGGGATATG-3’) and PSMalr2 (5’-ACGACGTCGTCGGGGGATTAC-3’) were used to amplify the pfe region from Pseudomonas aeruginosa. The 0.9-kb fragment and the vector pMAL-p2X were digested with EcoRI/Xhol, and ligated. To generate plasmid pM2Z43.

Plasmid pM2Z2108 was obtained by amplifying the McmK coding region from E. coli CA46 using primers MCMKsh01 (5’-GCTGTCCTCGATGATATGACATGTTGAAATGAAA-3’) and MCMKpSt1 (5’-ATGCTGTCCTGAGGATTACACACAAAGTTATT-3’), digesting the 1.2-kb fragment obtained and the vector pBAD/HisB with PstI/Xhol, and ligating them.
Isolation of fusion proteins. *E. coli* BL21(DE3)(pMZ2038) was grown in TY medium containing 50 μg ampicillin ml⁻¹ to a density of 5 x 10⁸ cells ml⁻¹. IPTG (1 mM) was then added, and the incubation was continued for 3 h. Cells were harvested, washed in 50 mM Tris/HCl pH 8.0, 2 mM EDTA, and stored as a frozen sediment at -70 °C. Cells from 50 ml cultures were suspended in 40 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9). After sonication, inclusion bodies and cellular debris were collected by centrifugation at 20 000 g for 15 min, and washed once in 20 ml binding buffer. The sediment was solubilized on ice in 5 ml binding buffer containing 6 M urea. After 1 h, the insoluble material was removed by centrifugation at 39 000 g for 20 min. A Ni-NTA column was loaded with the extract, and washed with 10 column volumes of binding buffer. After washing the column with six column volumes of binding buffer containing 20 mM imidazole, the protein was eluted with binding buffer containing 300 mM imidazole. Fractions were analysed by SDS-PAGE (see the supplementary figure S1 with the online journal). The purified NHis-IroD protein was dialysed against 50 mM Tris/HCl pH 7.5, 1 mM MgSO₄, 10 mM DTT for 18 h to renature the protein.

Plasmids pKH122 and pMZ243 were separately introduced into *E. coli* MC4100 by transformation. The transformants were grown in TY medium with 0.2% glucose and 50 μg ampicillin ml⁻¹ to a density of about 3 x 10⁸ cells ml⁻¹. IPTG (0.3 mM) was added to induce expression of the gene encoding the fusion protein. After 3 h, the cells were harvested, and the fusion protein was enriched from osmotic shock fluid, which mainly contains periplasmic proteins. The fusion protein was bound to amylose-agarose beads (New England Biolabs) and eluted with maltose as described by the manufacturer.

**Determination of IroD, IroE and PfeE activities.** Freshly dialysed NHis-IroD protein was used since the protein lost its enzymic activity within 48 h. The reaction mixture contained, if not indicated otherwise, 5 μl NHis-IroD (6-5 mg ml⁻¹ in renaturation buffer), 2 μl buffer (0.2 M sodium phosphate, pH 8.0), 8 μl salmochelin (5 mg ml⁻¹), final concentration 2 mM), 5 μl water. The mixture was incubated at room temperature. After 5, 10 and 30 min, 5 μl aliquots were withdrawn, and 55 μl stop buffer (6% acetonitrile/0.1% trifluoroacetic acid) was added. The sample was stored on ice until a 20 μl aliquot was analysed by HPLC.

The assay mixture for IroE or PfeE activity determination contained, if not indicated otherwise, 5 μl MalE–IroE (10 mg ml⁻¹), 2 μl buffer (0.2 M sodium phosphate buffer, pH 8.0), 8 μl salmochelin (5 mg ml⁻¹), 5 μl water. The assay mixture was incubated under the same conditions as above.

For *Kₘ* determinations, reaction mixtures contained salmochelin S₄ (0.125, 0.25, 0.5, 1, 2 and 4 mM), 0.8 μl buffer (200 mM phosphate buffer, pH 8.0), 2 μl MalE–IroE (10 mg ml⁻¹) or MalE–PfeE (10 mg ml⁻¹), and water up to 8 μl final volume. The mixture was incubated at room temperature for 1 minute, a sample of 5 μl was
withdrawn, and 55 μl stop buffer (6 % acetonitrile/0.1 % trifluoroacetic acid) was added. The samples were stored on ice until a 20 μl aliquot was analysed by HPLC.

HPLC analysis. Samples were analysed by HPLC (Shimadzu; LC10 pumps) on a reversed-phase column (Nucleosil C18, 5 μm, 4 × 250 mm) using a gradient increasing from 6 to 40 % acetonitrile in water (both with 0.1 % trifluoroacetic acid) with detection at 220 nm. The substrates were purified by preparative HPLC (Shimadzu; LC8 pumps) on a reversed-phase Nucleosil 100 C18 column (20 × 250 mm, 7 μm; Grom) using a gradient of 6 to 40 % acetonitrile in water, both containing 0.1 % trifluoroacetic acid, over 20 min (flow rate 5 ml min⁻¹, detection at 220 nm).

RESULTS

Enzymic activity of IroD

The iroD gene ligated into the vector pET19b allowed production of the NHis-IroD fusion protein as inclusion bodies. The esterase activity of the purified and renatured NHis-IroD was tested with various salmochelins as substrate. Salmochelin S4 was degraded completely within 5 min to form mostly the linear trimer salmochelin S2 and dimer salmochelin S1, which elute in close proximity in the HPLC analysis. Small amounts of the monomer salmochelin SX were also formed (Fig. 2). After 30 min incubation, higher amounts of salmochelin SX and 2,3-dihydroxybenzoylserine (DHBS) were formed, and the salmochelin S1/S2 double peak decreased in size. Similarly, when salmochelin S2 was used as substrate, salmochelin S1, salmochelin SX and DHBS were obtained as products (see the supplementary figure Fig. S2 with the online journal).

The K_m of NHis-IroD with deferri-salmochelin S4 was determined to be approximately 1.24 ± 0.4 mM.

Ga(NO_3)_3 was added to iron-free salmochelin S4 in equivalent amounts to allow formation of the Ga^{3+}-salmochelin S4 complex. Ga^{3+} was used because it is not redox active, and its dimensions are similar to those of Fe^{3+}. Degradation of Ga^{3+}-salmochelin S4, iron-free salmochelin S4 and Fe^{3+}-salmochelin S4 were compared. The iron-free form was hydrolysed within 5 min (Fig. 2), whereas Ga^{3+}-salmochelin S4 and Fe^{3+}-salmochelin were stable (see the supplementary figure Fig. S3 with the online journal). However, in the presence of the Fe^{2+}-complexing agent ferrozine, Fe^{3+}-salmochelin S4 was slowly cleaved by NHis-IroD (see the supplementary figure Fig. S4 with the online journal). The presence of DTT possibly allowed the reduction of the iron.

From the culture supernatants of salmochelin S4-producing strains, a compound, called S0, was isolated. S0 did not stimulate iron-dependent growth. However, biologically active salmochelin S4 was obtained from HPLC-pure salmochelin S0 by concentrating the HPLC fraction by evaporation. S0 was not cleaved by IroD (Supplementary Fig. S3), which indicated that salmochelin S0 has a structure that does not allow cleavage.

Enterobactin was also recognized by NHis-IroD and cleaved to linear oligomers of DHBS (see the supplementary figure Fig. S5 with the online journal). Fe-enterobactin was not cleaved by NHis-IroD (Supplementary Fig. S3).

IroD complements a fes mutant

Since IroD was able to cleave enterobactin, the iroD gene was tested for the ability to complement a fes mutant. E. coli AN273 fes was transformed with the plasmid pMZ2038 iroD. Colonies of E. coli AN273 fes were pink on TY plates, characteristic of enterobactin production, whereas colonies of E. coli AN273 fes(pMZ2038 iroD) were colourless, as typical of E. coli on these plates. On iron-limiting NTADE

![Fig. 2. Separation of the IroD reaction products by HPLC. The substrate salmochelin S4 (2 mM) was degraded within 5 min to salmochelin S2 and/or salmochelin S1, which elute in close proximity in HPLC. After 30 min, most of the salmochelin S2 was degraded to the monomers salmochelin SX (pacifarinic acid) and DHBS.](image-url)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enterobactin</th>
<th>Salmochelin S4</th>
<th>(DHBS)_3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN273 fes</td>
<td>15</td>
<td>+/-</td>
<td>12</td>
</tr>
<tr>
<td>(pMZ2038 iroD)</td>
<td>+/-</td>
<td>+/-</td>
<td>8</td>
</tr>
<tr>
<td>AN273 fes</td>
<td>+/-</td>
<td>+/-</td>
<td></td>
</tr>
</tbody>
</table>
Salmochelins and enterobactin produced by various *E. coli* K-12 strains

The strains were grown overnight in M63 glycerol medium with the appropriate supplements. The siderophores produced were concentrated on DEAE-cellulose, eluted, and analysed by HPLC. The peak areas were summed up, and the percentage of the compounds is given.

### Table 3. Salmochelins and enterobactin produced by various *E. coli* K-12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage catecholate siderophore</th>
<th>Enteroabctin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SX S1/2 S5 S0 S4 (DHBS) (DHBS)₂ (DHBS)₃</td>
<td></td>
</tr>
<tr>
<td>MG1655 (pKH18  iroBCDEN)</td>
<td>62 7 8 3 4 0</td>
<td>16</td>
</tr>
<tr>
<td>MG1655 (psP211  iroBCDEN)</td>
<td>– 36 – 3 28 0 3 0</td>
<td>30</td>
</tr>
<tr>
<td>AN273  fes</td>
<td>0 0 0 0 0 4</td>
<td>87</td>
</tr>
<tr>
<td>AN273  fes (pMZ2038  iroD)</td>
<td>0 0 0 0 0 0</td>
<td>18</td>
</tr>
<tr>
<td>AN311  fepB</td>
<td>0 0 0 0 0 0</td>
<td>52</td>
</tr>
<tr>
<td>AN311  fepB (pKH118  iroBCDEN)</td>
<td>17 38* 27 0 8 10 0 3</td>
<td></td>
</tr>
<tr>
<td>AN311  fepB (pKHI20  iroBCD)</td>
<td>6 34 35† 35† 19 3 0 0</td>
<td>3</td>
</tr>
<tr>
<td>AN311  fepB (pKHI21  iroBCDE)</td>
<td>5 59 12 0 8 16 0 0</td>
<td></td>
</tr>
</tbody>
</table>

*Compounds shown to be mainly S1.
†Salmochelins S0 and S5 were not separated; their sum is given.

plates, enterobactin and (DHBS)₃ had a strong growth-stimulating effect on *E. coli* AN273 fes (pMZ2038 iroD), whereas the fes mutant grew only weakly (Table 2). Salmochelin S4 caused only a very faint growth response (*E. coli* AN273 fes). The major peak was observed by mass spectrometry and was confirmed to be salmochlorin S2 (data not shown). S3 had been shown to be an oxidation product without siderophore activity (Hantke *et al.*, 2003). No further degradation of salmochlorin S2 by IroE was observed. In addition, IroE cleaved Fe⁴⁺-salmochlorin S4 and the iron-free form of salmochlorin S4 with nearly the same efficiency (Fig. 3). The *Kₘ* of MalE–IroE with deferri-salmochlorin S4 as a substrate was approximately 2·6 ± 0·5 mM.

Enterobactin was cleaved by MalE–IroE mainly into the linear trimer DHBS (Fig. 4). The Ga³⁺ complexes of salmochlorin S4 and enterobactin were not cleaved (Supplementary Fig. S3).

### PfeE of *P. aeruginosa* is an IroE homologue

Although *P. aeruginosa* does not produce enterobactin, the bacterium has an inducible transport system for this siderophore. The outer-membrane enterobactin receptor PfeA is regulated by the two-component system regulators PfeR and PfeS (Dean *& Poole*, 1993). Downstream of *pfeA*, an ORF (PA2689) with similarity to IroE (34% identity) was found and named PfeE. This gene was cloned, and the encoded protein was isolated as described for *iroE* (Supplementary Fig. S1). MalE–PfeE had an activity spectrum similar to that of IroE (Fig. 4, Supplementary Figs S6 and S7 with the online journal). MalE–PfeE degraded salmochlorin S4 to the linear trimer S2, but dimers and monomers were not observed (Supplementary Fig. S6). Enterobactin degradation products appeared in the position of the DHBS trimer, while in addition, low amounts of dimer were observed after 30 min (Fig. 4). The

### Enzymic activity of IroE

The *iroE* gene is predicted to encode a periplasmic enzyme. Therefore, IroE was fused with the periplasmic maltose-binding protein MalE. The resulting construct, pKH122, encoded a MalE–IroE fusion protein that was enriched from the osmotic shock fluid by binding to an amylose-agarose column. Part of the fusion protein was degraded, as shown by the elution of two proteins from the column by maltose. One protein had the expected molecular mass of 74 kDa; the double band apparently represented MalE fragments of approximately 48 kDa (Supplementary Fig. S1).
**Fig. 3.** Cleavage of salmochelin S₄ (4 mM) and Fe-salmochelin S₄ (2 mM) by MalE–IroE. HPLC chromatograms after 0, 5 and 30 min incubation are shown.

**Fig. 4.** Cleavage of enterobactin (6 mM) by MalE–IroE (a) and enterobactin (3 mM) by MalE–PfeE(b). The slope of the acetonitrile gradient (6 to 40 %) was different, 20 min in (a) and 30 min in (b), which explains the different retention times.
Ga\(^{3+}\)-salmochelin S4 complex was not degraded (data not shown), whereas the cyclic iron-containing complex of salmochelin S4 was linearized (Supplementary Fig. S7). *P. aeruginosa* K372, which is unable to produce the cognate siderophores pyochelin and poyoverdin (Heinrichs & Poole, 1993), grew with enterobactin or salmochelin on iron-limiting NTADE plates, which indicated that enterobactin and salmochelin are used as an iron supply.

**Function of IroE and IroD in iron-salmochelin uptake**

*S. enterica* serotype Typhimurium ATCC 14028 (Ba¨ umler et al., 1998) produces mainly the degradation products of salmochelin S4, namely salmochelins S2, S1, SX (pacifarinic acid) and DHBS. The mutant *S. enterica* H5547 iroD::kan produces mainly salmochelin S4 and DHBS (Hantke et al., 2003). This is in accordance with the observation that IroD degraded salmochelin S4 in vitro to the linear trimers, dimers and monomers. However, considering the operon organization and the phenotype of *S. enterica* H5547 iroD::kan, it is highly probable that this mutation has a polar effect on iroE; this could lead to salmochelin S4 not being cleaved in the periplasm, which possibly is a prerequisite for uptake. To test these possibilities, various mutants derived from plasmid pKHI18 iroBCDEN were constructed.

In the mutant plasmid pSP211 iroBCDEN, approximately 700 bp were removed from the iroE gene. *E. coli* K-12 MG1655 transformed with pSP211 iroBCDEN grew slightly slower in iron-poor M63 minimal medium (growth yield after 24 h 0.88 mg cell dry wt ml\(^{-1}\)) than *E. coli* MG1655 transformed with pKHI18 iroBCDEN (growth yield after 24 h 1.13 mg cell dry wt ml\(^{-1}\)). This might indicate that the uptake of Fe\(^{3+}\)-salmochelin S4 via IroN without degradation to Fe\(^{2+}\)-salmochelin S2 is not very efficient. Furthermore, *E. coli* MG1655(pSP211 iroBCDEN) produced more salmochelins S0 and S4 than *E. coli* MG1655(pKHI18 iroBCDEN), which produced mainly salmochelins S1 and S2 (Table 3).

The periplasmic enterobactin-binding protein FepB was tested to see whether it was necessary for salmochelin S4 uptake. *E. coli* AN311 fepB was transformed with the plasmids pKHI18 iroBCDEN and pSP211 iroBCDN. In minimal medium M63, strain AN311 produces mainly enterobactin and some trimeric 2,3-DHBS, which might result from enterobactin hydrolysis in the medium (Table 3). *E. coli* AN311 fepB(pKHI18 iroBCDEN) produced mainly the linear salmochelin S1, and the monomers salmochelin SX and DHBS, which indicated that salmochelin S4 uptake was independent of FepB. In addition, salmochelin S5, a dimer of salmochelin SX and DHBS, was found (Fig. 1), which had not been observed before in wild-type strains. Since the salmochelins were degraded to salmochelin SX and DHBS, they must have been taken up into the cytoplasm, where IroD is localized, since IroE in the periplasm degrades salmochelin only to the linear trimers salmochelins. Very similar results were obtained with fepC and fepD mutants (data not shown); therefore, uptake through the cytoplasmic membrane might be accomplished by IroC. In contrast, *E. coli* AN311 fepB(pKHI20 iroBCD) produced more undegraded salmochelins S4 and S0 than AN311 fepB(pKHI18 iroBCDEN) (Table 3), which indicated that cleavage of salmochelin S4 to salmochelin S2 is necessary for efficient uptake.

**Function of IroC in iron-salmochelin uptake**

IroC had been assumed to be an exporter for salmochelin S4 because of its similarities to eukaryotic multidrug resistance (MDR) proteins (Hantke et al., 2003). The results presented above indicated that IroC might be responsible for the uptake of iron-salmochelin. A test with *E. coli* AN311 fepB(pKHI18 iroBCDEN) showed that salmochelin S4 as well as salmochelin S2 stimulated growth on iron-limiting medium (Table 4). Since bacterial binding-protein-dependent ABC transporters are not functional without their cognate binding protein, it was presumed that the Fep-ABC-transporter would be inactive without FepB. Therefore, IroC was assumed to be the transporter of the salmochelins. Application of enterobactin to filter paper discs on agar seeded with *E. coli* H1882(pKHI18 iroBCDEN) produced a weak growth zone, which indicated that IroC allows not only the uptake of Fe\(^{3+}\)-salmochelin S2, but also the uptake of Fe\(^{3+}\)-(DHBS)\(_3\), the linear form of Fe\(^{3+}\)-enterobactin (Table 5). Strain *E. coli* H1882(pSP211 iroBCDEN) was not able to grow with enterobactin, possibly

### Table 4. Effect of catecholate siderophores on growth of *E. coli* AN311 on iron-deficient NTADE plates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Salmochelin S4</th>
<th>Salmochelin S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN311 fepB(pKHI18 iroBCDEN)</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>AN311 fepB(pSP211 iroBCDEN)</td>
<td>8 (weak growth)</td>
<td>19</td>
</tr>
<tr>
<td>AN311 fepB(pKHI23 iroDEN)</td>
<td>7 (weak growth)</td>
<td>16 (weak growth)</td>
</tr>
<tr>
<td>AN311 fepB</td>
<td>7 (weak growth)</td>
<td>16 (weak growth)</td>
</tr>
</tbody>
</table>
Table 5. Effect of catecholate siderophores on the growth of E. coli H1882, which is unable to produce enterobactin, on iron-deficient NTDAE plates

Filter paper discs with 10 μg enterobactin, salmochelin S4, linear enterobactin (DHBS)₃, or salmochelin S2 were applied to NTDAE plates seeded with the appropriate strain. The diameter of the growth zone is given in mm. Cell density in the growth zone: +++++ dense growth, +++ good growth, + faint growth, (+) poor growth, – no growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enterobactin</th>
<th>(DHBS)₃</th>
<th>Salmochelin S4</th>
<th>Salmochelin S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1882 Δ(fep ent)(pKH118 iroBCDEN)</td>
<td>16+</td>
<td>18+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H1882 Δ(fep ent)(pSP211 iroBCDN)</td>
<td>8+</td>
<td>25(+)</td>
<td>–</td>
<td>–</td>
</tr>
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<td>H1882 Δ(fep ent)</td>
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<td>H5687 ΔentC</td>
<td>12+++</td>
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because without IroE there is no degraded Fe³⁺-enterobactin in the periplasm.

To determine whether IroE functions as a binding protein for salmochelin uptake, growth tests were performed with AN311 fepB(pSP211 iroBCDN) (Table 4). The strain lacking iroE grew with salmochelin S2, which indicated that the major function of IroE is the linearization of salmochelin S4 to salmochelin S2. IroE does not seem to be a transport-relevant binding protein.

For reasons that are not understood, strains unable to produce enterobactin (e.g. those with mutations in entC, araB, araA) transformed with iro genes grew only poorly with various salarmehines. In addition, the strong growth response of E. coli H5687 ΔentC (Table 5) showed that the Fep uptake system is more efficient than the Iro proteins in the uptake of enterobactin.

**DISCUSSION**

To understand why two hydrolases – IroD and IroE – are present in the salmonchelin uptake system, both genes encoding them were cloned, and the proteins were studied in vitro. The IroD protein is related to the Fes protein (27% identity), which is known to cleave enterobactin to the monomer DHBS (Fig. 1) (Bryce & Brot, 1972; Brickman & McIntosh, 1992). IroD similarly degraded salmochelin S4 to the linear trimer salmochelin S2, the dimer salmochelin S1, and the monomers DHBS and salmochelin SX. Efficient cleavage of Fe³⁺-enterobactin by Fes is only observed in the presence of a reductant and an Fe²⁺ chelator; without addition of an Fe²⁺ chelator, hydrolysis of Fe³⁺-enterobactin is reduced to 20% (Brickman & McIntosh, 1992). Ferric-salmochelin S4 was cleaved slowly by IroD only in the presence of ferrozine, a Fe²⁺-complexing agent (the reductant DTT was always present in the buffer).

A major function of IroD might be to bind salmochelin in a conformation that allows reduction and removal of Fe²⁺. The second step might be cleavage, which may help to lower the affinity of the substrate deferri-salmochelin to IroD, thereby allowing the release of the degraded substrate. The mechanism of action of IroD might be very similar to that of Fes. Interestingly, IroD was also able to cleave enterobactin in vitro; this observation prompted us to test the complementation of an E. coli fes mutant with a cloned iroD gene. Complementation was observed, as predicted from the in vitro activity of IroD.

A third member of the Fes protein family, McmK, encoded in microcin biosynthesis operons (Patzer et al., 2003), is also able to complement Fes activity. Microcin E492 is modified by a linear enterobactin. One glucose residue bridges the C-terminal serine of the microcin peptide with the trimer of DHBS (Thomas et al., 2004). In the maturation process of microcins E492, M and H47, an IroB-like protein (MceC or McmK) might C-glycosylate enterobactin with a glucosyl residue, which is then linked to the C-terminal serine residue of the microcin peptide (Thomas et al., 2004). The Fes-like esterases MceD or McmK might cleave the cyclic triester of enterobactin, when bound via one glucose residue to the respective microcin peptide part. Cleavage of the cyclic enterobactin might be necessary for the export of the microcins via the TolC-coupled ABC exporter (MchEF or MceGH).

The IroE protein, isolated as a MalE–IroE fusion protein from the periplasm, had a different esterase specificity. It only cleaved the cyclic seryl-triester, and did not cleave the linear forms of salmochelin. Although it also cleaved the Fe³⁺-chelates, it did not cleave the Ga³⁺-salmochelin S4, which indicated that not only the ester ring is recognized.

A BLAST search revealed several IroE-related proteins in other bacteria. A dendrogram of some selected proteins is shown in Fig. 5. The PfeE protein, with 37% identity to IroE, is encoded downstream of the enterobactin reporter gene cluster pferSRA in P. aeruginosa and has functions similar to those of IroE. The cloned protein cleaved salmochelin S4, enterobactin and the iron-containing salmochelin S4. Similarly, a periplasmic protein with 32% identity to IroE is predicted downstream of bfeA, the enterobactin receptor of Bordetella parapertussis. Even in the Gram-positive bacterium Bacillus subtilis, two homologues are found. ybbA (25% identity) is downstream of feuABC, which encodes an iron-Fur-regulated ABC transporter with unknown substrate specificity, and yuil.
(28% identity) is downstream of the entA (2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase) homologue. It is tempting to speculate that all these proteins hydrolyse cyclic, ester-linked catecholate siderophores, such as salmochelin, enterobactin and corynebactin (the cognate siderophore of \textit{B. subtilis} [Budzikiewicz et al., 1997; May et al., 2001]).

It was surprising that \textit{E. coli} AN311 \textit{fepB} (pKH18 \textit{iroBCDEN}) produced mainly the dimeric and monomeric linear forms of salmochelin \([S1, S5, SX, (DHBS)2 and DHBS]\) since it was thought that IroC, as a relative of eukaryotic MDR proteins, would function in the export of salmochelin. Degradation of enterobactin and salmochelin to dimeric and monomeric forms indicated that IroD and/or Fes degraded these siderophores and that this could only take place after uptake into the cytoplasm. This indicated that salmochelin S2 and enterobactin were taken up by IroC, and degraded in the cytoplasm. Salmochelin stimulated growth of AN311 \textit{fepB} (pKH18 \textit{iroBCDEN}) (Table 4) and enterobactin stimulated growth of H1882 \textit{Δ(fep–ent)} (pKH18 \textit{iroBCDEN}) (Table 5), which confirmed the assumption that IroC complemented the defect Fep-ABC-transporter. This conclusion raised the question whether IroE, in addition to being an esterase, is also a periplasmic binding protein that interacts with the ATP-dependent IroC, as is known for bacterial ABC transporters. Growth of an \textit{iroE} mutant was stimulated by the linear salmochelin S2, which indicated that IroC without IroE was able to transport the linear forms of salmochelin. IroE is not a binding protein since in most bacterial ABC transporters, uptake is only possible in the presence of the binding protein. In addition, IroE has no sequence similarities to known binding proteins. The inability of the \textit{iroE} mutant to use cyclic salmochelin S4 indicated that IroC can only transport linear forms of the catecholate siderophores, and explains the necessity for IroE. The possibility that IroE functions as a binding protein for linear catechol-siderophores that is substituted in the \textit{iroE} mutant by another periplasmic protein cannot be excluded, but seems unlikely.

However, during these studies, we observed that strains with the complete set of \textit{iro} genes, which were unable to produce enterobactin owing to mutations in \textit{aroA}, \textit{aroB} or \textit{entC}, showed no growth response or only a faint response with salmochelin S4. This might indicate that in addition to iron and Fur (Bäumler et al., 1996), there is another, unknown, level of regulation.

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\textbf{Fig. 5.} Dendrogram of some selected proteins that have been shown to have, or which may have, a function in siderophore ester cleavage. McmK is encoded by the \textit{mcmK} gene of the \textit{E. coli} microcin H47 and M gene cluster (Patzer et al., 2003); MceD is encoded by the corresponding \textit{mceD} gene in the \textit{Klebsiella pneumoniae} microcin E492 gene cluster (Lagos et al., 2001).

\textbf{Fig. 6.} (a) Tentative model for the uptake of salmochelin S4 by \textit{E. coli}. For details and an explanation, refer to the text. Ent, enterobactin; DHBS, 2,3-dihydroxybenzoylserine; Glc, glucose. For the structures of the salmochelins S4, S2, S1 and SX, see Fig. 1. (b) The \textit{iro} gene cluster found in \textit{E. coli} and \textit{S. enterica} encodes IroB, a C-glucosyltransferase; IroC, an ABC transporter; IroD and IroE, two esterases; and IroN, the outer membrane receptor for salmochelin.
Based on the observations discussed above, we propose the following model of salmochelin-dependent iron uptake in *E. coli* (Fig. 6). Salmochelin S4 is synthesized from enterobactin by IroB, and excreted by an unknown mechanism into the medium, where itcomplexes Fe$^{3+}$. The complex is taken up preferentially by IroN in a TonB-dependent manner (Bister et al., 2004; Hantke et al., 2003). In the periplasm, Fe$^{3+}$-salmochelin S4 is cleaved by IroE to form the linear trimer Fe$^{3+}$-salmochelin S2, which is taken up via IroC. In the cell, Fe$^{3+}$-salmochelin S2 is bound by IroD, the iron is reduced and removed by an unknown mechanism, and salmochelin S2 is cleaved to the dimer salmochelin S1, the monomer salmochelin SX (pacifaric acid) and DHBS. The degradation products either are secreted by an unknown mechanism out of the cell and might act as low-affinity siderophores for further iron uptake, or are degraded by the periplasmic copper oxidase CueO (Grass et al., 2004), since salmochelin SX is often observed in the culture supernatant in amounts lower than expected.

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