

Salmonella typhimurium IroN and FepA Proteins Mediate Uptake of Enterobactin but Differ in Their Specificity for Other Siderophores

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***Salmonella typhimurium* possesses two outer membrane receptor proteins, IroN and FepA, which have been implicated in the uptake of enterobactin. To determine whether both receptors have identical substrate specificities, *fepA* and *iroN* mutants and a double mutant were characterized. While both receptors transported enterobactin, the uptake of corynebactin and myxochelin C was selectively mediated by IroN and FepA, respectively.**

Iron is essential for the multiplication of enterobacteria, since it is a component of enzymes (e.g., ribonucleotide reductase) which are required for the biosynthesis of macromolecules (e.g., DNA) and energy-generating electron transport processes. In order to obtain iron from insoluble Fe(III) complexes present under aerobic growth conditions, enterobacteria release low-molecular-weight compounds, designated siderophores, which bind this metal ion with high affinity (4). The Fe(III)-siderophore complexes are then internalized by iron-regulated outer membrane receptor proteins which display substrate specificity. The primary siderophore produced by enterobacteria is enterobactin, a cyclic trimer of *N*-(2,3-dihydroxybenzoyl)-L-serine (DBS). In *Escherichia coli*, transport of enterobactin across the outer membrane is mediated by the FepA outer membrane receptor protein (13).

An orthologue of *fepA* is present in *Salmonella enterica*, but the phenotype of a strain carrying a mutation in this gene has not been described to date (7, 19, 21). Recently, *iroN*, a gene absent from the *E. coli* K-12 genome, has been shown to encode an outer membrane siderophore receptor of *S. enterica* serotype Typhimurium (*S. typhimurium*) (3). When introduced into an *E. coli fepA aroB* mutant, the cloned *iroN* gene mediates the utilization of enterobactin as the sole source of iron (20a). However, an *S. typhimurium iroN aroA* mutant is not deficient in enterobactin uptake, presumably because it is still able to utilize this siderophore via FepA. These data suggest that *S. typhimurium* possesses two enterobactin receptor proteins, IroN and FepA.

The ability to produce siderophores is one of the main strategies by which microbes acquire Fe(III) in the environment. In addition to receptors for the utilization of enterobactin, *S. typhimurium* possesses several outer membrane receptors, including FhuA, FhuE, and FoxA, which are involved in the

utilization of siderophores that are not produced by this organism (12, 21). Thus, a second strategy apparently used by *S. typhimurium* to acquire Fe(III) is to steal siderophores produced by other microbes. This siderophore piracy (6) may explain why *S. typhimurium* possesses two receptors for the utilization of enterobactin. The presence of these two outer membrane enterobactin receptors may be advantageous under conditions when the available siderophores can be utilized only through either FepA or IroN. An important assumption in this line of reasoning is that although FepA and IroN both serve as enterobactin receptors, each receptor may in addition mediate the uptake of substrates that are not recognized and transported by any other siderophore receptor in *S. typhimurium*. To test this hypothesis, we constructed *S. typhimurium* strains lacking FepA or IroN or both receptors. Analysis of the siderophore profile utilized by these mutants enabled us to determine that the enterobactin receptors IroN and FepA differ in their substrate specificities.

Isolation of an *S. typhimurium fepA* mutant. The *fepA* gene is located at 14 min on the *E. coli* genetic map in a DNA region containing enterobactin biosynthesis (*ent*) genes (11, 13–15). According to a recent analysis of the 5' end of the *fepA* gene and the adjacent DNA region, this genetic organization is conserved in *S. typhimurium* with regard to gene order and map location (21). Since it is transcribed as a monocistronic messenger, mutational inactivation of *fepA* has no effect on the biosynthesis of enterobactin in *E. coli*. Based on these data, we reasoned that a selectable marker in *fepA* could be used to move enterobactin biosynthesis genes by cotransduction (16). Our strategy to identify a *fepA* mutant was therefore to characterize transposon insertions, which are cotransduced with enterobactin biosynthesis genes. A phage lysate of a random bank of *S. typhimurium* Tn10dTc mutants was transduced into an *S. typhimurium* mutant (TA2700) which is defective for enterobactin production (17). Transductions were performed by using the high-frequency generalized transducing phage mutant P22HT 105/1 int 201. Transductants were selected on chrome azurol S (CAS) agar plates, on which colonies producing enterobactin can be readily detected by their ability to form a halo (20). A total of 130 colonies producing a halo on CAS

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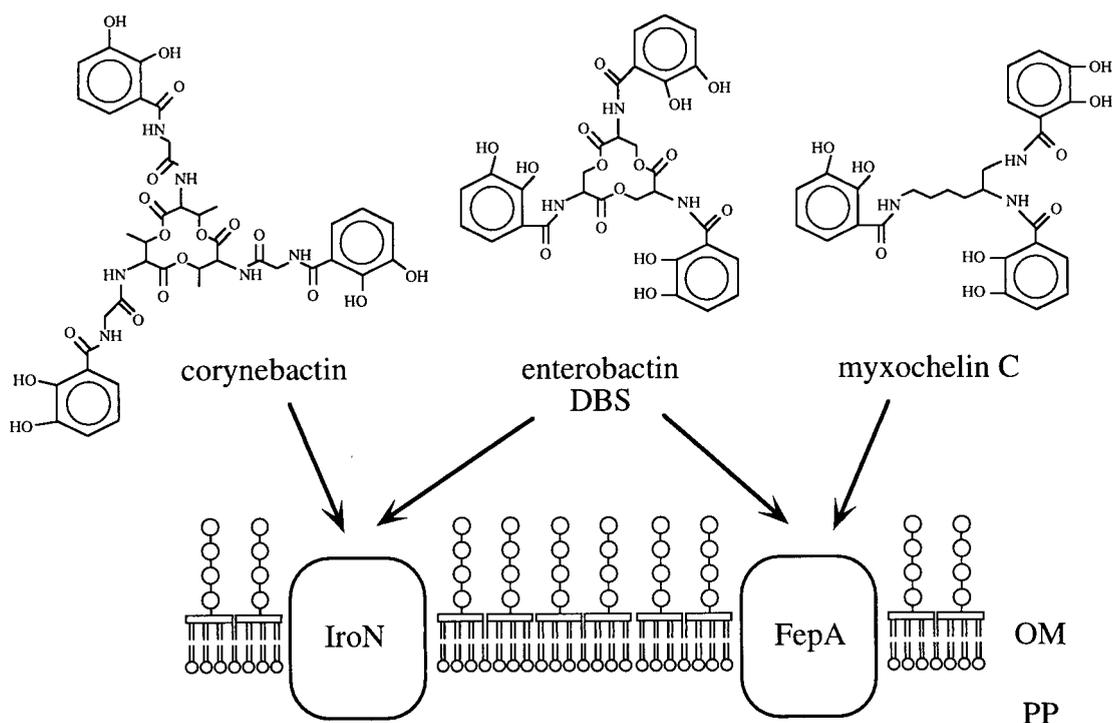


FIG. 1. Substrate specificity of the *S. typhimurium* IronN and FepA outer membrane (OM) receptor proteins. PP, periplasmic space. The structures of enterobactin, corynebactin, and myxochelin C are shown above.

agar plates were isolated, and enterobactin production was confirmed in a cross-feeding test with the *S. typhimurium* *ent* mutant (TA2700) (18). Outer membranes were isolated from these mutants as described previously by Hantke (8), and proteins were separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein bands were visualized by Coomassie blue staining. One mutant, WR1316, lacked an 83-kDa outer membrane protein, the size reported for FepA (data not shown). The Tn10dTc insertion in WR1315 was mapped to the enterobactin biosynthesis region by cotransduction with *entB::MudJ* (cotransduction frequency, 2%) and *ent::MudJ* from strain AR8439 (cotransduction frequency, 5%) (21). To confirm transposon insertion in *fepA*, a Southern blot of *Eco*RI-restricted genomic DNA of strains TA2700 and WR1315 was probed with the labeled (nonradioactive) labeling kit from NEN) insert of pFT17, a plasmid carrying the 5' ends of the *S. typhimurium* *fepA* gene (21). The probe hybridized with restriction fragments of 10 and 3 kb in TA2700 and WR1315, respectively. Thus, inactivation of *fepA* in strain WR1315 was suggested by Southern hybridization and the lack of an 83-kDa outer membrane protein.

Utilization of siderophores by FepA and IronN. In order to study siderophore utilization of the *fepA* mutant, the Tn10dTc insertion in WR1315 was transduced into TA2700 (*ent*), and a transductant (WR1316) which was tetracycline resistant and unable to produce enterobactin was selected on CAS agar plates. The ability of strain WR1316 to utilize enterobactin and structurally related siderophores was tested. For this purpose, enterobactin was prepared and purified from *E. coli* AN311 according to a protocol reported previously (22). Myxochelin derivatives were synthesized and characterized by W. Trovitzsch-Kienast and H. D. Ambrosi, Technische Fachhochschule Berlin, Berlin, Germany (1, 10). The siderophore corynebactin was isolated from *Corynebacterium glutamicum* (5) and

kindly provided by H. Budzikiewicz, Institute of Organic Chemistry, University of Cologne, Cologne, Germany. Utilization of siderophores was detected by an agar diffusion assay (18). To create iron-limiting growth conditions, 0.15 mM 2,2'-dipyridyl was added to Vogel Bonner medium (VBD) (18). The strain to be tested was poured into 3 ml of VBD top agar (VBD with 2% agar)-Noble agar onto a VBD agar plate. Filter paper disks impregnated with 5 μ l of a 1-mg/ml solution of the respective siderophores were laid onto the top agar and, after incubation overnight at 37°C, growth stimulation around the filter disk was recorded. Comparison of the profiles of the siderophores utilized by WR1316 (*ent fepA*) and its parent, TA2700 (*ent*), revealed that myxochelin C utilization was abolished in the *fepA* mutant (Table 1). To quantify growth stimulation by siderophores, strains TA2700 (*ent*) and WR1316 (*ent fepA*) were cultured in VBD broth, and bacterial growth was recorded by measuring the absorbance at 620 nm. Myx-

TABLE 1. Siderophore utilization of *S. typhimurium* mutants

Siderophore	Growth zone (mm) produced by indicated strain (genotype)				
	TA2700 (<i>ent</i>)	WR1316 (<i>ent fepA</i>)	WR1316 pITS449 (<i>ent</i> <i>fepA fepA</i> ⁺ on plasmid)	WR1223 (<i>ent iron</i>)	WR1332 (<i>ent fepA</i> <i>iron</i>)
Enterobactin	25	25	25	25	0
DBS	30	30	30	30	0
Myxochelin A	40	40	40	40	24
Myxochelin C	35	0	25	35	0
Corynebactin	35	35	ND ^a	0	0

^a ND, not determined.

ochelin C stimulated the growth of strain TA2700, but not that of strain WR1316, in VBD broth.

We next tested whether the defect in myxochelin C utilization in WR1316 could be restored by introducing the cloned *fepA* gene from *E. coli*. A plasmid (pITS449) (2) carrying the *E. coli fepA* gene was kindly provided by Phil E. Klebba, University of Oklahoma. Introduction of plasmid pITS449 (*E. coli fepA*) restored the ability of strain WR1316 to utilize myxochelin C as an iron source (Table 1). These data showed that FepA can mediate the uptake of myxochelin C in *S. typhimurium*. Furthermore, the inability of WR1316 (*ent fepA*) to utilize myxochelin C suggested that FepA is the only outer membrane receptor involved in the transport of this substrate in *S. typhimurium*.

Unlike an *E. coli fepA* mutant, the *S. typhimurium fepA* mutant (WR1316) was able to utilize enterobactin, presumably because of the presence of a second enterobactin receptor (Table 1). To test the hypothesis that *S. typhimurium* possesses two outer membrane enterobactin receptors, strain TA2700 (*ent*) was used to generate isogenic *ent iroN* (WR1223) and *ent iroN fepA* (WR1332) mutants. A *iroN* allele which carries an insertion of suicide vector pGP704 (9) was moved by P22 transduction from *S. typhimurium* AJB64 (3). The parent (TA2700, *ent*), the *ent fepA* mutant (WR1316), and the *ent iroN* mutant (WR1223) were able to utilize enterobactin. However, utilization of enterobactin and DBS were abolished in the *ent iroN fepA* mutant (WR1332), suggesting that both IroN and FepA can mediate uptake of these siderophores in *S. typhimurium* (Table 1).

In contrast to the *ent* mutant (TA2700) and the *ent fepA* mutant (WR1316), strains WR1223 (*ent iroN*) and WR1332 (*ent iroN fepA*) were unable to utilize corynebactin, suggesting that this siderophore is transported via IroN in *S. typhimurium* (Table 1). To quantify growth stimulation by siderophores, bacterial strains were cultured in VBD broth supplemented with corynebactin, myxochelin C, or enterobactin. As a control, VBD broth was supplemented with myxochelin B, a siderophore, which is not transported by FepA or IroN. VBD broth supplemented with corynebactin did not support the growth of strain WR1223 (*ent iroN*) or WR1332 (*ent iroN fepA*). Furthermore, strain WR1332 was unable to grow in VBD broth supplemented with myxochelin C. While strain TA2700 and its derivatives (WR1332 and WR1223) were unable to grow in VBD broth lacking siderophore supplements, all strains were able to grow in VBD broth supplemented with myxochelin B. Enterobactin promoted the growth of strains TA2700 (*ent*), WR1223 (*ent iroN*), and WR1316 (*ent fepA*) but not that of strain WR1332 (*ent iroN fepA*). While cultures of strains TA2700 (*ent*) and WR1223 (*ent iroN*) reached the same density (optical density at 620 nm [OD₆₂₀] = 0.5), the growth of strain WR1316 (*ent fepA*) was reduced (OD₆₂₀ = 0.4), suggesting that enterobactin is utilized more effectively by FepA than by IroN.

Our data show that *S. typhimurium* possesses two outer membrane enterobactin receptor proteins, IroN and FepA. These receptor proteins differ in their specificities for other substrates, such as myxochelin C and corynebactin (Fig. 1). Corynebactin is excreted by *Corynebacterium glutamicum*, an organism found in soil, an environment frequently encountered during the fecal-oral transmission of *S. typhimurium*. Growth in this environment may require the ability to utilize a wide spectrum of siderophores produced by the bacteria in the soil. It could therefore be speculated that IroN and FepA facilitate growth in soil, since the presence of these receptor proteins may increase the capability of *S. typhimurium* to obtain iron by siderophore piracy.

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