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Iron Transport Systems of *Serratia marcescens*

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***Serratia marcescens* W225 expresses an unconventional iron(III) transport system. Uptake of Fe^{3+} occurs in the absence of an iron(III)-solubilizing siderophore, of an outer membrane receptor protein, and of the TonB and ExbBD proteins involved in outer membrane transport. The three SfuABC proteins found to catalyze iron(III) transport exhibit the typical features of periplasmic binding-protein-dependent systems for transport across the cytoplasmic membrane. In support of these conclusions, the periplasmic SfuA protein bound iron chloride and iron citrate but not ferrichrome, as shown by protection experiments against degradation by added V8 protease. The cloned *sfuABC* genes conferred upon an *Escherichia coli aroB* mutant unable to synthesize its own enterochelin siderophore the ability to grow under iron-limiting conditions (in the presence of 0.2 mM 2.2′-dipyridyl). Under extreme iron deficiency (0.4 mM 2.2′-dipyridyl), however, the entry rate of iron across the outer membrane was no longer sufficient for growth. Citrate had to be added in order for iron(III) to be translocated as an iron citrate complex in a FecA- and TonB-dependent manner through the outer membrane and via SfuABC across the cytoplasmic membrane. FecA- and TonB-dependent iron transport across the outer membrane could be clearly correlated with a very low concentration of iron in the medium. Expression of the *sfuABC* genes in *E. coli* was controlled by the Fur iron repressor gene. *S. marcescens* W225 was able to synthesize enterochelin and take up iron(III) enterochelin. It contained an iron(III) aerobactin transport system but lacked aerobactin synthesis. This strain was able to utilize the hydroxamate siderophores ferrichrome, coprogen, ferrioxamine B, rhodotorulic acid, and schizokinen as sole iron sources and grew on iron citrate as well. In contrast to *E. coli* K-12, *S. marcescens* could utilize heme. DNA fragments of the *E. coli fhuA*, *iut*, *exbB*, and *fur* genes hybridized with chromosomal *S. marcescens* DNA fragments, whereas no hybridization was obtained between *S. marcescens* chromosomal DNA and *E. coli fecA*, *fhuE*, and *tonB* gene fragments. The presence of multiple iron transport systems was also indicated by the increased synthesis of at least five outer membrane proteins (in the molecular weight range of 72,000 to 87,000) after growth in low-iron media. *Serratia liquefaciens* and *Serratia ficaria* produced aerobactin, showing that this siderophore also occurs in the genus *Serratia*.**

Fe^{3+} is insoluble at neutral pH, so iron supply in microbes occurs via siderophore compounds (12, 39). The Fe^{3+} siderophores are taken up in an energy-coupled process across the outer and cytoplasmic membranes of gram-negative bacteria (7, 10). Transport across the outer membrane requires receptor proteins which specifically recognize the various Fe^{3+} siderophores. These receptors are synthesized under iron-limiting growth conditions in amounts similar to those of the major outer membrane proteins, whereas they are barely detectable on Coomassie-stained polyacrylamide gels after growth at sufficient iron concentrations (8, 15, 33, 41). Outer membrane translocation of the Fe^{3+} siderophores depends in addition on the TonB and ExbBD proteins, which have been localized in the cytoplasmic membrane. The TonB protein extends into the periplasmic space and is thought to be involved in the energy-dependent vectorial release of the Fe^{3+} siderophores from the receptors into the periplasm (19, 20, 23, 27, 29, 43). Uptake across the cytoplasmic membrane seems to follow a periplasmic binding-protein (PBP)-dependent transport mechanism, since the sequences and locations of the characterized proteins of the *Escherichia coli* Fe^{3+} hydroxamate (13, 14) and Fe^{3+} citrate (44, 49, 53) transport systems are characteristic of PBP systems (1, 2).

Recently, we described an Fe^{3+} transport system of *Serratia marcescens* which differed from the hitherto studied transport systems of members of the family *Enterobacteriaceae* by the lack of a siderophore and an outer membrane receptor protein (3, 52). Both compounds could have been missed if present in very low amounts, but since Fe^{3+} transport mediated by the cloned genes in genetically well-defined *E. coli* strains was independent of the TonB and ExbBD proteins, we suggested a novel type of Fe^{3+} uptake mechanism across the outer membrane. The sequences of the three transport proteins, derived from the nucleotide sequences of the *sfuABC* genes, and their localizations in the periplasm and cytoplasmic membrane were typical for PBP transport systems.

In this paper, we report physiological data on the Sfu transport system that support our previous conclusion of an unusual Fe^{3+} uptake across the outer membrane and a conventional PBP mechanism across the cytoplasmic membrane. Furthermore, we obtained evidence for additional iron supply systems in *S. marcescens*, indicating multiple ways for the difficult acquisition of this essential element.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *S. marcescens* and *E. coli* strains used are listed in Table 1. They were grown in tryptone-yeast extract (TY) medium and nutrient broth (NB) as described previously (52). To reduce the available iron in NB medium, 2.2′-dipyridyl (0.2 mM for NBD and 0.4 or 0.5 mM for NBD*) or ethylenediamine-di(*o*-hydroxy)phenylacetic acid (EDDA; 0.1 mM) was added. Antibiotics were added to maintain the plasmids (ampicillin,

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TABLE 1. Strains and plasmids used

Strain or plasmid	Genotype	Source or reference
S. marcescens		
W225	Wild type	42
DF1 to DF4	*aro-1, -2, -3, -4* derivatives of W225	This study
DF2R	Rough derivative of DF2	S. Gaisser
DF2R3	*tonB* derivative of DF2R	S. Gaisser
NB22	*fep-1* derivative of W225	This study
ba$^+$17	Wild type	46
ba$^+$33	Wild type	46
DSM1608	Wild type	46
S. liquefaciens DSM30125	Wild type	46
S. ficaria G4028	Wild type	46
E. coli K-12		
AB2847	*aroB malT tsx thi*	26
BR158	AB2847 *tonB*	26
ZI323	AB2847 *fecA zag*::Tn*10*	44
KO280	AB2847 *fhuB metE zif*::Tn*10*	34
KO295	AB2847 *fhuD metE zif*::Tn*10*	W. Köster
IR20	AB2847 *fepA*	31
H1388	*exbB*::Tn*10 aroB pro lac malT tsx*	19
W3110	Wild type	This institute
W3110-6	W3110 *exb*	23
H1443	*aroB araD139 ΔlacU169 rpsL150 relA1 deoC1 ptsF25 rbsR thi flbB5301*	9
MS172	H1443 *fhuE*::λp*lac*Mu53	47
ZI325	H1443 *fecA zag*::Tn*10 fecB*::Mud1(Ap*lac*)	44
ZI314	H1443 *fecB zag*::Tn*10*	44
ZI418	H1443 *fecB*::Mud1(Ap*lac*)	44
ZI342	H1443 *fecB*::Mud1(Ap*lac*) *tonB zch*::Tn*10*	44
H1876	H1443 *cir fepA*::Tn*10 fiu*::MudX	28
H1875	H1443 *cir*::MudX *fepA*::Tn*10*	28
H1877	H1443 *fiu*::MudX *fepA*::Tn*10*	28
H1728	H1443 *fiu*::MudX *cir*	28
JB1691	H1443 *fepB*::λp*lac*Mu	K. Hantke
H1717	H1443 *fhuF*::λp*lac*Mu53	28
SC11	H1717 *fur31*	11
AN260	*fepC aroB proA argE pheA tyrA trp supE44 rpsL lacY galK xyl mtl*	31
WM1576 (K38)	*thr leu lacY thi supE hsdR fhuA trxA*; pGP1-2	51
Plasmids		
pACYC184		36
pLG339		50
pT7-5		51
pGP1-2		51
pSZ1	*sfuABC* on pBR322	52
pAA100	*sfuABC* on pACYC184	This study
pLA200	*sufABC* on pLG339	This study
pAA1	*sfuABC* on pT7-5	This study
pST18	*fecABCDE* of *E. coli* B on pACYC184	44
pLZ30	*'fecA fecBCDE* on pACYC184	44
pSV100	*fecCDE* on pACYC184	S. Veitinger
pFB103-1	*fhuA* on pBR322	21
pMS157	*fhuE* on pACYC184	47
pAN302	*iut* on pACYC184	This study
pBJM2	*tonB* on pACYC177	32
pKE7	*exbB exbD* on pUC18	19
pSV5	*fur* on pUC19	11

50 μg/ml; chloramphenicol, 40 μg/ml; neomycin, 50 μg/ml; tetracycline, 15 μg/ml).

Mutants of *S. marcescens* W225 deficient in enterochelin synthesis were obtained by incubating log-phase cells with 0.2 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) per ml for 55 min at 37°C, after which 99% of the cells had been killed. Cells were harvested by centrifugation, washed twice with M9 salt solution (38), and then incubated for 2 h in TY medium at 37°C. They were then transferred into M9 minimal medium (38) and incubated for 3 h and then overnight in the presence of 0.1 mg of cycloserine per ml. After being plated on M9 medium supplemented with tryptophan, tyrosine, and phenylalanine (0.1 mg/ml each) and *p*-amino benzoic acid and *p*-hydroxybenzoic acid (0.04 mM each), they were replica plated on M9 minimal medium. Strains DF1 to DF4 were able to grow only on M9 medium supplemented with all of the above-mentioned nutrients, indicating a biosynthetic block prior to chorismate. Overnight cultures (10 μl) of strains DF1 to DF4 placed on filter paper disks supported growth of strain H1443 *aroB* seeded on M9 minimal medium agar, which had been supplemented with tryptophan, tyrosine, and phenylalanine at a concentration (0.004 mM) which was not sufficient to support growth of H1443. Strains DF1 to DF4 secreted a compound that fed H1443, placing the DF1 to DF4 mutations beyond the *aroB* mutation. Shikimate did not support growth, suggesting mutation sites in genes between shikimate and chorismate encoding biosynthesis functions (or inability to take up shikimate).

In a further experiment, *S. marcescens* was treated with MNNG, and selection for iron supply mutants was performed in NB medium in the presence of streptonigrin (5 μg/ml) as described previously (9). The resulting strain, NB22, grew weakly on NBD agar plates and showed a large yellow zone around colonies grown on chrome azurol S plates (see below).

The plasmids used are listed in Table 1. Their construction was described previously with the exception of the following plasmids. pAA2 was obtained by *Mlu*I digestion of pAA1 followed by circularization. pAA100 contains the *Hin*dIII fragment of pSZ1 in pACYC184. pLA200 was constructed by cloning the larger *Eco*RI-*Eco*RV fragment of pSZ1 into pLG339 cut with *Eco*RI and *Sma*I. pSV100 contains the *fecCDE* genes of plasmid pST18, from which the smaller *Sal*I fragment was excised (obtained from S. Veitinger). pAN302 contains the *Bam*HI fragment of pEN2 carrying the *iut* gene (25) in pACYC184.

Iron supply assays. Growth was determined on NBD or NBD* agar plates (17.5 ml) seeded with 0.2 or 0.1 ml of an overnight culture of the strain to be tested in 3 ml of overlay NBD or NBD* agar. Filter paper disks (8-mm diameter) were placed onto the NBD or NBD* agar, which contained 15 μl of the following 1 mM solutions: enterochelin, ferrichrome, coprogen, schizokinen, ferrioxamine B, and rhodotorulic acid. Several concentrations were used for assaying growth promotion on plates by dihydroxybenzoate (1 and 10 mM), sodium citrate (100, 50, 10, 5, and 1 mM), and iron citrate (5, 1, 0.5, and 0.05 mM iron). Growth in liquid cultures was measured in NBD medium and in NBD medium supplemented with 0.1 mM sodium citrate. Iron citrate solutions contained a 40-fold citrate surplus. Growth stimulation of the Sfu system with sodium citrate via FecA-TonB was also determined on NB plates containing 0.1 mM EDDA. Cells (2×10^7) were layered onto the NB-EDDA plates in 3 ml of NB soft agar (0.7%). To assay growth promotion by hemin, hemin (Sigma) was washed with 10 mM

HCl and water and then dissolved (1 mM) in 10 mM NaOH. Sizes of growth zones around the disks were estimated by measuring their diameters (9, 24, 25).

Production of siderophores was tested by placing 15 μl of an overnight culture of the producer strain in NB medium onto filter paper disks which were placed on NBD agar plates seeded with an indicator strain which could grow only when supplied with a siderophore that the indicator strain was able to use (24, 25). Siderophore production was also tested on agar plates supplemented with chrome azurol S (48). Blue dye turned yellow around siderophore-producing colonies. For the quantitative determination of catecholate siderophores in culture supernatants of cells grown overnight, the method of Arnow (4) was employed.

Determination of iron transport rates. Cells were grown in 20 ml of NB medium or in NB medium supplemented with 0.1 mM sodium citrate to a density of 3×10^8 cells per ml. They were harvested by centrifugation and washed twice with 40 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) adjusted to pH 7.2 with KOH, 0.5 mM $CaCl_2$, 50 mM NaCl, 100 mM $MgSO_4$, 0.2% glucose, or M9 medium in the case of iron transport studies using sodium PP_i. Cells were suspended in 5 ml of the wash solution supplemented with 0.1 mM nitrilotriacetate to a density of 5×10^8 cells per ml and incubated for 5 min prior to the addition of 50 μl of the radioactive $^{55}Fe^{3+}$ solution. The latter solution for the iron transport experiment in the presence of sodium PP_i consisted of 1.5 μl of 10.5 mM $^{55}FeCl_3$ (57 kBq), 30 μl of 250 mM sodium PP_i, and 284 μl of M9 salt solution, which were all incubated for 20 min at 37°C. For the determination of iron transport in the presence of sodium citrate, 1 μl of 1.05 mM $^{55}FeCl_3$ (74 kBq), 14 μl of 1 mM $FeCl_3$ in 0.02 N HCl, and 40 μl of 1 M sodium citrate were incubated for 20 min at 37°C and then diluted with 260 μl of water. The cultures were incubated at 27°C. Samples (1 ml each) were withdrawn after 0, 5, 10, 15, and 20 min; filtered; washed twice with 5 ml of LiCl; dried; and counted in a liquid scintillation counter.

Protection against protease V8 hydrolysis. Cells (3×10^8) of *E. coli* WM1576 transformed with plasmids pAA1 (*sfuABC*) and pGP1-2 were labeled with 0.37 MBq of [^{35}S]methionine for 5 min at 30°C. For preparation of spheroplasts (35), the sedimented cells were suspended in 0.4 ml of 0.2 M Tris hydrochloride–0.5 M sucrose (pH 8), after which 50 μl of 5 mM EDTA (pH 8), 50 μl of a lysozyme solution in water (6.4 mg/ml), and 0.5 ml of 0.2 M Tris hydrochloride–0.5 mM EDTA (pH 8) were added. The suspension was incubated for 15 min on ice, 20 μl of 1 M $MgSO_4$ was added, and the spheroplasts were sedimented for 30 min in an Eppendorf centrifuge. The supernatant fraction contained the periplasm, of which 0.4-ml samples were supplemented with 40 μl of water, 1 mM $FeCl_3$ (in 0.02 N HCl), 1 mM Fe^{3+}–20 mM citrate, 1 mM Fe^{3+}–200 mM citrate, or 1 mM ferrichrome. After incubation with V8 protease of *Staphylococcus aureus* (30 μg/ml) at 56°C for 0, 60, 90, and 120 min, samples (0.1 ml) were taken, and 50 μl of 30% trichloroacetic acid was added. The precipitates were washed with 0.1 ml of acetone and 0.1 ml of acetone-water (1:1) and then dissolved in the sample buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Electrophoresis of outer membrane proteins. Outer membranes were prepared by lysis of cells with lysozyme followed by solubilization of the cytoplasmic membrane with Triton X-100 and differential centrifugation, as has been previously described in detail (19). The proteins were separated by electrophoresis on an 11% polyacrylamide gel in the presence of 0.1% SDS (19).

Recombinant DNA techniques. Isolation of chromosomal DNA and plasmids, use of restriction enzymes, ligation, agarose gel electrophoresis, recovery of fragments from agarose, transformation, and Southern DNA-DNA hybridization were done as previously described (49).

RESULTS

Sfu-mediated iron uptake in *E. coli*. Because of the lack of defined transport mutants of *S. marcescens*, the Sfu transport system was studied with *E. coli* K-12 mutants transformed with cloned *sfu* genes. The *sfuABC* genes confer upon *E. coli aroB* mutants unable to produce enterochelin the ability to grow on iron-limiting NBD. We have previously shown that *E. coli* single mutants lacking the outer membrane iron siderophore receptor proteins FhuA, FepA, FecA, FhuE, Cir, and Fiu were not impaired in Sfu-mediated iron transport (52). Since Fe^{3+} transport via dihydroxybenzoyl serine can alternatively utilize three receptors (Fiu, FepA, and Cir) (28) and catechol-substituted cephalosporins can utilize two receptors (Cir and Fiu) (16, 40), we measured growth of the triple-receptor mutant H1876 *fiu cir fep* transformed with plasmid pAA100 *sfuABC* on NBD agar plates. No growth inhibition was observed, which indicates that these receptors are not involved in Sfu-mediated iron transport.

In order to inhibit growth of *E. coli sfuABC* transformants on nutrient broth, the dipyridyl concentration had to be increased to 0.4 mM (NBD*). The Sfu system failed to enable *E. coli* KO280 *fhuB*, which lacks the cytoplasmic membrane protein FhuB, and KO295 *fhuD*, which lacks the periplasmic FhuD protein, to grow around filter paper disks containing ferrichrome or coprogen on NBD* plates. This means that iron carried as ferrichrome and coprogen across the outer membrane could not be transported via the Sfu system across the cytoplasmic membrane. The same results were obtained with dihydroxybenzoate, which did not support growth of the *fepC* mutant AN260 (26). This mutant cannot transport ferric dihydroxybenzoate across the cytoplasmic membrane and for this reason is also unable to synthesize enterochelin from dihydroxybenzoate.

In contrast to the above results, iron citrate was able to support Sfu-mediated iron transport. Strain ZI418 *fecB*::Mud1, which is devoid of the periplasmic FecB protein required for citrate-mediated iron transport (44, 49), showed after transformation with plasmid pAA100 on NBD* medium a distinct growth zone around filter paper disks containing citrate. The Mud1 insertion exhibits a strong polar effect on the expression of the downstream *fecCDE* genes, as shown by complementation experiments with cloned *fecBCDE* genes (51a). Since the *fecBCDE* gene products are required for citrate-mediated iron transport across the cytoplasmic membrane, iron transport in this transformant was not catalyzed by the Fec system but rather by the Sfu system. However, uptake of iron dicitrate into the periplasm depends on the FecA outer membrane receptor protein and the TonB protein (33, 44). Therefore, we tested whether the Sfu-catalyzed, citrate-mediated iron uptake also required the FecA and TonB proteins. Indeed, no growth of strain ZI323 *fecA*(pAA100) or strain BR158 *tonB*(pSZ1) on NBD* plates was observed. No distinct growth zones appeared around filter paper disks with sodium citrate after 2 days of incubation at 27°C. Growth rates and the final cell density of strain ZI418 *fecA*$^+$ *tonB*$^+$(pAA100) in NBD liquid culture supplemented with 0.1 mM citrate were also twice as high as those of strains ZI323 *fecA*(pAA100) and BR158 *tonB*(pSZ1).

Apparently, iron uptake across the outer membrane under the extreme iron starvation conditions in NBD* medium requires the FecA receptor and the TonB protein (and presumably energy, which has not been examined).

Since dipyridyl is primarily an Fe^{2+} chelator, additional experiments have been done with the Fe^{3+} chelator EDDA. The experimental conditions had to be adapted to this very strong iron chelator (stability constant, 33 for EDDA compared with 17 for dipyridyl). In fact, we could not observe any improved growth of *E. coli* H1443 transformed with plasmid pAA100 compared with growth of the untransformed strain on NB agar containing 0.1 mM EDDA. However, when EDDA was given only to the solid NB nutrient agar (1.5%) and the number of cells seeded in 3 ml of NB soft agar (0.7%) over the solid agar was reduced from 1×10^8 to 2×10^7, a clear growth promotion zone around filter paper disks with 100 mM sodium citrate could be discerned. Growth promotion was dependent on a functional FecA-TonB outer membrane transport system, since growth of *fecA* and *tonB* mutants was not supported by citrate.

Since iron dicitrate served as an iron donor for the Sfu system, we attempted to ascertain whether the SfuBC proteins could be replaced by the functionally analogous FecCDE proteins. The SfuB protein, like the FecCD proteins, is very hydrophobic and is located in the cytoplasmic membrane. The SfuC protein and the FecE protein are hydrophilic but associated with the cytoplasmic membrane and contain nucleotide-binding domains, so that both could drive the Sfu-mediated iron transport through ATP hydrolysis, as has been shown for histidine (5) and maltose transport (17). Strain H1443 transformed with the two plasmids pAA2 *sfuA* and pSV100 *fecCDE* did not grow on NBD, excluding transfer of iron from the periplasmic SfuA protein to the FecCDE proteins in the cytoplasmic membrane.

In order to obtain quantitative data, transport rates with $^{55}Fe^{3+}$ citrate were measured. Cells were grown in NB medium supplemented with citrate. The available iron in this medium is low but sufficient for normal growth in contrast to the extremely iron-deficient conditions in NBD*. All *fec* mutants, including *fecA* mutants, which were transformed with pAA100 *sfuABC*, were able to transport iron at high rates (Fig. 1, curves 1, 2, and 3). Under conditions of low iron supply, the FecA receptor is not required, which also holds true for the TonB protein. No transport into untransformed cells was seen (Fig. 1, curves 5, 6, and 7). Iron transport via the Sfu system was faster than that via the Fec system of strain AB2847 *fec*$^+$ (Fig. 1, curve 4). This may be caused by the multiple *sfuABC* gene copies in the transformants in contrast to the single *fecABCDE* copy in AB2847. However, transformation of ZI418 *fecB* (polar on *fecCDE* expression) with plasmid pLZ30, which carries a 5'-truncated *'fecA fecBCDE*, did not increase the citrate-mediated iron transport rate but instead slightly decreased it (data not shown).

Previously, it has been shown by growth and transport assays that sodium PP_i serves as an iron donor for the Sfu system at low iron concentrations (52). At the extremely iron-deficient conditions in NBD*, sodium PP_i up to 30 mM did not support growth. At 40 mM sodium PP_i, growth was observed; this growth, however, did not depend on the Sfu system, since it also occurred to the same extent in the untransformed H1443 *aroB* strain and the untransformed BR158 *aroB tonB* strain. The sodium PP_i-mediated iron transport rates (measured at 0.25 mM sodium PP_i) of cells grown in NB were similar to those of citrate-mediated transport via the Sfu system, indicating that more than one

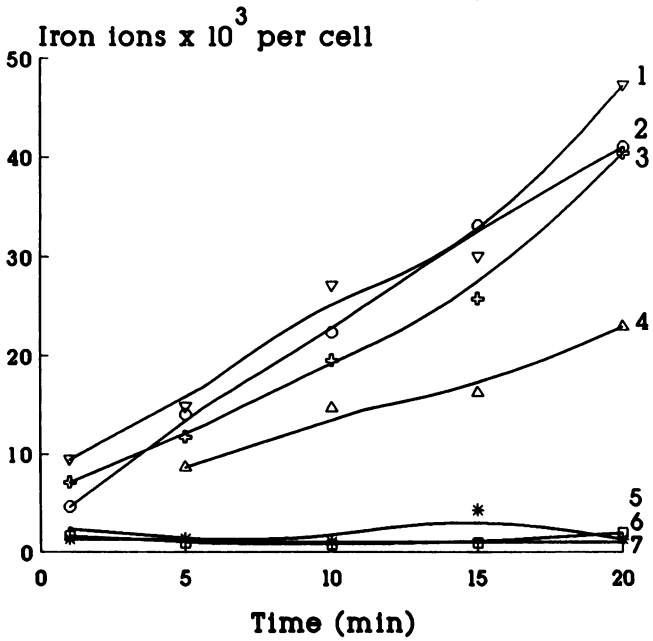

FIG. 1. Citrate (10 mM)-mediated $^{55}Fe^{3+}$ transport into cells of *E. coli* ZI342 *fecB tonB*(pAA100 *sfuA*$^+$*B*$^+$*C*$^+$) (curve 1), ZI418 *fecB*(pAA100) (curve 2), ZI325 *fecA fecB*(pAA100) (curve 3), and the untransformed strains ZI342 (curve 5), ZI418 (curve 6), and ZI325 (curve 7) lacking plasmid pAA100. For comparison, citrate-mediated iron transport into *E. coli* AB2847 via the Fec system has been included (curve 4).

ligand could solubilize Fe^{3+} and donate it to the Sfu system (Fig. 2, curves 1 and 2, transformants carrying pAA100). The same strains transformed with plasmid pLZ30 *'fecABCDE* were unable to transport Fe^{3+} supplied as sodium PP_i salt (curves 3 and 4).

Iron regulation of *sfu* gene expression. A potential consensus sequence for the Fur iron repressor protein (11, 18) was found at nucleotides 54 to 72 upstream of the *sfuA* gene (3), suggesting iron-regulated transcription of the *sfuABC* genes. To examine this notion, plasmid pLA200 containing the *sfuABC* genes on the low-copy-number vector pLG339 was transferred into *E. coli* SC11 *fur*. The iron transport rates obtained in the presence of sodium PP_i were three times higher than those obtained for the parent strain H1717 *fur*$^+$(pLA200) (data not shown).

Attempts to identify the substrate for the Sfu system. The only transport protein which can readily be isolated under mild conditions and therefore in an active state is the periplasmic SfuA protein. It was released by transferring cells from a medium of high osmolarity into a medium of low osmolarity (osmotic shock treatment [35]). The *E. coli* cells contained the *sfuABC* genes on plasmid pAA1 downstream of the phage T7 gene 10 promoter, which was transcribed by the T7 RNA polymerase encoded on plasmid pGP1-2 under the control of a temperature-sensitive repressor (51). The cellular RNA polymerase was inhibited by rifampin. Under these conditions, only the SfuA protein of the periplasmic fraction was labeled with [^{35}S]methionine, as revealed by SDS-PAGE (Fig. 3). We then tried to degrade SfuA by using proteases to see whether binding of the substrate would alter the peptide degradation pattern. This approach has been successful in the case of the FhuD protein, where only the substrates that were transported (aerobactin, ferrichrome,

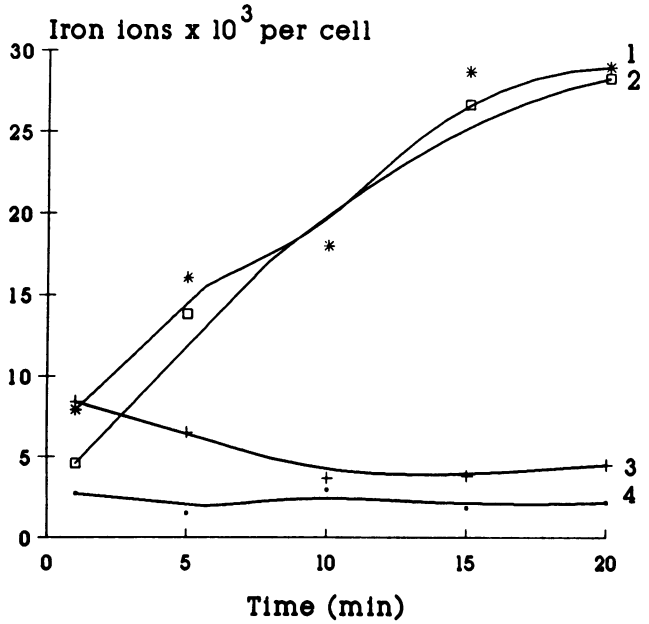

FIG. 2. Sodium PP_i (0.25 mM)-mediated $^{55}Fe^{3+}$ transport into cells of *E. coli* ZI418 *fecB*(pAA100 *sfuA*$^+$*B*$^+$*C*$^+$) (curve 1), ZI325 *fecA fecB*(pAA100) (curve 2), ZI325(pLZ30) (curve 3), and ZI418(pLZ30 '*fecABCDE*) (curve 4).

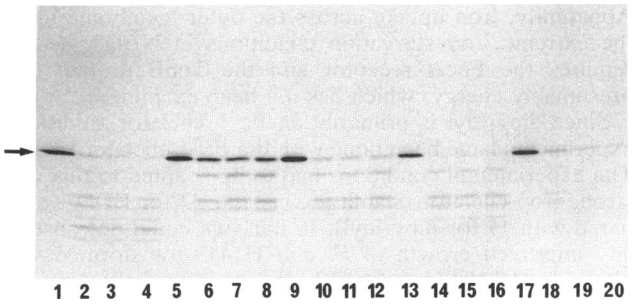

FIG. 3. Protection of the SfuA protein against V8 proteinase digestion by Fe^{3+} ions. SfuA protein from the osmotic shock fluid of *E. coli* WM1576(pAA1 *sfuA*$^+$*B*$^+$*C*$^+$) was treated at 56°C with V8 proteinase (30 μg/ml) for 0, 60, 90, and 120 min in the presence of H_2O (lane 1 to 4), 0.1 mM $FeCl_3$ (lanes 5 to 8), 0.1 mM Fe^{3+} citrate (1:20; lanes 9 to 12), 0.1 mM Fe^{3+} citrate (1:200; lanes 13 to 16), or 0.1 mM ferrichrome (lanes 17 to 20). Cells were labeled with [^{35}S]methionine (370 kBq/ml) in the presence of rifampin after induction of the phage T7 RNA polymerase.

and coprogen) protected the periplasmic protein from being degraded (35). The SfuA protein was largely resistant to degradation by trypsin and proteinase K. Only the staphylococcal V8 protease at 56°C degraded part of SfuA (Fig. 3, lanes 1 to 4). Degradation could be partially prevented by the addition of 0.1 mM iron chloride (lanes 5 to 8) and to a lesser extent but reproducibly by 0.1 mM iron citrate (ratio of Fe^{3+} to citrate, 1:20) (lanes 9 to 12). When the citrate concentration was increased (ratio of Fe^{3+} to citrate, 1:200) to further reduce the free-iron concentration, no protection was observed (lanes 13 to 16). There was also no inhibition of SfuA degradation by ferrichrome (lanes 17 to 20). These data suggest that Fe^{3+} may bind directly to the SfuA protein and that citrate may function as an iron carrier from which iron dissociates to bind to the SfuA protein. Iron chloride did not inhibit the V8 protease, since the FhuD protein was degraded as fast in its presence as in its absence (data not shown).

Siderophore production of *S. marcescens* W225. Studies to see whether the unusual Sfu system is the only iron transport system of *S. marcescens* have been undertaken. A yellow zone appeared around colonies of *S. marcescens* grown on chrome azurol S nutrient agar plates, indicating that the cells secreted a compound that withdrew iron from the blue dye and turned it yellow. To identify the presumed siderophore(s), growth promotion of *E. coli* indicator strains on NBD medium was tested around filter paper disks containing samples of the *S. marcescens* culture. The *E. coli* indicator bacteria produced no siderophore and were either impaired in the uptake of Fe^{3+} enterochelin (IR20 *fepA*, IB1691 *fepB*) and its degradation product dihydroxybenzoyl serine and its precursor dihydroxybenzoate (H1876 *fepA fiu cir*), other catecholates (H1875 *fepA cir*, H1877 *fepA fiu*, H1728 *fiu cir*), Fe^{3+} aerobactin (H1443 *iut*), ferrichrome (KO280 *fhuB*), coprogen (MS172 *fhuE*), and Fe^{3+} dicitrate (ZI314 *fecB*) or were deficient in all iron uptake routes (BR158 *tonB*, W3110-6 *exbBD*). Stimulation of growth by secretion products of *S. marcescens* W225 was observed only with *E. coli* cells which took up enterochelin. The *fep* mutants showed no growth zone (*fepB*) or a reduced growth zone (*fepA*). The weak growth of the latter strain was not observed with strain H1876, indicating utilization of enterochelin degradation products or other catecholate compounds as iron sources. Only the triple mutant H1876 showed no growth, while growth of the *E. coli* double mutants carrying *fepA cir*, *fepA fiu*, and *fiu cir* mutations was supported. *E. coli* mutants with mutations in *tonB* and *exbBD* did not respond to *S. marcescens* secretion products. Apparently, enterochelin was the only secretion product of *S. marcescens* which could serve as a siderophore for *E. coli*.

Determination of catecholates in the culture supernatant by using the Arnow procedure resulted in A_{564}/A_{578} ratios of 1.92 for *S. marcescens* W225 and 0.14 for *E. coli* W3110. There was no enterochelin-specific absorbance for AB2847 *aroB*. These data show that *S. marcescens* secretes much larger amounts of enterochelin into the culture supernatant than *E. coli* W3110.

Secretion of aerobactin was tested with *E. coli* H1876(pAN302) *iut*$^+$, which takes up but does not synthesize aerobactin. No growth stimulation was observed with *S. marcescens* W225, excluding aerobactin synthesis by this strain. The additional *S. marcescens* strains tested (Table 1) were all aerobactin negative. In contrast, *Serratia liquefaciens* and *Serratia ficaria* stimulated growth of *E. coli* H1876(pAN302), indicating synthesis and secretion of aerobactin.

Use of Fe^{3+} siderophores by *S. marcescens* W225. To test whether *S. marcescens* takes up Fe^{3+} siderophores, which it does not synthesize itself, *aro* mutants were isolated to avoid competition with the genuine enterochelin. Four such strains were obtained after MNNG mutagenesis and cycloserine selection. They showed no yellow zones around colonies on chrome azurol S plates, and the Arnow test also revealed no catecholate compound in the culture supernatant. However, strains DF1 and DF4 growing on filter paper disks supported somewhat reduced growth of *E. coli* indicator strains able to take up enterochelin. The diameters of the growth zones were 11 mm; those of the parent strain were 16 mm (diameter of the filter paper disk, 8 mm). Since the diameter of the

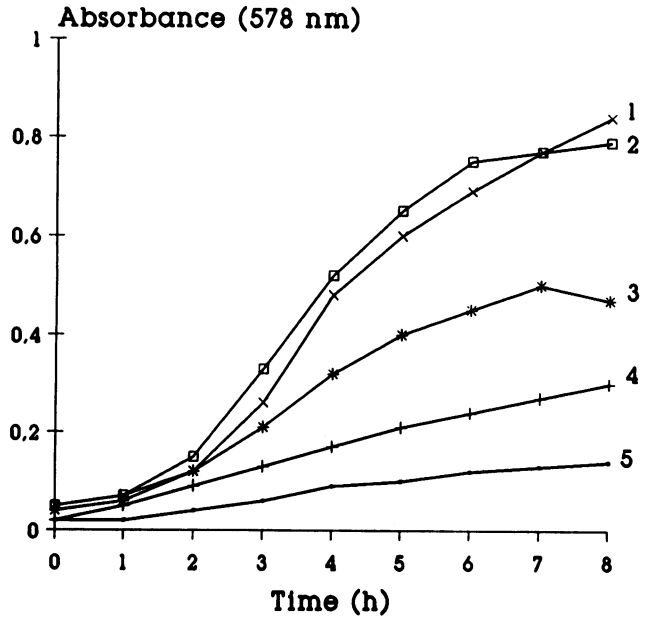

FIG. 4. Growth rates after incubation in NBD liquid culture of *E. coli* H1443 *aroB* (curve 5) and H1443 *aroB*(pSZ1) (curve 1), *S. marcescens* wild-type strain W225 (curve 2), and *S. marcescens aro* mutants DF1 (curve 4) and DF2 (curve 3).

growth zone is logarithmically related to the concentration of the compound to be tested, the difference between wild type and mutants is substantial. Mutants DF2 and DF3 did not support growth of any of the *E. coli* indicator strains, so they are enterochelin negative according to this very sensitive assay. However, upon prolonged incubation, low growth stimulation was also obtained with DF2 and DF3. They apparently secreted an enterochelin precursor which was taken up and converted to enterochelin by the *E. coli aroB* indicator strains (H1875, H1877, and H1728). Growth of the triple mutant H1876 and of BR158 *tonB*, which do not take up catecholate compounds, was not supported.

Incubation in NBD liquid cultures also demonstrated the different growth rates of strains DF1 and DF2 (Fig. 4). It also showed the growth-enhancing role of the *sfu* genes when *E. coli* H1443 was transformed with pSZ1 *sfu*.

Strains DF1 to DF4 were used to determine growth stimulation on NBD* agar plates (containing 0.5 mM dipyridyl), onto which the siderophores enterochelin, aerobactin, ferrichrome, schizokinen, coprogen, rhodotorulic acid, ferrioxamine B, and hemin at concentrations of 1 mM (15 μl) on filter paper disks were placed. All siderophores supported growth of the *S. marcescens aro* mutants to a similar extent. *E. coli sfuABC* transformants were unable to use heme iron, so the heme uptake system in *S. marcescens* remains unknown.

Citrate-mediated iron uptake into *S. marcescens* DF2R showed a complex behavior. On NBD* plates (containing 0.5 mM dipyridyl) seeded with DF2R, growth zones of 3, 2, and 1.6 cm appeared around filter paper disks soaked with 15 μl of 50, 10, and 5 mM sodium citrate, respectively. No growth zone was observed with 1 mM sodium citrate. A *tonB* mutant of DF2R exhibited no growth, suggesting a receptor- and TonB-dependent uptake of iron across the outer membrane. Under these conditions, citrate had to compete for iron with the high concentration of dipyridyl in the NBD* medium. When iron citrate instead of sodium citrate was used, growth of DF2R was stimulated down to a concentration of 0.05 mM. Growth of the *tonB* mutant was also supported but only down to a concentration of 0.5 mM. At all iron concentrations used (5, 1, 0.5, and 0.05 mM), the growth zones of the *tonB* mutant were smaller than those of the *tonB*$^+$ parent strain (3.4 and 2.5, 2.5 and 1.5, 2.0 and 1.1, 0.9 and 0 cm, respectively). Of the two control strains (*E. coli* H1443 and BR158 *tonB*) employed, only growth of H1443 *tonB*$^+$ was supported by sodium and iron citrate, whereas the *tonB* mutant did not grow. Growth of H1443 was supported down to a sodium citrate concentration of 5 mM and down to an iron citrate concentration of 0.5 mM.

An additional mutant (NB22) obtained after MNNG mutagenesis and streptonigrin selection formed a larger yellow zone on chrome azurol S agar plates than the *aro*$^+$ parent strain. However, NB22 was unable to grow on NBD* plates (0.5 mM dipyridyl), and growth was not enhanced by the addition of enterochelin. Apparently, strain NB22 synthesized and secreted enterochelin but was unable to take up Fe^{3+} enterochelin. This conclusion was supported by the positive Arnow test, in which an A_{564}/A_{578} ratio of 0.7 was obtained. Failure to utilize the secreted enterochelin puts the cells under severe iron deficiency, since the iron remains trapped as an enterochelin complex in the medium. This explains the streptonigrin resistance, which kills cells only in the presence of sufficient intracellular iron.

Hybridization of DNA fragments from *E. coli* iron transport genes with chromosomal DNA fragments of *S. marcescens*. To corroborate the data on *S. marcescens* iron transport systems and to obtain an indication of how closely those systems are structurally related to the *E. coli* transport systems, we hybridized fragments excised from *E. coli* transport genes with fragments of the chromosome of *S. marcescens* W225, which was cleaved with the same restriction enzymes as the *E. coli* genes. The plasmids used for isolating the DNA fragment are listed in Table 1. The *Dra*I fragment of *fhuA* composed about half of the gene, the *Kpn*I fragment of *fhuE* was 90% of the gene, the *Pvu*II-*Eco*RV fragment was 95% of *iut*, the *Hin*dII fragment was the entire *tonB* gene plus 800 bp of flanking *E. coli* DNA, the *Eco*RI fragment was the entire *exbB* gene, the *Hin*dIII-*Bgl*I fragment was half of the *fur* gene plus 0.2 kb of the vector, and the *Sal*I-*Bam*HI fragment was the entire *fecA* gene plus a portion of the *fecB* gene. The 4.8-kb *Hin*dIII fragment of *sfu* served as a positive control. Hybridization with *fhuA*, *iut*, *exbB*, and *fur* was obtained (data not shown). The sizes of the fragments of *S. marcescens* and *E. coli* were the same for *fhuA* (1.5 kb) and *iut* (2 kb) and differed for *exbB* (3.7 and 2 kb, respectively) and *fur* (1 and 2 kb, respectively). No hybridization between a 4.8-kb *Hin*dIII DNA fragment of the *S. marcescens sfu* region and *Hin*dIII-digested chromosomal DNA of *E. coli* H1443 was observed.

Iron-regulated outer membrane proteins. The large variety of Fe^{3+} siderophores that supported growth of *S. marcescens* W225 prompted an examination of iron-regulated outer membrane proteins, which in *E. coli* and many other gram-negative bacteria serve as receptors for Fe^{3+} siderophores. We used the rough derivative DF2R of W225 to avoid wavy protein bands after SDS-PAGE caused by the O antigens. Synthesis of *S. marcescens* proteins in the molecular weight range of 70,000 to 85,000 was low in TY medium, which contains sufficient amounts of iron (Fig. 5, lane 1). Reduction of the available iron by addition of dipyridyl (1 mM) resulted in a large increase in the amounts of these proteins (Fig. 5, lane 2). Growth on nutrient broth medium, which contains

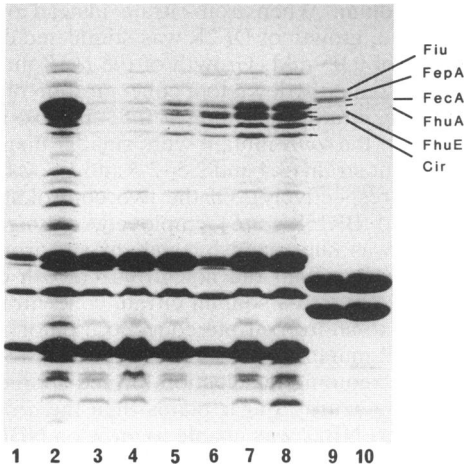

FIG. 5. Protein pattern of outer membranes after SDS-PAGE. Cells of an *S. marcescens* DF2R *aro* mutant were grown in TY medium (lane 1), TY medium complemented with 1 mM dipyridyl (lane 2), NB medium (lane 3), or NB medium complemented with 10 mM sodium citrate (lane 4), 50 μM dipyridyl (lane 5), 100 μM dipyridyl (lane 6), 50 μM dipyridyl and 1 mM citrate (lane 7), or 100 μM dipyridyl and 1 mM citrate (lane 8). *E. coli* H1443 *aroB* was grown in NB medium (lane 9) and NB medium supplemented with 1 mM citrate (lane 10).

less iron than TY, led to a slight increase in these proteins (lane 3) which became progressively stronger upon addition of increasing concentrations of dipyridyl (from 50 to 100 μM; Fig. 5, lanes 5 to 6). Addition of citrate did not abolish derepression of the iron-regulated outer membrane protein synthesis, nor did it induce a protein (lane 4, 10 mM citrate; lanes 7 and 8, 1 mM citrate with 50 and 100 μM dipyridyl, respectively). The iron-regulated *E. coli* proteins were more strongly expressed in nutrient broth (lane 9) than were the *Serratia* proteins (lane 3), which agrees with the superior iron supply of the latter via the Sfu transport system. Addition of citrate suppressed synthesis of these proteins except for the FecA protein (lane 10). The electrophoretic mobilities of the iron-regulated proteins and the major outer membrane proteins differed in *Serratia* spp. and *E. coli*.

DISCUSSION

The Sfu iron transport system of *S. marcescens* accepts Fe^{3+} solubilized with oxaloacetate, sodium PP_i, and citrate (52). This excludes a ligand specificity for uptake across the outer membrane and stands in sharp contrast to all the siderophore-mediated iron transport systems hitherto characterized, which exhibit a high ligand specificity that is even narrower for iron transport across the outer membrane than for transport across the cytoplasmic membrane (7, 8). We therefore conclude that the Sfu system transports iron without a specific ligand. However, not all Fe^{3+} siderophores served in iron uptake via Sfu. Ferrichrome, coprogen, and dihydroxybenzoate were inactive in experiments with *E. coli sfuABC* transformants that were lacking components required to transport these siderophores across the cytoplasmic membrane (*fhuB* and *fepC*). This also held true under extremely iron-deficient conditions (0.4 mM dipyridyl or 0.1 mM EDDA), under which citrate was an active iron donor for the Sfu system. Interestingly, this extreme iron deficiency required the FecA iron citrate receptor and the TonB protein for transport across the outer membrane, since *E. coli sfuABC* transformants mutated in *fecA* and *tonB* could not grow with sodium citrate. *E. coli sfuABC* transformants mutated in the *fecBCDE* genes, which are required for citrate-mediated iron transport across the cytoplasmic membrane, took up iron, showing that citrate carried iron across the outer membrane and the Sfu system carried it across the cytoplasmic membrane. Apparently, at high dipyridyl concentrations and in the presence of the strong iron chelator EDDA, entry of iron through the outer membrane is too low to support growth. For this reason, transport catalyzed by FecA and TonB is necessary. The iron citrate-mediated transport for the Sfu system across the outer membrane clearly demonstrates receptor and TonB dependence under conditions of strong iron deficiency and no such requirement under a lower iron limitation. We take this as evidence that under the latter conditions, the iron diffusion rate is high enough to fulfill the iron requirement. At very low iron concentrations, the energy-coupled receptor and TonB-dependent uptake across the outer membrane are necessary.

The reason for the inability of ferrichrome, coprogen, and dihydroxybenzoate to serve as iron donors for the Sfu system, despite their efficient uptake across the outer membrane, may be sought in the very high stability constants of these iron complexes (ferrichrome, 29.1; coprogen, 30.2; dihydroxybenzene, 43.7 for dihydroxybenzoate) (30), which presumably exceed that of iron citrate by several orders of magnitude (numbers at pH 7 are for various reasons not available [22, 37]). The iron citrate complex has just the right properties in that it is strong enough that an outer membrane transport system has evolved for transport of the complex and sufficiently weak that it donates iron to the Sfu system. The other siderophores tested are apparently inactive in donating iron to the Sfu system in the periplasm. Also, EDDA is less suitable than dipyridyl for characterizing the Sfu iron transport system because of its very high iron complex formation constant, so that iron is not transferred from EDDA to Sfu.

The primary acceptor for iron in the Sfu system is probably the periplasmic SfuA protein. Indeed, binding of iron supplied as iron chloride and iron citrate could be demonstrated by showing an altered V8 protease degradation in their presence compared with the pattern obtained in their absence. In contrast, the transport-inactive ferrichrome exerted no effect on protease degradation of SfuA. These results are similar to the results obtained with the FhuD protein, where only the Fhu transport-active iron siderophores of the hydroxamate type (aerobactin, ferrichrome, and coprogen) prevented degradation by proteinase K and trypsin, whereas the transport-inactive ferrichrome A, iron citrate, and iron sulfate were without effect (35). We found no metal-binding motif in the SfuA protein.

The SfuABC-mediated iron transport rate in *E. coli sfuABC* transformants is higher than the iron citrate transport rate via the Fec system. This indicates a high efficiency of transport across the cytoplasmic membrane which is sufficient to support growth despite the presumably low entry rate of iron through the outer membrane.

A highly conserved region has been identified in the cytoplasmic membrane components of a number of PBP-dependent uptake systems, including the family of iron uptake proteins (33b). If a matrix reflecting this pattern is generated and used for a search in a data base (PC/GENE

program MATSCAN, SwissProt Release 17), it is possible to distinguish between members of the iron and noniron uptake systems. Interestingly, the corresponding region of SfuB (amino acids 419 to 450) is detected only with the noniron matrix. The possibility of distinguishing between iron and noniron uptake systems with such a matrix is in agreement with the fact that all the iron uptake systems possess an outer membrane TonB-dependent receptor, whereas the other uptake systems do not need one. Furthermore, significant homologies between all the cytoplasmic membrane components of the iron uptake systems except SfuB (33a) have been detected (PC/GENE program PCOMPARE). These findings support the unique properties of the Sfu iron transport system.

Enterobacteriaceae usually express more than one iron transport system (7, 8, 15, 41). *E. coli* K-12 has at least seven Fe^{3+} and one Fe^{2+} transport systems. To obtain a more complete view of the iron transport systems of *S. marcescens*, of which we had studied only the Sfu system because of its unusual properties, we performed a survey study, without going into detail, when the systems seem to be similar to those of *E. coli*. The results obtained indicate the existence of Fe^{3+} enterochelin, Fe^{3+} aerobactin, ferrichrome, Fe^{3+} coprogen, ferrioxamine B, Fe^{3+} citrate, and hemin transport systems. Enterochelin was the only siderophore synthesized by the *S. marcescens* strains tested. Aerobactin was not formed by *S. marcescens* but was formed by *S. liquefaciens* and *S. ficaria*. However, *S. marcescens* contained an Fe^{3+} aerobactin transport system. *S. marcescens* and *S. liquefaciens* are the two clinically relevant species of the genus *Serratia*. Apparently, *S. marcescens* contains *fep*, *fes*, *ent*, *fhu*, *fec*, *iut*, and *fiu* (*cir*) genes, which are equivalent to those characterized in *E. coli*. *S. marcescens* also expresses a number of iron-regulated outer membrane proteins. Five protein bands could be identified on gels, indicating at least five iron receptors. Unfortunately, it was not possible to correlate the proteins with the various iron transport systems by isolating drug-resistant mutants. Growth inhibition by albomycin and the rifamycin derivative CGP4832 (45) (both in *E. coli*, indicative for the FhuA receptor) and by catecholate-substituted cephalosporins (Cir and Fiu) (16, 40) occurred at higher concentrations than in *E. coli*, and no inhibition was observed with the colicins (M for FhuA, and B and D for FepA), so that resistant, frequently receptor deletion mutants were not obtained with these agents. In these assays, a rough derivative (DF2R) has been used to avoid partial resistance due to the long lipopolysaccharide O antigens, which may inhibit access to the receptors. Therefore, we have to conclude that the transport components of *S. marcescens* differ from those of *E. coli* so that no or only weak cross-sensitivity to these agents exists. On the other hand, hybridization with chromosomal DNA fragments of *S. marcescens* and a number of DNA fragments of *E. coli* iron transport genes (*fhuA*, *iut*, *exbB*, and *fur*) was obtained, despite the difference in the GC content of the two organisms (*S. marcescens*, 58%; *E. coli*, 50%). Although no hybridization with the *tonB* gene was found, sequencing of the *S. marcescens* gene revealed a high homology to the *E. coli* protein but too low a DNA homology to be detected at the stringent conditions used in the hybridization experiments (23a). Therefore, the failure to find additional DNA hybridizations does not necessarily demonstrate large structural and functional differences from the *E. coli* transport systems. Growth on Fe^{3+} coprogen also suggests an FhuE-equivalent coprogen receptor in *S. marcescens*, despite the lack of hybridization with the *E. coli fhuE* gene. A wide distribution of FhuE equivalent receptor proteins among gram-negative bacteria is suggested by the occurrence of such a protein in the plant growth-promoting *Pseudomonas putida* strain WCS358 (6). The protein, designated PupA, shows the greatest homology (31.4%) to FhuE among all the iron receptor proteins. It serves as a receptor for pseudobactin 358, which contains both a hydroxamate and a catecholate group.

Two systems are probably involved in citrate-mediated iron uptake in *S. marcescens*. The experiments with *E. coli sfuABC* transformants clearly indicated that citrate served as an iron donor for the Sfu system. The same mechanism was probably operating in the TonB-independent uptake of added iron citrate in *S. marcescens* DF2R *tonB*. On the other hand, when iron had to be withdrawn by sodium citrate from NBD* medium, growth of DF2R depended on TonB activity. The low amounts of iron citrate formed under these conditions required a receptor and TonB-dependent uptake through the outer membrane. However, we were unable to induce an outer membrane iron citrate receptor protein by adding citrate, as has been done in *E. coli* (44). Therefore, other than the physiological data, we have no protein analysis or genetic indications of a citrate-mediated iron transport system in *S. marcescens*.

Threefold enhancement of the Sfu-mediated iron transport rate in an *E. coli fur* mutant compared with uptake in the fur^+ parent strain suggests a Fur-regulated expression of the *sfuABC* genes. In addition, synthesis of the iron-related outer membrane proteins of *S. marcescens* was strongly increased after cells were grown in iron-limiting dipyridyl-containing medium. Interestingly, the *S. marcescens* hemolysin genes (*shlA* and *shlB*) are also derepressed by a factor of 10 when cloned into an *E. coli fur* mutant (42). Sequences typical of Fur-binding regions do occur in the −35 region upstream of the *sfuA* (3) and *shlB* (42) genes, and they are apparently recognized by the *E. coli* Fur repressor. It seems that *S. marcescens* shares several iron transport systems as well as their regulation with *E. coli* and other gram-negative bacteria and that only the Sfu system is unique for *S. marcescens*.

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