Mol Gen Genet (1987) 210:135–139

MGG

Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K 12: *fur* not only affects iron metabolism

Klaus Hantke
Lehrstuhl für Mikrobiologie II, Universität Tübingen, Auf der Morgenstelle 28, D-7400 Tübingen, Federal Republic of Germany

Summary. A selection procedure using Mn^{2+} is described. A high percentage of the Mn^{2+} resistant mutants had constitutive iron transport systems. By P1 transduction, and complementation with the cloned *fur* gene it could be shown that nearly all the mutants constitutive in the expression of the operon fusion *fiu*::λ*plac*Mu were only defective in *fur*. High concentrations of manganese inhibited the derepression of an iron-regulated *lac* operon fusion. In another iron-regulated *lac* operon fusion that was inducible by iron, manganese also induced the production of β-galactosidase. Most of the *fur* mutants isolated (80%) were not able to grow on succinate, fumarate or acetate. After transformation with a *fur*$^+$ plasmid all 39 mutants tested were able to grow on succinate. In *fur* mutants the presence of succinate in the growth medium reduced succinate uptake rates by 50%–70%. Succinate dehydrogenase activity was reduced to 10% of that of the parent strain.

Key words: *fur* selection with Mn^{2+} – Mn^{2+} and iron transport – Succinate utilization

Introduction

The iron uptake systems of *Escherichia coli* are regulated by the gene product of *fur* although in some iron transport systems superimposed regulatory systems exist (Hantke 1985). The regulation by *fur* was studied using *lac* operon fusions in several iron transport genes (Hantke 1981). By selection for constitutive expression of such an operon fusion, Fur$^-$ mutants were selected which were constitutive for all five known ferric iron transport systems. The gene *fur* was mapped at 15.5 min on the genetic map of *E. coli* (Hantke 1984) and the cloned gene was sequenced (Schäffer et al. 1985).

A positive selection procedure for Fur mutants in *E. coli* K12, *Klebsiella* and *Serratia* is described. The procedure is based on the isolation of manganese resistant mutants described previously (Silver et al. 1972). The ready isolation of *fur* mutants permitted further characterization of the Fur phenotype.

Materials and methods

Media and growth conditions. The selection medium for manganese resistant mutants was as described (Silver et al. 1972) with one modification: one litre of medium contained 4 g tryptone, 2.5 g NaCl, 10 mM $MnCl_2$ and in addition 50 μM desferral. The solid medium contained 15 g agar (Difco) per litre. It was essential to use fresh plates, as observed by Silver et al. (1972).

For the membrane preparations of *Pseudomonas aeruginosa* M-2 (Montie et al. 1982), the strains were grown either on CDM medium (Anwar et al. 1984) or on tryptone yeast extract medium (8 g tryptone, 5 g yeast extract and 5 g NaCl per litre). The membranes were prepared as described (Anwar et al. 1984).

The iron-rich MacConkey lactose plates were prepared by adding 40 μM $Fe(NH_4)_2(SO_4)_2$. β-Galactosidase activity was determined as described (Miller 1972). One unit of β-galactosidase was defined as the cleavage of 1 nmol *o*-nitrophenyl-β-D-galactoside by 10^9 cells per min.

The minimal medium E (Vogel und Bonner 1956) was used. The carbon sources used were: 0.4% glucose or 0.6% succinate, fumarate, glycerol, and acetate, respectively.

Strains and plasmids. The strains used are listed in Table 1.

The P1 transductions and the Hfr crosses were as described (Miller 1972). The Tn*10* insertions were selected on tryptone yeast-extract medium supplemented with 15 mg tetracycline per litre. Tests for *hsdR* were done with phage λ grown on an *hsdR hsdM* mutant, *tsr* was tested on tryptone swarm plates (Hedblom and Adler 1980) and *fhuF*::λ*plac*Mu was tested on MacConkey lactose plates supplemented with iron.

The plasmids pMH15*fur*$^+$ and pMH16*fur*$^+$ have been described (Hantke 1984; Schäffer et al. 1985).

Succinate transport. The cells were pregrown overnight in tryptone yeast extract medium. For induction of the succinate uptake system, cells were resuspended in minimal medium to an absorbance of 0.4 at 578 nm and then incubated for 3 h with 0.4% glycerol and 0.2% succinate (Bewick and Lo 1979) or with 0.5% acetate (Herbert and Guest 1971). Cells were washed in minimal medium E with no added carbon source and resuspended in the same medium to an absorbance of 0.5 at 578 nm. Transport was started by the addition of 0.11 mM ^{14}C-succinate (0.5 μCi/ml). The rate of uptake was calculated over the first 1–3 min of transport.

Succinate dehydrogenase and NADH oxidase assay. Cells were grown under the same conditions as described for the succinate transport. The cells were opened in a French

Table 1. Strains of *Escherichia coli* K12

Strain	Parent	Genotype	Source
W3110		wild type	This institute
MC4100		*araD139* Δ*(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	M. Casadaban
H1443	as MC4100	but *aroB*	Hantke 1983
H1632	as MC4100	but *zbf*::Tn*10*	This study
H1645	as H1443	but *ird*::λ*plac*Mu53	This study
H1717	as H1443	but *fhuF*::λ*plac*Mu53	This study
H1780	as JB1698	but *fur*	This study
H1782	as H1443	but *zji*::Tn*10 hsdR*	This study
SC11	as H1717	but *fur31*	This study
JB1698	as MC4100	but *fiu*::λ*plac*Mu53	This study
F2116		HfrH *thi relA mdoB*::Tn*10*	Fiedler and Rotering (1985)
AW518	AW405	*thr ara leu lac his rpsL xyl tsr*	Hedblom and Adler (1980)

pressure cell at 10 MPa. Remaining whole cells were separated by a short centrifugation at $6000 \times g$ and discarded. Membranes were collected after centrifugation at $45000 \times g$ for 1.5 h. Succinate dehydrogenase and NADH oxidase activities in the membranes were tested as described (Osborn et al. 1972).

Results

Characterization of mutants

Some mutants resistant to Mn^{2+} (Silver et al. 1972) showed an outer membrane protein profile with derepressed iron-regulated proteins reminiscent of the Fur phenotype (Hantke 1981). For a further investigation of the selection procedure, the strain JB1698 *fiu*::λ*plac*Mu was used. The addition of desferral to the selection medium accelerated the growth of Mn^{2+} resistant mutants. After 3 days, many mutants were observed on the plates with desferral, while fewer mutants appeared one or more days later on the plates without desferral. The *lac* fusion in strain JB1698 is regulated by iron (Hantke 1983). On iron-rich MacConkey lactose plates Fur mutants appear as red colonies while in Fur^+ strains repression leads to white colonies. From 174 manganese resistant colonies tested, 102 exhibited the Fur phenotype on MacConkey plates. Of these supposed Fur mutants, 38 were transduced with a P1 lysate from H1632 *zbf15*::Tn*10* *fur*$^+$. All mutations, except one, mapped at the *fur* gene locus. The one exception (strain H1776) was mapped to the *ent-fep* gene cluster. However, in the latter mutant, enterochelin synthesis and uptake did not seem to be impaired. Some *fur*$^+$ transductants were tested and found to be as sensitive to manganese as the parent strain, indicating that *fur* was the only gene altered in these mutants.

To verify that no gene other than *fur* was mutated, another 40 of the above mentioned 102 mutants were transformed with the plasmids pMH15*fur*$^+$ and pMH16*fur*$^+$ (Hantke 1984). From each mutant five transformants were tested, and in each case a Fur^+ phenotype was found. This indicated that the mutants tested could be complemented by the cloned gene.

Selection for iron-deregulated mutants in *Serratia marcescens* and *Klebsiella pneumoniae* was attempted. Some of the manganese resistant mutants were found to overproduce the iron-regulated outer membrane proteins (data not shown). On the same medium, manganese resistant mutants of *Pseudomonas aeruginosa* M-2 were isolated. Since *P. aeruginosa* produces fluorescent iron chelators, the mutants were streaked on tryptone yeast-extract medium where production of the siderophores is repressed. Out of 100 manganese-resistant mutants, one produced a fluorescent compound on this medium. After growth on the same liquid medium, the outer membranes of this mutant strain showed derepressed iron-regulated outer membrane proteins, in contrast to the parent strain (Fig. 1). However, in minimal medium the mutant phenotype was repressible by iron. The mutant grew very slowly compared to the parent strain. Further work is necessary to determine whether this method is useful for isolating mutants of *Pseudomonas* with deregulated iron metabolism.

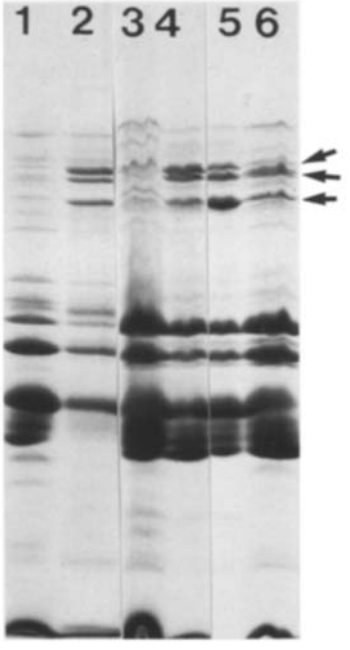

Fig. 1. Polyacrylamide gel electrophoresis of outer membranes of *Pseudomonas aeruginosa* M-2 (lanes 1, 3, 4) and the manganese resistent mutant H1762 (lanes 2, 5, 6). The membranes were prepared after growth on tryptone yeast-extract medium (lanes 1, 2), on minimal medium (lanes 4, 5) and on minimal medium with 62 μM iron added (lanes 3, 6). The *arrows* indicate the iron-regulated outer membrane proteins

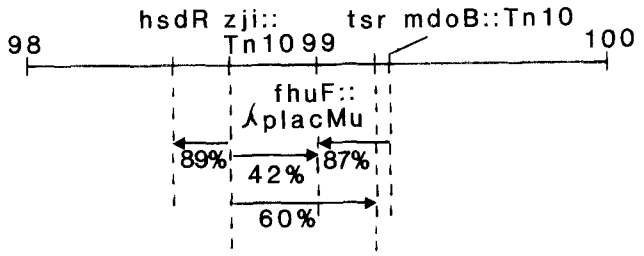

Fig. 2. Linkage of *fhuF* and nearby markers as determined by P1 transductions. Cotransduction frequencies are given in percent and the *arrow* indicates the unselected marker

Influence of manganese on iron regulated lac *fusions*

The selection of mutants derepressed in their iron uptake systems can be explained by the assumption that Mn^{2+} at high concentrations mimics Fe^{2+} and represses the iron uptake systems. To test this hypothesis manganese was added to strains with iron-regulated *lac* operon fusions.

For this test strain H1717 was used. It contained a fusion which was regulated like an iron uptake system. The fusion was found after λ*plac*Mu53 mutagenesis (Bremer et al. 1985) and screening of the mutants as described (Hantke 1983). The gene was named *fhuF* since the growth response to desferral on nutrient broth dipyridyl plates was specifically diminished. Also, the mutant was partially resistant to bacteriophage ∅80 (E. Fischer, unpublished observation).

The mutation was mapped by P1 transductions and the most probable gene order is *hsdR zji::*Tn*10 fhuF::*λ*plac*Mu *tsr mdoB::*Tn*10* (Fig. 2). The *lac* fusion produced high amounts of β-galactosidase under low iron stress.

The other strain used, H1645, contained a *lac* operon fusion which, in contrast to all iron-regulated genes hitherto studied, was induced rather than repressed by high concentrations of iron. The mutation was mapped near *his* by Hfr crosses but the exact position is unclear. Until now no phenotype of the mutant has been observed, and the gene locus has provisionally been named *ird* (*ir*on in*d*ucible). Strain H1645 *ird*::λ*plac*Mu produced low amounts of β-galactosidase under low iron growth conditions and high amounts when sufficient iron was supplied. It was not possible to induce the *lac* fusion in a Fur^- mutant of this strain by addition of iron.

In strain H1717 repression of β-galactosidase was observed after addition of manganese (Fig. 3). In the Fur^- derivative of this strain, SC11, no repression was observed. Interestingly production of β-galactosidase in H1645 increased after addition of manganese. As a control, induction of the chromosomal *lac* operon by isopropyl β-D-thiogalactoside was then carried out. The addition of 1 mM manganese had very little influence, indicating that the low Mn^{2+} concentration did not generally disturb cell metabolism. The manganese concentration used on the selection plates (10 mM) impaired the derepression, or synthesis, of β-galactosidase in W3110.

Fur not only affects iron transport

It has been observed that *fur* mutants are unable to grow on succinate (Hantke 1985). However, since the genes for succinate dehydrogenase (*sdh*, 16.7 min) and dicarboxylic acid transport (*dctB, cbt*, 16.4 min) map close to *fur* (15.5 min), double mutants could not be excluded. To determine whether or not *fur* also regulates the use of succinate, 49 *fur* mutants were tested for their ability to grow on succinate as sole carbon source. Two mutants showed growth, 8 showed weak growth and 39 were unable to grow on succinate plates. However, all of the 39 mutants transformed with a fur^+ plasmid were able to grow at a normal rate on succinate plates. This indicated that *fur* not only regulates iron uptake but also influences the utilization of succinate.

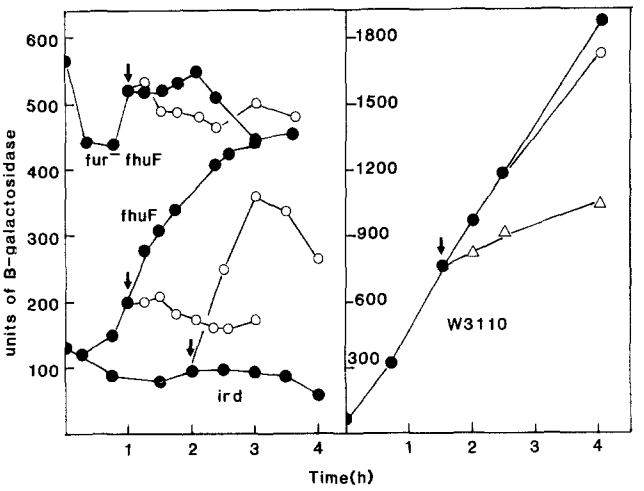

Fig. 3. Influence of Mn^{2+} on iron-regulated *lac* fusions. The strains H1717 *fhuF::*λ*plac*Mu, SC11 *fur fhuF::*λ*plac*Mu, H1645 *ird::*λ*plac*Mu and W3110 were pregrown overnight on 4 g tryptone and 2.5 g NaCl per litre. Cells were inoculated into the same medium with 20 μM desferral to derepress the iron-regulted genes and incubated at 37 °C. The W3110 culture medium contained, in addition, 1 mM isopropyl β-D-thiogalactoside to derepress the *lac* genes. At the times indicated by the *arrows*, 1 mM $MnCl_2$ was added to one half of the cultures. β-Galactosidase was measured in the cultures without Mn^{2+} (•) and with Mn^{2+} (○). To the W3110 culture, 10 mM Mn^{2+} was also added (△)

Phenotypic revertants of these *fur* mutants appeared after some days on succinate minimal plates. These revertants still overproduced the iron-regulated outer membrane proteins but these mutants have not been further characterized.

One of the mutants unable to grow on succinate, strain H1780 *fur*, was grown in liquid medium containing different carbon sources. The mutant consistently grew more slowly than the parent strain. In fact, on succinate, fumarate and acetate no growth was observed. The generation times in glucose-supplemented medium were 39 min for the parent strain compared with 66 min for H1780, with glycerol 87 min compared with 129 min, and with glycerol and succinate (as used in the transport experiment, see below) 86 min compared with 162 min. The faster growth rate of H1780 in glycerol alone (129 min per generation) compared with glycerol (0.4%) and succinate (0.2%) (162 min per generation) indicated that succinate had an inhibitory effect.

The transport of succinate was also measured. After a short incubation with acetate (3 h) to induce succinate uptake (Herbert and Guest 1971), the mutant exhibited only about 30% of the uptake rate of the parent strain (Table 2). In contrast to our expectations, after growth on glycerol and succinate, succinate uptake was reduced compared with uptake rates after growth on glycerol alone. In the Fur^- mutant, H1780, following growth on glycerol and succinate

Table 2. Uptake rates of succinate (nmol/min per milligram dry weight) and succinate dehydrogenase and NADH oxidase activities (nmol/min per milligram protein). The strains JB1698 and H1780 *fur* were incubated for 3 h on minimal medium with the carbon sources indicated, prior to being tested

Carbon source	Succinate transport		Succinate dehydrogenase		NADH oxidase	
Strain	JB1698	H1780	JB1698	H1780	JB1698	H1780
Succinate	6.3	2.1	97	14.7	896	373
Acetate	22.6	6.8	112	12.4	485	407
Glycerol + succinate	11.1	5.3	84	12.4	644	315
Glycerol	16.3	15.3	115	11.8	644	390

the uptake rate was 50% lower than that of JB1698. However, after growth on glycerol the uptake rates of both strains were nearly the same. This indicated that in Fur^- mutants the uptake of succinate is inhibited in the presence of acetate or succinate.

Succinate dehydrogenase was tested in cells grown under the same conditions. In the *fur* mutant only about 10% of the succinate dehydrogenase activity was observed compared with the parent strain. In contrast, NADH oxidase activity was diminished by a factor of two in the mutant strain.

Discussion

Screening for manganese-resistant mutants selected, with high incidence, mutants in the central regulatory gene of iron metabolism, *fur*.

P1 transductions, and transformations with fur^+ plasmids, showed that in all selected mutants the *fur* gene was affected. The only exception, H1776, showed a faint red colour in contrast to the strong red colour of the Fur mutants on the iron-rich MacConkey agar plates. This mutant may be partially constitutive for the enterochelin uptake system, although firm evidence for this is lacking.

The selection procedure also worked with other Enterobacteriaceae, indicating that in these strains a similar type of iron regulation may be found. In *Pseudomonas* many mutants could be isolated, but most of them seemed not to be changed in their iron regulation. The one mutant isolated with an altered iron regulation showed very slow growth on complex and minimal media. In *E. coli*, *fur* mutants are often unable to use succinate. Since succinate is a preferred carbon source of *P. aeruginosa*, such mutations may not be healthy or may even be lethal, perhaps because they disturb this central pathway.

The selective medium contains a relatively low concentration of Mg^{2+}. This is one of the reasons for the growth inhibition observed with Mn^{2+}, since addition of Mg^{2+} could restore growth (Silver et al. 1972). In the selective medium this inbalance between Mg^{2+} and Mn^{2+} leads to relatively high concentrations of Mn^{2+} inside the cell. It is astonishing that Mn^{2+} represses the iron transport systems and induces a positively regulated iron-dependent gene. From these observations it seems possible that manganese directly interacts with the Fur protein, thus leading to a repression of the iron transport systems. However, an indirect mechanism is also possible where iron is mobilized in the cell by Mn^{2+}, thus leading to a high concentration of free Fe^{2+} and to a repression of the iron transport systems. Whatever the mechanism of manganese action is, the constitutive mutants were not repressed by Mn^{2+} and this allowed the cells to grow.

The other mutants that were not *fur* have not been characterized further. They may be altered in their magnesium metabolism, which may lead to lower internal Mn^{2+} levels (Silver et al. 1972). The early observations that *fur* mutants were often unable to use succinate as carbon source (Hantke 1985) could be confirmed and were extended to fumarate and acetate which also could not be used. The complementation by the fur^+ plasmids indicates that it is the *fur* mutation that leads to the observed phenotype. In the *fur* mutants able to grow on succinate, perhaps only a domain of the Fur protein necessary for iron regulation was changed.

Succinate uptake after growth on glycerol was nearly the same in Fur^- and Fur^+ strains. However under the other growth conditions (succinate, succinate and glycerol, acetate) where an induction of the transport system was expected, a reduction of the succinate uptake rates was observed in the Fur^- strain. In addition, succinate dehydrogenase was assayed under the same growth conditions and in all cases very low activities were observed. This indicates that *fur* influences succinate uptake and metabolism.

Rainnie and Bragg (1973) reported that under low-iron growth conditions on succinate, the respiration of the cells was impaired, possibly because the iron for the respiratory chain with its cytochromes and non-haem iron was lacking. However, there may also be a regulatory signal via *fur* that somehow hinders the ability of the cell to synthesize proteins for the utilization of succinate. In Fur^- mutants this incorrect signal may hinder the cells' ability to grow on succinate.

Acknowledgements. I thank K. Maschke for her expert technical assistance, K. Poole and R. Harkness for reading the manuscript, and L. Zimmermann, E. Bremer and J. Brandt for strains. I am also very grateful to V. Braun for support and discussions. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 323).

References

Anwar H, Brown MRW, Day A, Weller PH (1984) Outer membrane antigens of mucoid *Pseudomonas aeruginosa* isolated directly from the sputum of a cystic fibrosis patient. FEMS Microbiol Lett 24:235–239

Bewick MA, Lo TCY (1979) Dicarboxylic acid transport in *Escherichia coli* K12: involvement of a binding protein in the translocation of dicarboxylic acids across the outer membrane of the cell envelope. Can J Biochem 57:653–661

Bremer E, Silhavy TS, Weinstock GM (1985) Transposable λ*plac*Mu bacteriophages for creating *lacZ* operon fusions and

kanamycin resistance insertions in *Escherichia coli*. J Bacteriol 162:1092–1099
Fiedler W, Rotering H (1985) Characterization of an *Escherichia coli mdoB* mutant strain unable to transfer *sn*-1-phosphoglycerol to membrane-derived-oligosaccharides. J Biol Chem 260:4799–4806
Hantke K (1981) Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol Gen Genet 182:288–292
Hantke K (1983) Identification of an iron uptake system specific for coprogen and rhodotorulic acid in *Escherichia coli*. Mol Gen Genet 191:301–306
Hantke K (1984) Cloning of the repressor protein gene of iron regulated systems in *Escherichia coli* K12. Mol Gen Genet 197:337–341
Hantke K (1985) Iron transport in bacteria. In: Spik G, Montreuil J, Crichton RR, Mazurier J (eds) Proteins of iron storage and transport in bacteria. Elsevier, Amsterdam, pp 231–243
Hedblom ML, Adler J (1980) Genetic and biochemical properties of *Escherichia coli* mutants with defects in serine chemotaxis. J Bacteriol 144:1048–1060
Herbert AA, Guest JR (1971) Two mutations affecting the utilization of C_4-dicarboxylic acids by *Escherichia coli*. J Gen Microbiol 63:151–162
Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
Montie TC, Doyle-Huntzinger D, Craven RC, Holder IA (1982) Loss of virulence associated with absence of flagellum in an isogenic mutant of *Pseudomonas aeruginosa* in the burned mouse model. Infect Immun 38:1296–1298
Osborn MJ, Gander JE, Parisi E, Carson J (1972) Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J Biol Chem 247:3962–3972
Rainnie DJ, Bragg PD (1973) The effect of iron deficiency on respiration and energy coupling in *Escherichia coli*. J Gen Microbiol 77:339–349
Schäffer S, Hantke K, Braun V (1985) Nucleotide sequence of the regulatory gene *fur*. Mol Gen Genet 200:110–113
Silver S, Johnseine P, Whitney E, Clark D (1972) Manganese-resistant mutants of *Escherichia coli*: Physiological and genetic studies. J Bacteriol 110:186–195
Vogel HJ, Bonner DM (1956) A convenient growth medium for *Escherichia coli* and some other micro-organisms. Microbiol Genet Bull 13:43–44

Communicated by J. Lengeler

Received February 25, 1987 / June 25, 1987

Note added in proof

A recent publication (de Lorenzo V, Wee S, Herrero M, Neilands JB (1987) J Bacteriol 169:2624–2630) gives further evidence that Mn^{2+} may substitute for Fe^{2+} with the Fur protein. In vitro Mn^{2+} dependent binding of the Fur protein to operator sequences of the iron regulated aerobactin operon was observed.