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## To cite this version:

Daniel Vinella, Christian Albrecht, Michael Cashel, Richard d'Ari. Iron limitation induces SpoT-dependent accumulation of ppGpp in Escherichia coli.. Molecular Microbiology, 2005, 56, pp.958-970. 10.1111/j.1365-2958.2005.04601.x . hal-00069437

**HAL Id: hal-00069437**

**https://hal.science/hal-00069437**

Submitted on 8 Jun 2006

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# Iron limitation induces SpoT-dependent accumulation of ppGpp in *Escherichia coli*

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Running Title: **Iron starvation and ppGpp synthesis**

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**Summary**

In *Escherichia coli* the β-lactam mecillinam specifically inhibits penicillin-binding protein 2 (PBP2), a peptidoglycan transpeptidase essential for maintaining rod-shape. We have previously shown that PBP2 inactivation results in a cell division block and that an increased concentration of the nucleotide ppGpp, effector of the RelA-dependent stringent response, confers mecillinam resistance and allows cells to divide as spheres in the absence of PBP2 activity. In this study we have characterized an insertion mutation which confers mecillinam resistance in wild type and Δ*relA* strains but not in Δ*relA* Δ*spoT* strains, devoid of ppGpp. The mutant has an insertion in the *fes* gene, coding for enterochelin esterase. This cytoplasmic enzyme hydrolyzes enterochelin-$Fe^{3+}$ complexes, making the scavenged iron available to the cells. We show that inactivation of the *fes* gene causes iron limitation on rich medium plates and a parallel SpoT-dependent increase of the ppGpp pool, as judged by the induction of the iron-regulated *fiu*::*lacZ* fusion and the repression of the stringently controlled $P1_{rrnB}$::*lacZ* fusion, respectively. We further show, by direct ppGpp assays, that iron starvation in liquid medium produces a SpoT-dependent increase of the ppGpp pool, strongly suggesting a role for iron in the balance of the two activities of SpoT, synthesis and hydrolysis of (p)ppGpp. Finally, we present evidence that ppGpp exerts direct or indirect positive control on iron uptake, suggesting a simple homeostatic regulatory circuit: iron limitation leads to an increased ppGpp pool, which increases the expression of iron uptake genes, thereby alleviating the limitation.

## Introduction

The Gram negative bacterium *Escherichia coli* has evolved various adaptive mechanisms which enable it to respond rapidly to a large number of changes in its growth conditions. The stringent response is one such mechanism. In *relA*$^+$ cultures starved for a required amino acid the transcription of ribosomal and transfer RNA genes is severely limited and accumulation of stable RNA decreases markedly (Stent and Brenner, 1961; Cashel *et al*., 1996). This response depends on the ribosome-associated RelA protein, which, in the presence of an uncharged cognate tRNA in the ribosomal A site, pyrophosphorylates GTP to produce pppGpp, which is rapidly converted to ppGpp [guanosine 3′-pyrophosphate 5′-pyrophosphate (Wendrich *et al*., 2002)]. Co-crystallization studies have revealed that the nucleotide ppGpp binds RNA polymerase near the catalytic center in the secondary channel (Artsimovitch *et al*., 2004). Its binding is proposed to be stabilized by DksA, a GreA/GreB-like protein thought to stabilize bound ppGpp by weakening initiating NTP binding and destabilizing open promoter complexes (Perederina *et al*., 2004; Paul *et al*., 2004).

The ppGpp pool increases not only during amino acid starvation (the stringent response) but also after a number of other stresses, including starvation for carbon, nitrogen, phosphate or fatty acids (Murray and Bremer, 1996; Seyfzadeh *et al*., 1993; Spira *et al*., 1995; Villadsen and Michelsen, 1977). In most if not all cases, this increase is independent of RelA. It depends on the SpoT protein, which both synthesizes and hydrolyzes (p)ppGpp, and it is brought about by an altered balance of the SpoT synthetic and hydrolytic activities, even in the absence of RelA. The signal or regulator to which SpoT responds during SpoT-dependent ppGpp accumulation after various stresses remains unknown (see Cashel *et al*., 1996, for review). In the complete absence of SpoT, ppGpp will accumulate to levels that block ribosome synthesis and prevent cell growth when the RelA protein is present; Δ*relA* Δ*spoT* strains are viable and have no detectable ppGpp (Xiao *et al*., 1991).

In *E. coli* the β-lactam antibiotic mecillinam (Lund and Tybring, 1972) specifically inactivates penicillin-binding protein 2 (PBP2), a peptidoglycan transpeptidase essential for the maintenance of rod shape and cell elongation (Spratt, 1975; Spratt and Pardee, 1975;

Spratt, 1977). When PBP2 is inactivated, either genetically or by mecillinam, the cells become spherical and, in the case of wild type strains in rich media, they die (Aono *et al*., 1979). We have shown that death results from a failure of the cocci to divide (Vinella *et al*., 1993), although PBP2 is not involved in the process of cell division. We further showed that division can be restored in the absence of PBP2 activity by a moderate increase of the ppGpp pool (Vinella *et al*., 1992; Joseleau-Petit *et al*., 1994). Under these conditions, the cells are resistant to mecillinam, growing and dividing as spheres. This seemed to provide a convenient selection for identifying regulators of SpoT activity. We therefore selected mecillinam resistant mutants of a Δ*relA* strain, able to grow in rich medium in the presence of the antibiotic. We report here the characterization of one such mutant obtained by random insertion mutagenesis. The mutant has an increased ppGpp pool. Molecular analysis shows that it is affected in iron uptake; the results suggest the involvement of iron in the regulation of SpoT activity.

Iron is an essential element, but in ærobic environments its acquisition poses a problem since ferrous ions are readily oxidized to ferric ions and ferric salts are generally insoluble at neutral pH. To solve this problem, bacteria have evolved several mechanisms of iron uptake. The most efficient involves siderophores, small molecules with extraordinarily high affinity for ferric ions (see Earhart, 1996, for review). The *E. coli* siderophore enterochelin (or enterobactin) is synthesized in the cytoplasm by the *ent* gene products and secreted into the medium, where it scavenges ferric ions. The Ent-$Fe^{3+}$ complex is then brought back into the cell via a specific uptake system (Fep). It is hydrolyzed by the product of the *fes* gene, enterochelin esterase, releasing the iron in the cytoplasm where it is reduced to $Fe^{2+}$ (Earhart, 1996).

Other iron uptake systems of *E. coli* (Earhart, 1996) include the use of exogenous citrate, which can chelate $Fe^{3+}$ ions and deliver them to a specific membrane transport system (the Fec system); a permease for $Fe^{2+}$ ions (Feo); and specific transport systems for siderophores synthesized by other microorganisms (Fhu, Iut). The regulation of iron uptake is under negative control by the Fur protein, which, in the presence of its corepresssor $Fe^{2+}$, represses the transcription of all genes involved in iron metabolism; Fur-$Fe^{2+}$ also represses

the expression of the small RNA RyhB (Masse and Gottesman, 2002; Masse *et al*., 2002), a translational repressor of genes coding for a number of iron-containing and iron storage proteins (Geissmann and Touati, 2004).

In the present work we analyze the connection between iron starvation and ppGpp levels. Our results indicate that iron limitation causes SpoT-dependent ppGpp accumulation, which in turn stimulates the cell's capacity to take up iron. This negative feedback loop provides homeostatic regulation of the internal iron concentration, mediated by the SpoT protein and the nucleotide ppGpp.

## Results

*Selection and characterization of a new mecillinam resistant mutant*

A simple selection for mecillinam resistant insertion mutants was described previously (Vinella *et al*., 2000). It was carried out with a mini-Tn*10*($Cm^R$) element in the Δ*relA* Δ*lacZ* $P1_{rrnB}$::*lacZ* strain CF6301. One of the mutants so isolated was *mcr-14*::*cat.* We backtransduced the $Cm^R$ determinant into strain CF6301 and verified that it was stable and that chloramphenicol and mecillinam resistance were 100% cotransducible (Vinella *et al*., 2000), showing that the mecillinam resistant phenotype is indeed due to the mini-Tn*10*($Cm^R$) insertion. On LB plates the *mcr-14*::*cat* mutant cells grow and divide as spheres in the presence of mecillinam, as rods in its absence.

The ppGpp pool, normally low in rich media, was deduced to be increased in the *mcr-14*::*cat* mutant, as evaluated by the $P1_{rrnB}$::*lacZ* fusion. This promoter of the ribosomal RNA operon *rrnB* is under negative control by ppGpp. It had lower expression in the *mcr-14*::*cat* mutant than in the parental strain, as evidenced by a pale blue coloration of the colonies on LB plates containing X-Gal compared to darker blue for the parent (Vinella *et al*., 2000). Furthermore, unlike mecillinam resistant mutants affected in aminoacyl-tRNA synthetases (Vinella *et al*., 1992), the decreased expression of $P1_{rrnB}$::*lacZ* in the *mcr-14*::*cat* mutant was RelA-independent since strain CF6301 is Δ*relA*. This suggested that SpoT activity was altered in the mutant or, alternatively, that the mutant cells were more sensitive then the parental strain to a given concentration of ppGpp.

In addition to its mecillinam resistance and decreased $P1_{rrnB}$::*lacZ* expression, the *mcr-14*::*cat* mutant had several other phenotypes. On rich medium plates (LB or LB*, *cf.* Experimental Procedures) it formed smaller colonies than the parental strain, these colonies were pinkish-brown in color, and on M9 glucose plates it could barely grow, forming tiny colonies visible only after 3 days of incubation at 37°C and unable to grow further (Table 1). As described below, the *mcr-14*::*cat* mutation also increased the expression of a *fiu*::*lacZ* fusion, indicating iron limitation (Table 2B).

The *mcr-14*::*cat* mutation, transduced into the isogenic *relA*$^+$ strain DV352, conferred a similar phenotype: mecillinam resistance on LB* plates, lowered expression of the P1$_{rrnB}$::*lacZ* fusion on LB X-Gal plates, small pink colonies on LB plates and near absence of growth on M9 glucose plates.

*Identification of the* mcr-14::cat *mutation as a* fes::cat *allele*

To localize the mini-Tn*10*(Cm$^R$) element present in the *mcr-14*::*cat* mutant, we cloned the *cat* gene and flanking chromosomal DNA in the vector pKS+(Amp$^R$) (see Experimental Procedures). From an Amp$^R$ Cm$^R$ transformant we extracted the plasmid DNA and sequenced it using the CmD and CmF primers. The insertion was found to interrupt the 359th codon (of 375) of the *fes* gene, coding for enterochelin esterase. The *mcr-14*::*cat* mutation was therefore renamed *fes*::*cat*.

The *fes* gene, coding for enterochelin esterase, is the first in an operon including the *entF* and *fepE* genes, whose products are involved in enterochelin synthesis and the transport into the cells of the Ent-Fe$^{3+}$ complex, respectively (Earhart, 1996). To determine whether the phenotypes of the *fes*::*cat* mutant were due to inactivation of the *fes* gene itself or to polar effects, we cloned just the wild type *fes* gene with its natural promoter in the vectors pBAD18(Km$^R$) and pKS+(Amp$^R$) (see Experimental Procedures), producing pDV51 and pDV52, respectively. We transformed the mecillinam resistant strain DV377 (*fes*::*cat relA1*) with pDV51 and showed that it became mecillinam sensitive on LB* plates. When pDV52 was introduced into strains CF6301 (*fes*::*cat* Δ*relA* P1$_{rrnB}$::*lacZ* Δ*lacZ*) and DV310 (*fes*::*cat* Δ*relA fiu*::*lacZ* Δ*lacZ*), it restored normal colony size on M9 glucose, LB and LB* plates and eliminated the pink coloration of the colonies on LB and LB* plates. The plasmid also restored a normal level of expression of the P1$_{rrnB}$::*lacZ* and *fiu*::*lacZ* fusions on LB and LB* plates containing X-Gal, as judged by the intensity of the blue coloration. In conclusion, all phenotypes of the *fes*::*cat* mutant were due to inactivation of the *fes* gene, not to polar effects on *entF* or *fepE*.

*Genetic and physiological analysis*

It seemed possible that the slow growth (small colony size) of the *fes*::*cat* mutant on rich medium plates reflected iron limitation, since the mutant is unable to use the enterochelin system, which is the cell's most efficient pathway of iron acquisition. We therefore looked at the effect on colony size of additives that increase iron availability. Indeed, citrate and ascorbate increased colony size on LB and LB* plates (*cf*. Experimental Procedures); citrate activates the Fec system of iron uptake and ascorbate reduces ferric ions to $Fe^{2+}$, which can then enter the cell via the Feo permease (Earhart, 1996). In the presence of ascorbate, a complete parental phenotype was restored (Table 1). The addition of $FeCl_3$ (0.1 mM) also restored a parental phenotype; in contrast, the addition of $MgSO_4$, $MnCl_2$, $ZnSO_4$, $CuSO_4$ or $CoCl_2$ (each 10 mM) did not improve the growth of the mutant on LB plates (data not shown).

These results are consistent with the hypothesis of iron limitation of the *fes*::*cat* mutant on rich medium plates. We next looked at the phenotypic effects of other mutations affecting iron uptake. The FepB protein specifically binds the Ent-$Fe^{3+}$ complex in the periplasm, after it has crossed the outer membrane, and presents it to an inner membrane transporter that takes it into the cytoplasm; the FepC protein is one component of this transporter (Earhart, 1996). The *fepB* and *fepC* single mutants behaved similarly to the *fes*::*cat* mutant on rich medium and M9 glucose plates (Table 1), except that their colonies on LB plates, although small, lacked the pink coloration. The *fes*::*cat fepB* and *fes*::*cat fepC* double mutants were also small but of normal color on LB and LB* plates. The pink coloration thus seems to require entry of the Ent-$Fe^{3+}$ complex into the cytoplasm; this may reflect oxidation of periplasmic enterochelin in the *fep* mutants by CueO (Grass *et al*., 2004). The phenotypes of *fepB* and *fepC* strains, like those of the *fes*::*cat* mutant, were suppressed by $FeCl_3$ (but not by other metal ions) and by ascorbate and citrate, the latter two being more effective in rich media.

The *entB* single mutant, specifically blocked in enterochelin synthesis, formed large colonies nearly identical to those formed by the parental strain on both rich medium and M9 glucose plates, indicating that, in the absence of enterochelin, alternative uptake systems are able to provide enough iron to support normal growth. Interestingly, the *entB* mutation is epistatic to the *fes*::*cat* mutation: the *fes*::*cat entB* double mutant formed colonies of almost

normal size and normal coloration on LB, LB* and M9 glucose plates (Table 1 and data not shown). Similar epistasis was observed on rich medium plates when the *entB* mutation was replaced by an *aroA*::Tn*10* allele, which blocks enterochelin synthesis upstream of chorismic acid synthesis (data not shown). This suppression is explained below.

All the above genes (except *aroA*) are repressed by the Fur protein when the iron supply is sufficient and derepressed when iron is limiting. Consistent with this, introduction of a *fur* mutation into the above strains had little effect on colony size for all strains on all plates (Table 1).

It thus appears that enterochelin, when it is synthesized but cannot be used by the cells, as in the *fes* and *fep* mutants, causes slow growth on rich plates and near absence of growth on M9 glucose plates, which have a low iron concentration; this slow growth is not observed in the absence of enterochelin synthesis. The presence of secreted enterochelin around the colonies probably reduces the efficiency of alternative iron uptake systems (see below). In addition, the *fes*::*cat* (*ent*$^+$) mutant accumulates a substance – most likely cytoplasmic Ent-$Fe^{3+}$ – which confers a pink color on the colonies. Suppression of the *fes*::*cat* mutant phenotypes by increasing the iron concentration or activating alternative iron uptake pathways could reflect repression of enterochelin synthesis under these conditions, thereby eliminating its inhibitory effect on the growth of the *fes* and *fep* mutants.

Our results can be explained by a double hypothesis: first, that the *fes* mutation causes iron limitation during growth on rich medium plates, and second, that iron limitation causes SpoT-dependent ppGpp accumulation, resulting in mecillinam resistance. The experiments below were designed to test these ideas.

*Iron limitation and increased ppGpp levels in the* fes::cat *mutant: gene fusion studies*

We first wished to quantify the growth rates of our strains and their levels of expression of the $P1_{rrnB}$::*lacZ* and *fiu*::*lacZ* fusions, used to estimate ppGpp levels and iron availability, respectively. Surprisingly, however, when we grew the *fes*::*cat* mutant strains in liquid LB or LB*, they all had a generation time of 19-20 minutes, similar to the parental strains CF6301 and DV310, and there was no further improvement of their growth by the addition of citrate, ascorbate or $FeCl_3$ (data not shown; *cf.* below and Discussion).

Expression of the two *lacZ* fusions was strongly affected in the *fes* mutant when grown on LB or LB* plates supplemented with X-Gal. $P1_{rrnB}$::*lacZ* was expressed at a lower level than in the parental strain (pale blue colonies), indicating an increased pool of ppGpp, and *fiu*::*lacZ* expression was higher than in the parental strain (dark blue colonies), indicating iron limitation. However, when grown in liquid LB or LB*, the *fes* mutant strains exhibited the same level of expression as the parental strains for the two fusions (data not shown). Since the *fes*::*cat* mutant had a clear growth impairment and was strongly affected in the expression of the two fusions when grown on solid media, we carried out our β-galactosidase assays on bacteria recovered directly from colonies on plates (*cf.* Experimental Procedures).

Using these two transcriptional fusions and assaying the β-galactosidase activity in colonies picked from plates, we quantified the degree of iron limitation and the ppGpp increase in the *fes*::*cat* mutant. Expression of the $P1_{rrnB}$::*lacZ* fusion is reduced fourfold on LB plates (Table 2A), indicating a substantially higher ppGpp pool in the *fes*::*cat* mutant than in the parental strain. Expression of the *fiu*::*lacZ* fusion is increased more than tenfold in the mutant on both LB and LB* plates (Table 2B and data not shown), indicating iron limitation.

Mutants blocked in enterochelin synthesis (*entB* and *fes*::*cat entB*) or in the entry of the Ent-$Fe^{3+}$ complex (*fepB*, *fepC*, *fes*::*cat fepB* and *fes*::*cat fepC*) were as strongly derepressed as *fes*::*cat* for *fiu*::*lacZ* expression on LB and LB* plates (Table 2B and data not shown). Furthermore, this seems to be the maximum level since inactivation of the Fur repressor did not increase it. Thus all mutants lacking a functional enterochelin system are iron limited when growing on LB or LB* plates. Even the *entB* and *fes*::*cat entB* strains, which seem to have a normal growth rate as judged by colony size, are derepressed for the Fur regulon, indicating that the alternative iron uptake systems are barely able to maintain rapid growth on these plates.

The *fepB* and *fepC* mutants were also repressed for $P1_{rrnB}$::*lacZ* expression, approximately to the same extent as *fes*::*cat* (Table 2A), indicating a swollen ppGpp pool during growth on LB plates. Repression of the $P1_{rrnB}$::*lacZ* fusion does not depend on the Fur protein because it was also observed in the *fur fes*::*cat*, *fur fepB* and *fur fepC* double mutants (Table 2A and data not shown). The *entB* mutant, in contrast, had a near normal level of

P1$_{rrnB}$::*lacZ* expression, as did the *fes*::*cat entB* double mutant (Table 2A). Thus these latter strains, although iron limited, seem to have a normal ppGpp pool, in keeping with their apparently normal growth rate on LB plates. The swelling of the ppGpp pool thus seems to occur when enterochelin is synthesized and secreted into the solid medium but the Ent-$Fe^{3+}$ complex cannot be utilized by the cells.

We next quantified the effect on the fusions of the addition of citrate, $FeCl_3$ or ascorbate to LB and LB* plates. For the *fes*::*cat*, *fepB* and *fepC* single mutants, these additions restored an almost normal level of expression of the P1$_{rrnB}$::*lacZ* fusion (Table 2A) and partially (citrate) or totally (ascorbate) suppressed iron limitation as estimated from the expression of the *fiu*::*lacZ* fusion (Table 2B).

The observed RelA-independent increase in the ppGpp pools was confirmed to be SpoT-dependent. Introduction into strain DV301 (*fes*::*cat* Δ*relA* P1$_{rrnB}$::*lacZ* Δ*lacZ*) of a Δ*spoT* deletion, which eliminates ppGpp, increased the colony size and restored high P1$_{rrnB}$::*lacZ* expression (Table 2A). This confirmed that the low level of P1$_{rrnB}$::*lacZ* expression observed in the *fes*::*cat* strains was indeed due to an increased ppGpp pool. In contrast, the Δ*spoT* mutation did not prevent derepression of *fiu*::*lacZ* expression in *fes*::*cat* strains (Table 2B).

*Iron starvation, ppGpp synthesis and mecillinam resistance*

The *fes*::*cat* mutant was originally selected for its mecillinam resistant phenotype on LB plates. Analysis of its overall phenotype led us to hypothesize that it is iron limited and that iron limitation results in SpoT-dependent ppGpp synthesis and hence mecillinam resistance. The data presented above confirm these speculations. In the course of our analysis, we observed that other mutants affected in iron acquisition are also iron limited on LB or LB* plates (*cf.* Table 2B). We therefore investigated their plating efficiency in the presence of mecillinam.

No single mutant other than *fes*::*cat* was mecillinam resistant on LB or LB* plates. Even the *fepB* and *fepC* mutants were sensitive (Table 3 and data not shown), although our results with the *fiu*::*lacZ* and P1$_{rrnB}$::*lacZ* fusions indicate that they are iron limited and have a higher ppGpp pool than the parental strain (Table 2). Moreover, the *fes*::*cat fepB* and

*fes*::*cat fepC* double mutants had only 1% plating efficiency in the presence of mecillinam, compared to over 50% for the *fes*::*cat* single mutant, indicating partial suppression of the *fes*::*cat* mutation in the absence of uptake of the Ent-$Fe^{3+}$ complex. The *entB* mutant, which did not show repression of $P1_{rrnB}$::*lacZ* (Table 2A), was also mecillinam sensitive on LB* plates and, in addition, the *entB* mutation completely suppressed the mecillinam resistance of *fes*::*cat*, showing that the synthesis of enterochelin is actually required for this resistance, as it is for the pink coloration of the colonies, slow growth, iron limitation and ppGpp accumulation. Inactivation of the *fur* gene did not confer mecillinam resistance, indicating that derepression of all genes involved in iron uptake is not in itself sufficient to confer this phenotype. Finally, as expected, the *fes*::*cat* mutation failed to confer mecillinam resistance in the absence of ppGpp, *i.e.*, when *spoT* is deleted (Table 3).

The presence of ascorbate (10 mM) or $FeCl_3$ (0.1 mM) completely abolished the mecillinam resistance of the *fes*::*cat* mutant, whereas the addition of $MgSO_4$, $MnCl_2$, $ZnSO_4$, $CuSO_4$ or $CoCl_2$ (each 10 mM) did not (Table 3 and data not shown). The presence of citrate (10 mM) did not suppress the mecillinam resistance of the *fes*::*cat* mutant on LB* mecillinam plates, although it decreased the colony size; however, citrate abolished the partial resistance of the *fes*::*cat fepB* and *fes*::*cat fepC* double mutants (Table 3 and data not shown). We conclude that reestablishment of an adequate iron supply suppresses the mecillinam resistance of the *fes*::*cat* mutant and that this is accomplished efficiently by adding ascorbate or iron to the medium but only partially by adding citrate.

*Effect of iron chelation*

The presence of chemical chelators can considerably reduce the availability of iron. Addition of the iron chelator dipyridyl (0.25 mM) to plates had several effects. First, it improved the partial mecillinam resistance of the *fes*::*cat* mutant in classical LB, although it did not confer resistance on any of the other strains (Tables 3 and 4). Second, when dipyridyl was present in M9 glucose Casaminoacids plates, it prevented all mutants affected in enterochelin metabolism from forming colonies, although the parental strain grew well on this medium (Table 4). Third, unexpectedly, when present in classical LB plates, dipyridyl prevented the growth of the *fes*::*cat entB*$^{\pm}$ mutants completely lacking ppGpp (Δ*relA* Δ*spoT*), although all

the other strains were able to form colonies (Table 4). These two strains were also the only ones unable to grow on M9 glucose Casaminoacids plates in the absence of dipyridyl. Finally, the *fes*$^+$ ppGpp deficient strain, although able to grow in the presence of dipyridyl on both LB and M9 glucose Casaminoacids plates, formed smaller colonies in its presence than its Δ*relA spoT*$^+$ parent (Table 4). These observations show that the enterochelin system is required for growth on M9 glucose Casaminoacids dipyridyl plates and provide a clue that ppGpp somehow activates enterochelin synthesis (see below). Overall, these results are also taken to show that SpoT responds to iron limitation, causing ppGpp accumulation by increasing the ratio of (p)ppGpp synthesis to hydrolysis.

*Stimulatory effect of ppGpp on iron accumulation systems*

On M9 glucose Casaminoacids plates containing dipyridyl, the enterochelin system is absolutely required for colony formation: the parental strain grows well whereas no growth is observed with all mutants lacking this pathway (*fes*::*cat*, *fep*, *entB*, *fes*::*cat fep*, *fes*::*cat entB*; Table 4). On these plates a strain with an intact enterochelin system but lacking ppGpp (Δ*relA* Δ*spoT*) is severely handicapped, forming microcolonies visible only after two days' incubation. This suggests that the level of enterochelin expression is considerably lower in the absence of ppGpp than in its presence. We confirmed this hypothesis by the use of CAS plates containing chrome azurol S, glucose and Casaminoacids. On these plates, secreted enterochelin forms a halo surrounding the colonies, the diameter of which is a measure of the quantity secreted. As seen in Fig. 1, in the absence of ppGpp the halo is much smaller than in its presence, in both *fur*$^+$ and *fur* strains, confirming the predicted positive effect of ppGpp on enterochelin production and further showing that this regulation is Fur-independent.

Our data also suggest a stimulatory role of ppGpp on the expression of one or more alternative (non-enterochelin) iron uptake systems. This is seen by the growth patterns on M9 glucose Casaminoacids without dipyridyl and on LB plates containing dipyridyl. On these plates the enterochelin system is not required, as evidenced by growth of the *fes*::*cat*, *fep* and *entB* mutants (Table 4); the alternative systems work efficiently. In the absence of ppGpp, however, the *fes*::*cat* (Δ*relA* Δ*spoT*) and *fes*::*cat entB* (Δ*relA* Δ*spoT*) strains are no longer able

to form colonies (Table 4), strongly suggesting lower expression of one or more alternative systems of iron acquisition in the absence of ppGpp.

*Assays of ppGpp in liquid culture*

We next wished to quantify the SpoT-dependent increase in the ppGpp pool during iron limitation. Unlike the β-galactosidase assay, our ppGpp assay, involving $^{32}P$, cannot be carried out on bacteria recovered from colonies. We therefore looked for conditions causing iron limitation in liquid medium, first exploiting the iron chelator dipyridyl. In MOPS medium supplemented with glucose, all 20 amino acids and $FeS0_4$ (1 μM), the presence of 0.25 mM dipyridyl did not affect the growth of the Δ*relA* parental strains. However, the growth of the *fes*::*cat*, *fep* and *entB* mutants was inhibited about 60 minutes after the addition of the chelator. Under these conditions there was rapid induction of the *fiu*::*lacZ* fusion, showing iron starvation, and slower repression of the $P1_{rrnB}$::*lacZ* fusion, indicative of an increase in the ppGpp pool (data not shown); since this repression was observed in the *entB* mutant, it cannot be due to the accumulation of unusable Ent-$Fe^{3+}$ complexes; we presume it is a direct result of iron limitation.

Using strain DV301 (*fes*::*cat* Δ*relA* ), we assayed the ppGpp pool at different times after the addition of dipyridyl. In our medium containing 0.3 mM phosphate, iron was limiting instead of phosphate when added at concentrations lower than 0.5 μM. In exponential phase in the presence of excess $FeSO_4$ (1 μM), the generation time of the strain was 45 min, the ppGpp concentration was 4 pmol $ml^{-1}$ $OD^{-1}$ and the ppGpp/(ppGpp + GTP) ratio was less than 0.005; the ratio remained at this low level until phosphate became limiting in the medium (Fig. 2). After addition of the chelator, as growth slowed down we observed a slow but regular increase of the ppGpp pool (Fig. 2). Five hours after the addition of dipyridyl, the ppGpp concentration had increased about 10-fold and the ppGpp/(ppGpp + GTP) ratio reached 0.073 (and 0.079 after 6 h). These observations show that the *fes*::*cat* mutation can indeed cause an increased ppGpp pool, as indicated by the $P1_{rrnB}$::*lacZ* fusion, making it unlikely that the mutant is more sensitive then the parental strain to a given concentration of ppGpp.

We next followed the evolution of the ppGpp pool during iron depletion without the addition of a chelator, simply by growing the strain in a medium to which no iron had been added. We again observed a continuous increase of the ppGpp pool as the iron was depleted and growth slowed down (Fig. 3A). The addition at this point of $5\times10^{-5}$ M $FeSO_4$ led to a rapid drop of the ppGpp pool to barely detectable levels, with the ppGpp/(ppGpp + GTP) ratio falling from about 0.040 to less than 0.005. This drop took less than 30 min and was complete even before growth had resumed.

When the cells were incubated overnight in medium without added iron, the ppGpp/(ppGpp + GTP) ratio was even higher, about 0.100 (Fig. 3B). Under these conditions of prolonged starvation, the culture never regained its maximal growth rate after iron readdition. The addition of iron nevertheless led to a rapid drop in the ratio, reaching 0.025 in less than 30 min (Fig. 3B).

These results show conclusively that during iron limitation, there is a RelA-independent SpoT-dependent accumulation of ppGpp in *E. coli*.

## Discussion

The level of the regulatory nucleotide ppGpp is elevated during many different physiological stresses. During amino acid starvation the RelA protein is the principal synthetase but during carbon starvation and most other stresses leading to ppGpp accumulation, the SpoT protein is responsible for the increase. The opposing activities of this bifunctional enzyme apparently are coordinately regulated by substrate induced conformational antagonism (Hogg *et al*., 2004; Mechold *et al*., 2002), but it is not known how stress mediates changes in the ratio of the synthetase to hydrolase activities of SpoT to bring about ppGpp accumulation. Our previous work established that an expanded ppGpp pool in *E. coli* allows it to grow and divide in rich medium in the absence of a functional PBP2, whether the inactivation is by mutation or by the highly specific β-lactam mecillinam. We therefore thought that, starting with a Δ*relA* strain, a selection for mecillinam resistant derivatives might produce mutants affected in a putative regulator of SpoT activity, causing a higher ppGpp pool.

In the present work we describe a mecillinam resistant mutant carrying a *fes*::*cat* mutation, inactivating enterochelin esterase. The characterization of this strain showed that it is iron limited when growing on rich medium plates, and under these conditions its ppGpp pool is high and SpoT-dependent. This led us to hypothesize that iron starvation leads to SpoT-dependent ppGpp accumulation. We confirmed our hypothesis by direct ppGpp measurements on cultures during iron starvation, caused either by the presence of the iron chelator dipyridyl or through iron depletion by the bacteria.

The Fur protein is the general repressor of genes involved in iron metabolism; repression is relieved when the corepressor $Fe^{2+}$ is scarce. However, Fur is not involved in the detection of iron limitation by SpoT, since the *fes*::*cat* mutation causes the same ppGpp increase independently of the presence or absence of the Fur protein, as judged by the level of expression of the $P1_{rrnB}$::*lacZ* fusion. Conversely, a *fur* mutant, with high expression of all iron related genes even in the presence of excess iron, has an essentially normal ppGpp pool as judged by the same fusion (Table 2). Thus the SpoT protein must detect iron limitation through some as yet undefined element, possibly the internal concentration of $Fe^{2+}$ or $Fe^{3+}$.

All mutants affected in the enterochelin pathway are iron limited on LB plates, whether the block is in biosynthesis (*entB*), uptake of the Ent-$Fe^{3+}$ complex (*fepB*, *fepC*), or release of the iron in the cytoplasm (*fes*::*cat*); in all cases, the *fiu*::*lacZ* fusion was completely derepressed (Table 2). However, the severity of the iron limitation does not seem to be the same for the different mutants, as judged by colony size on LB plates; according to this criterion, the *fes*::*cat* mutant is more strongly limited than the *fep* mutant, which is more limited than the *entB* mutant. The discrepancy between colony size and fusion expression is presumably because the *fiu*::*lacZ* fusion is simply too sensitive, being fully induced even during mild iron limitation.

It is striking that iron limitation is observed only on plates, not in LB or M9 glucose Casaminoacids liquid culture. A likely explanation for this apparent paradox has to do with the fate of the secreted enterochelin, which traps iron in the medium. If the enterochelin remains near the bacteria that produced it, as would be the case on plates, this would deplete iron locally; the cell's alternative pathways of iron acquisition cannot compete with enterochelin, so that mutants like *fes* and *fep*, unable to recover iron from the Ent-$Fe^{3+}$ complexes, would be iron limited. Furthermore, the resulting iron limitation would aggravate the situation further since it would stimulate the cells to produce even more enterochelin; indeed, *fes* and *fep* mutants have been shown to secrete large quantities of enterochelin (Langman *et al*., 1972). In liquid LB culture, in contrast, the secreted enterochelin is dispersed throughout the culture and does not reach a concentration sufficient to titrate out all iron in the medium, so iron is not limiting. In *entB* mutants, of course, there can be no local depletion on plates since no enterochelin is made; the large colony size of *entB* strains shows that alternative iron uptake pathways readily provide sufficient iron if it is not trapped by secreted enterochelin. Thus the *entB* mutation relieves the severe iron limitation caused (on plates) by the *fes*::*cat* mutation, allowing faster growth, abolishing the increase in the ppGpp pool and suppressing mecillinam resistance.

The *fepB* and *fepC* strains, like the *fes*::*cat* mutant, are iron limited on LB plates (small colonies, high *fiu*::*lacZ* expression) and have a high ppGpp pool (low $P1_{rrnB}$::*lacZ* expression) (Table 2), yet they are not mecillinam resistant (Table 4). We do not have an

explanation for this observation. It is conceivable that mecillinam resistance is somehow favoured by the presence of unhydrolyzed Ent-$Fe^{3+}$ complexes in the cytoplasm, or discouraged by their presence in the periplasm. Alternatively, it is possible that CueO-dependent oxidation of the enterochelin moiety of Ent-$Fe3^{+}$ complexes in the periplasm releases a small amount of ferric ions that the cells can use. Any of these hypotheses would explain the partial epistasis of the *fepB* mutation over *fes*::*cat* (*cf.* Table 3).

SpoT-dependent ppGpp accumulation has been observed after many different stresses (Cashel *et al*., 1996), but in no case do we know the nature of the signal to which SpoT responds. One speculative scenario draws on the suggestion that SpoT activity responds to the level of uncharged tRNA in the cell (Murray and Bremer, 1996). It is known that iron starvation leads to undermodification of several tRNAs (Björk, 1996); if this results in a lower charge ratio for some species, it might generate a signal that increases the synthesis to hydrolysis ratio of SpoT. Alternatively, one could imagine that iron limitation reduces cytochrome production, thereby reducing respiration and ATP generation and possibly triggering SpoT-dependent ppGpp accumulation, as during carbon starvation.

Our data show that ppGpp stimulates the expression of the enterochelin system and strongly suggest that it stimulates the expression of at least one alternative (non-enterochelin) iron uptake system. Although we have not identified specific targets responsible for this stimulation, it is interesting to note that expression of the *fiu*::*lacZ* fusion, completely derepressed in the *fes*::*cat* Δ*relA* and *fur* Δ*relA* mutants (360 and 370 units, respectively; Table 2B), is significantly reduced in the absence of ppGpp (190 and 240 units; Table 2B). This indicates a positive, Fur-independent effect of ppGpp on the Fur-regulated *fiu* promoter.

These observations imply the existence of a homeostatic negative feedback loop governing iron acquisition: iron limitation stimulates SpoT-dependent ppGpp accumulation, ppGpp stimulates the synthesis of iron uptake systems, and these in turn help relieve the iron limitation.

The role of iron in bacterial pathogenicity is well documented (see (Ratledge and Dover, 2000) for review). In certain cases, iron-acquisition systems have been shown to be important for virulence whereas, in other cases, iron starvation increases the expression of virulence

determinants and toxins. The nucleotide ppGpp has also been shown to be involved in virulence. *Salmonella* strains devoid of ppGpp have been shown to be defective in expression of a number of virulence genes (Pizarro-Cerda and Tedin, 2004), and the gene *hilA*, coding for the central virulence regulator, absolutely requires ppGpp in order to be expressed, both on entry into stationary phase and during carbon starvation (Song *et al*., 2004). It will be interesting to determine whether ppGpp is involved in the regulation of siderophore synthesis in pathogenic bacteria and whether it is required for the increased expression of virulence genes caused by iron starvation. It is interesting to note that Merrell *et al.* (Merrell *et al*., 2003), studying the effect of iron starvation in the pathogen *Helicobacter pylori*, observed that it caused the repression of the synthesis of ribosomal proteins and the derepression of genes involved in aminoacid biosynthesis. *H. pylori* does not accumulate ppGpp in response to aminoacid starvation (Scoarughi *et al*., 1999), but Merrell *et al*'s results strongly suggest that ppGpp accumulates in response to iron limitation, just as in *E. coli.*

## Experimental procedures

*Bacterial strains and phages*

All the strains used in this work are *E. coli* K-12 derivatives; some are described in Table 5. The *entB*::*kan* (Staab and Earhart, 1990), the *fiu*::*lacZ* fusion (Hantke, 1987) and the Δ*nagEBACD*::$Tc^R$ allele (Plumbridge, 1992) were kindly provided by C. Earhart, D. Touati and J. Plumbridge, respectively. Strains AN311 (Young and Gibson, 1979) and AN260 (Pierce and Earhart, 1986), kindly provided by K. Hantke, were the donors of the *fepB* and *fepC* mutations, respectively.

The $P1_{rrnB}$::*lacZ* fusion is integrated within the *malEFK* operon and has an associated kanamycin resistance (Hernandez and Bremer, 1990). Low values of β-galactosidase activity are typical for this fusion, which was constructed with a low efficiency ribosome binding site derived from phage ϕX174 (Hernandez and Bremer, 1990).

Strain DV310 (Δ*relA fiu*::*lacZ* Δ*lacZ*) was constructed in a similar way to CF6301 (Δ*relA* $P1_{rrnB}$::*lacZ* Δ*lacZ*) (Vinella *et al*., 2000): the $P_{fiu}$::*lacZ* fusion was generated by insertion of a λp*lac*Mu53$Km^R$ in the *fiu* gene (Hantke, 1987) and transduced by selecting its associated kanamycin resistance. To introduce the *fepB* and *fepC* mutations into our strains, we first transduced the linked insertion *zbe-601*::Tn*10* into AN311 and AN260; small $Tc^R$ clones were used as donors to transduce strain CF1742 (*relA1*) to tetracycline resistance; $Tc^R$ transductants were screened for inability to grow on minimal glucose plates unless $10^{-2}$ M citrate was added; these were used, in turn, as donors of the *fepB and fepC* mutations by cotransduction with *zbe-601*::Tn*10*. This marker was also used to introduce the *entB*::*kan* allele into $Km^R$ backgrounds, selecting $Tc^R$ transductants that were unable to grow on M9 glucose plates in the presence of 0.25 mM dipyridyl. To construct the *fes*::*cat fepB* and *fes*::*cat fepC* derivatives of CF1742, we transduced strains *fepB zbe-601*::Tn*10* and *fepC zbe-601*::Tn*10* using a P1 stock grown on a *fes*::*cat entB*::*kan* strain and selecting for chloramphenicol resistance; $Cm^R$ $Km^S$ transductants were of two types: clones forming pink colonies (presumed to be *fes*::*cat* single mutants) and clones forming colonies of normal coloration (presumed to be *fes::cat fep* double mutants). To confirm the genotype of the

strains, P1 stocks were grown on the two types of transductants and used to transduce strain CF1742 to chloramphenicol resistance; P1 grown on the presumed *fes*::*cat* single mutant gave only pink $Cm^R$ transductants while P1 grown on the presumed *fes*::*cat fep* double mutant gave both pink and normal transductants in a ratio consistent with the genetic map (gene order *fes..fepC...fepB...entB*). The *fes*::*cat entB*::*kan* derivatives of CF6301 and DV310 were made by transducing these parental strains (which are $Km^R$) with a P1 stock grown on a *fes*::*cat entB*::*kan* donor strain and selecting $Cm^R$ transductants on LB plates; we obtained small $Cm^R$ pink colonies (*fes*::*cat* single mutants) and bigger clones of normal coloration (*fes*::*cat entB*::*kan* double mutants). These genotypes were confirmed by the respective inability or ability of P1 stocks grown on the transductants to cotransduce kanamycin resistance with chloramphenicol resistance.

The *fur*::*kan* mutation was introduced into strains CF6301 and DV310 (which are kanamycin resistant) by first introducing a Δ*nagEBACD*::$Tc^R$ allele, then transducing to $Nag^+$ on minimal medium containing 0.2% N-acetylglucosamine as carbon source, using a *fur*::*kan* donor; cotransduction of the *fur*::*kan* mutation was confirmed by the ability to retransfer kanamycin resistance by cotransduction with $nag^+$.

The Δ*spoT207*::*cat* mutation was introduced into $Cm^R$ recipients by cotransduction with the *zib-563*::Tn*10*($Tc^R$) marker; *spoT* cotransductants were detected by their inability to grow on minimal glucose plates containing $10^{-2}$ M citrate. This allele was from strain CF1693.

*Media and growth conditions*

All bacteria used in this work were grown at 37°C. Our mecillinam resistant mutants were originally selected on classical LB plates (Miller, 1992) in one laboratory but, for an unknown reason, this phenotype was not reproducible in a second laboratory unless 1% KCl was added to standard LB (called LB* medium); we therefore included experiments on LB* medium in our subsequent analysis. Minimal medium was M9 (Miller, 1992); glucose (0.4%), Casaminoacids (0.4% ) or aminoacids (0.01%) were added as noted. For ppGpp assays, MOPS (Neidhardt *et al.*, 1974) was used, supplemented with 0.2% glucose, 0.3 mM phosphate and all 20 amino acids (40 µg $ml^{-1}$ each); the concentration of iron and additional supplements are specified in the text. Estimates of enterochelin secretion were made on

chrome azurol S (CAS) plates (Schwyn and Neilands, 1987) supplemented with 0.4% glucose and 0.3% Casaminoacids. Solid media contained 1.5% agar. Antibiotics were used at the following concentrations: chloramphenicol (Cm) 20 μg $ml^{-1}$; kanamycin (Km) 40 μg $ml^{-1}$; mecillinam (Mec) 10 μg $ml^{-1}$; tetracycline (Tc) 20 μg $ml^{-1}$; ampicillin (Amp) 100 μg $ml^{-1}$. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was added at 40 μg $ml^{-1}$ when indicated. All cultures and plates were incubated at 37°C. X-Gal and 2, 2′-dipyridyl were from Sigma Chemical Co.

*Test for mecillinam resistance*

The susceptibility of strains to mecillinam was tested by plating on LB or LB* plates supplemented with 10 μg $ml^{-1}$ mecillinam, a generous gift of Laboratoires Léo (France); for plasmid-bearing strains, antibiotics required for plasmid maintenance were also added; additional supplements are indicated in the text. Plates were incubated three nights. Cultures were plated in parallel on identical plates lacking mecillinam and the ratio of the two titers was calculated. When this efficiency of plating was greater than 0.3 the strain was considered resistant, when less than $3\times10^{-4}$, sensitive.

*β-galactosidase assays*

Tests on liquid cultures were carried out as described (Miller, 1992). For assays on colonies, we prepared plates with about 50 colonies, then picked colonies into 1.5 ml of Z buffer, using several colonies if necessary to reach a final $OD_{600}$ of 0.1 to 0.2; 1 ml was used for β-galactosidase assays. For both fusions used in this work, the ratio of β-galactosidase activity in the parental strain to that in the *fes* mutant changes with the incubation time of the plates but remains approximately constant between 20 and 30 hours (Fig. 4). All measurements presented here were made after 24 to 26 h incubation of the plates. Numbers shown in the tables are mean values obtained from at least 10 measurements; in all cases, the observed deviation did not exceed 10%.

*Estimates of enterochelin secretion*

Overnight LB cultures were centrifuged and washed twice in $10^{-2}$ M $MgSO_4$; 1 μl of each suspension was deposed on a CAS plate, which was then incubated for 48 hours.

*ppGpp assay*

The ppGpp concentration was measured in cells grown in MOPS glucose minimal medium with 40 μg $ml^{-1}$ (each) of all 20 amino acids and containing $^{32}P$ (0.1 mCi/0.4 μmol $ml^{-1}$) and various concentrations of $FeSO_4$. Nucleotides were extracted by adding ice cold formic acid to 6.5 M. Samples were frozen and thawed at least twice before being spotted on PEI plates (Polygram CEL 300, Macherey-Nagel). One-dimensional chromatography was carried out in 1.5 M $KH_2PO_4$. Quantification was with a Storm PhosphorImager.

*DNA techniques and plasmids*

Plasmids were extracted and transformation was carried out as described by Sambrook *et al.* (Sambrook *et al*., 1989). The cloning vectors used in this work were pKS+ (high copy $Amp^R$, Stratagene) and pBAD18 (high copy $Km^R$ (Guzman *et al*., 1995)). All clonings were carried out in strain XL1 Blue (Stratagene).

To localize the mini-Tn*10*($Cm^R$) element present in the *mcr-14*::*cat* mutant, chromosomal DNA was extracted from strain DV53 (CF6301 *mcr-14*::*cat*) and digested with *Bst*UI, a restriction enzyme that does not cut in the inserted sequence. From this digest we cloned the *cat* gene in the vector pKS+ linearized with *Sma*I. To sequence the chromosomal DNA adjacent to the ends of the mini-Tn*10* insertion element, we used synthetic primers (5′ to 3′) CmD = TTATTCTGCCTCCCAGAGCC and CmF = AACGGCAAAAGCACCGCCGG. PCR amplification of the *fes*$^+$ gene and promoter was carried out with Taq polymerase (Perkin Elmer) using the primers (5′ to 3′) FesUP = AAGAATTCCCATTACGCTATTGCAAAT-GCAAAT and FesDO = AAACTGCAGATTACTGAATGCCATATTCAACTCC. The 1.25 kb amplified fragment was digested by *Eco*RI and *Pst*I, then cloned into pBAD18, generating pDV51, whence it was subcloned into pKS+, generating pDV52.

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**Table 1. Growth on plates**

| Strain | Growth[a] on plates of | | | |
|---|---|---|---|---|
| | M9 glucose | LB | LB+Cit[b] | LB+Asc[b] |
| Parental[c] | ++ | ++ | ++ | ++ |
| *fes::cat (mcr-14::cat)* | ± | +(p) | ++(p) | ++ |
| *fepB*[c] | ± | + | ++ | ++ |
| *entB* | ++ | ++ | ++ | ++ |
| *fes::cat fepB*[d] | ± | + | ++ | ++ |
| *fes::cat entB* | ++ | ++ | ++ | ++ |
| *fur* | ++ | ++ | ++ | ++ |
| *fur fes::cat* | ± | +(p) | ++(p) | ++ |
| *fur fepB*[d] | ± | + | ++ | ++ |
| *fur entB* | ++ | ++ | ++ | ++ |

[a]The strains were cultivated overnight in liquid LB and washed twice with M9 medium before plating. Similar results were obtained in the CF6301 (Δ*relA* $P1_{rrnB}$*::lacZ*) and CF1742 (*relA1*) strain backgrounds. "++" indicates normal colony size and "+" indicates small colonies after overnight incubation (LB plates) or two nights' incubation (M9 glucose plates); "±" represents microcolonies visible only after three days; "p" indicates pink coloration of the colonies.

[b]"Cit" is 10 mM citrate, "Asc" is 10 mM ascorbate; addition of 0.1 mM $FeCl_3$ gave the same results as ascorbate.

[c]The parental strain was CF6301 (Δ*relA*).

[d]The same results were obtained when the strain carried a *fepC* mutation instead of *fepB*.

**Table 2. Expression of the P1$_{rrnB}$::*lacZ* and *fiu*::*lacZ* fusions in mutants affected in enterochelin-dependent iron uptake**

**A. Expression of the *P1$_{rrnB}$*::*lacZ* fusion in Δ*relA* strains[a]**

| Strain | LB | LB + Cit[b] | LB + Asc[b] |
|---|---|---|---|
| Parental[c] | 17 | 15 | 14 |
| *fes*::*cat* | 4.1 | 10 | 13 |
| *fepB* | 4.5 | 9 | 15 |
| *entB* | 12 | 13 | 14 |
| *fes*::*cat fepB* | 3.2 | 7.5 | 9.5 |
| *fes*::*cat entB* | 12 | 13 | 13 |
| *fur* | 11 | 9 | 9.5 |
| *fur fes*::*cat* | 2.3 | 4.1 | 3.4 |
| *fur fepB* | 2.1 | 9.6 | 5.5 |
| *fur entB* | 11 | 8 | 8.2 |
| Δ*spoT* | 18 | 16 | 16 |
| Δ*spoT fes*::*cat* | 20 | 17 | 22 |
| Δ*spoT fes*::*cat entB* | 18 | 19 | 18 |
| Δ*spoT fur* | 15 | 10 | 15 |

[a]Assayed on suspensions of colonies picked from LB plates and expressed in Miller units (Miller, 1992).

[b]"Cit" is 10 mM citrate, "Asc" is 10 mM ascorbate; addition of 0.1 mM $FeCl_3$ gave the same results as ascorbate.

[c]The parental strain was CF6301 (Δ*relA*).

**B. Expression of the *fiu*::*lacZ* fusion in Δ*relA* strains[a]**

| Strain | LB | LB + Cit[b] | LB + Asc[b] |
|---|---|---|---|
| Parental[c] | 20 | 17 | < 5 |
| *fes*::*cat* | 360 | 82 | < 5 |
| *fepB* | 320 | 82 | < 5 |
| *entB* | 300 | 89 | < 5 |
| *fes*::*cat fepB* | 340 | 87 | < 5 |
| *fes*::*cat entB* | 360 | 81 | < 5 |
| *fur* | 370 | 390 | 360 |
| *fur fes*::*cat* | 330 | 360 | 360 |
| *fur ent* | 360 | 350 | 360 |
| *fur fep* | 320 | 360 | 320 |
| Δ*spoT* | 21 | 17 | < 5 |
| Δ*spoT fes*::*cat* | 190 | 20 | < 5 |
| Δ*spoT fes*::*cat entB* | 140 | 20 | < 5 |
| Δ*spoT fur* | 240 | 300 | 200 |

[a] Assayed on suspensions of colonies picked from LB plates and expressed in Miller units (Miller, 1992).

[b]"Cit" is 10 mM citrate, "Asc" is 10 mM ascorbate; addition of 0.1 mM $FeCl_3$ gave the same results as ascorbate.

[c]The parental strain was DV310 (Δ*relA*).

**Table 3. Mecillinam susceptibility of enterochelin mutants**

| Strain | Mecillinam resistance[a] on | | | | |
|---|---|---|---|---|---|
| | LB* | | | LB | |
| | – | + Cit[b] | + Asc[b] | – | + Dip[b] |
| Parental[c] | S | S | S | S | S |
| *fes*::*cat* | R | R | S | S/R | R |
| *fepB* | S | S | S | S | S |
| *entB* | S | S | S | S | S |
| *fes*::*cat fepB* | S/R | S | S | S | S |
| *fes*::*cat entB* | S | S | S | S | S |
| *fur* | S | S | S | S | S |
| *fur fes*::*cat* | R | R | S | S/R | R |
| *fur fepB* | S | S | S | S | S |
| *fur entB* | S | S | S | S | S |
| Δ*spoT* | S | S | S | S | S |
| Δ*spoT fes*::*cat* | S | S | S | S | S |

[a]Symbols: "R", plating efficiency > 50% in the presence of 10 μg $ml^{-1}$ mecillinam; "S", plating efficiency < 0.01% in the presence of 10 μg $ml^{-1}$ mecillinam; "S/R", plating efficiency of 1% in the presence of 10 μg $ml^{-1}$ mecillinam.

[b]"Cit" is 10 mM citrate, "Asc" is 10 mM ascorbate and "Dip" is 0.25 mM dipyridyl; addition of 0.1 mM $FeCl_3$ gave the same results as ascorbate.

[c]The parental strain was CF6301 (Δ*relA*); the same results were obtained with derivatives of DV310 (Δ*relA*).

**Table 4. Iron starvation in enterochelin mutants**

| | Growth on plates | | | | | |
|---|---|---|---|---|---|---|
| | M9 glucose Casaminoacids | | LB | | | |
| Strain | – | +Dip[a] | – | +Dip[a] | +Mec[a] | +Dip Mec[a] |
| Parental[b] | + | + | + | + | – | – |
| *fes*::*cat* | + | – | + | + | – | + |
| *fepB* | + | – | + | + | – | – |
| *entB* | + | – | + | + | – | – |
| *fes*::*cat fepB* | + | – | + | + | – | – |
| *fes*::*cat entB* | + | – | + | + | – | – |
| *fur fes*::*cat* | + | – | + | + | – | + |
| Δ*spoT* | + | ±[c] | + | ±[c] | – | – |
| Δ*spoT fes*::*cat* | – | – | + | – | – | – |
| Δ*spoT fes*::*cat entB* | – | – | + | – | – | – |

[a]"Dip" is 0.25 mM dipyridyl, "Mec" is 10 μg $ml^{-1}$ mecillinam.

[b]The parental strain was CF6301 (Δ*relA*).

[c]Microcolonies visible only after two days' incubation.

Table 5. Bacterial strains

| Strain | Genotype | Origin or reference |
|---|---|---|
| MG1655 | Wild type | (Bachmann, 1996) |
| CF1742 | As MG1655, relA1 | M. Cashel, lab collection |
| DV206 | As MG1655, lacIpoZΔ(Mlu) | (Vinella et al., 2000) |
| DV352 | As DV206, P1rrnB::lacZ | This work |
| CF6301 | As DV206, ΔrelA251::kan P1rrnB::lacZ | (Vinella et al., 2000) |
| DV310 | As DV206, ΔrelA 251::kan fiu::lacZ | This work |
| DV301 | As CF6301, fes::cat | This work |
| DV311 | As DV310, fes::cat | This work |
| DV377 | As CF1742, fes::cat | This work |
| CF1693 | As MG1655, ΔrelA251::kan ΔspoT207::cat | (Xiao et al., 1991) |
| XL1 Blue | *endA1 hsdR17* ($r_K^-$, $m_K^+$) *supE44 thi-1 recA1 gyrA96 relA1* Δ*lac*/F′ *proAB*$^+$ *lacI*$^q$ *lacZDM15* Tn*10* | (Bullock *et al.*, 1987) |

**Figure Legends**

**Fig. 1.** Enterochelin secretion in the presence and absence of ppGpp. Bacteria were spotted on a CAS plate and incubated for 48 hours. The wild type strain is DV352; the other strains are CF6301 and its derivatives, as in Table 2A.

**Fig. 2.** Increase of the ppGpp pool induced by dipyridyl. Strain DV301 (*fes*::*cat* Δ*relA*) was grown in MOPS medium containing glucose, all 20 amino acids (40 μg $ml^{-1}$ each), 1 μM $FeSO_4$ and $^{32}P_i$. At time zero, dipyridyl (0.25 mM) was added to a portion of the culture and the ppGpp and GTP pools and optical density were monitored. Squares: no dipyridyl; diamonds: + dipyridyl.

**Fig. 3.** ppGpp pool increase induced by iron depletion. Strain DV301 (Δ*relA fes*::*cat*) was grown at 37°C in MOPS medium supplemented with glucose (0.2 %), all 20 aminoacids (40 μg $ml^{-1}$ each), $P_i$ (0.3 mM) and $FeSO_4$ (1 μM) to $OD_{600}$ = 0.5, then diluted 250-fold in the same medium but lacking iron and containing $^{32}P_i$. $FeSO_4$ (50 μM) was added to part of the culture after 5 h incubation (time zero in Panel A) or after 14 h incubation (time zero in Panel B). Squares: no addition of $FeSO_4$; diamonds: addition of $FeSO_4$.

**Fig. 4.** β-galactosidase activity on plates of the *fes* mutant (diamonds) and its $fes^+$ parent (squares) as a function of time of incubation of the plates. Upper Panel: *fiu*::*lacZ*; lower panel: $P1_{rrnB}$::*lacZ*.

Fig. 1

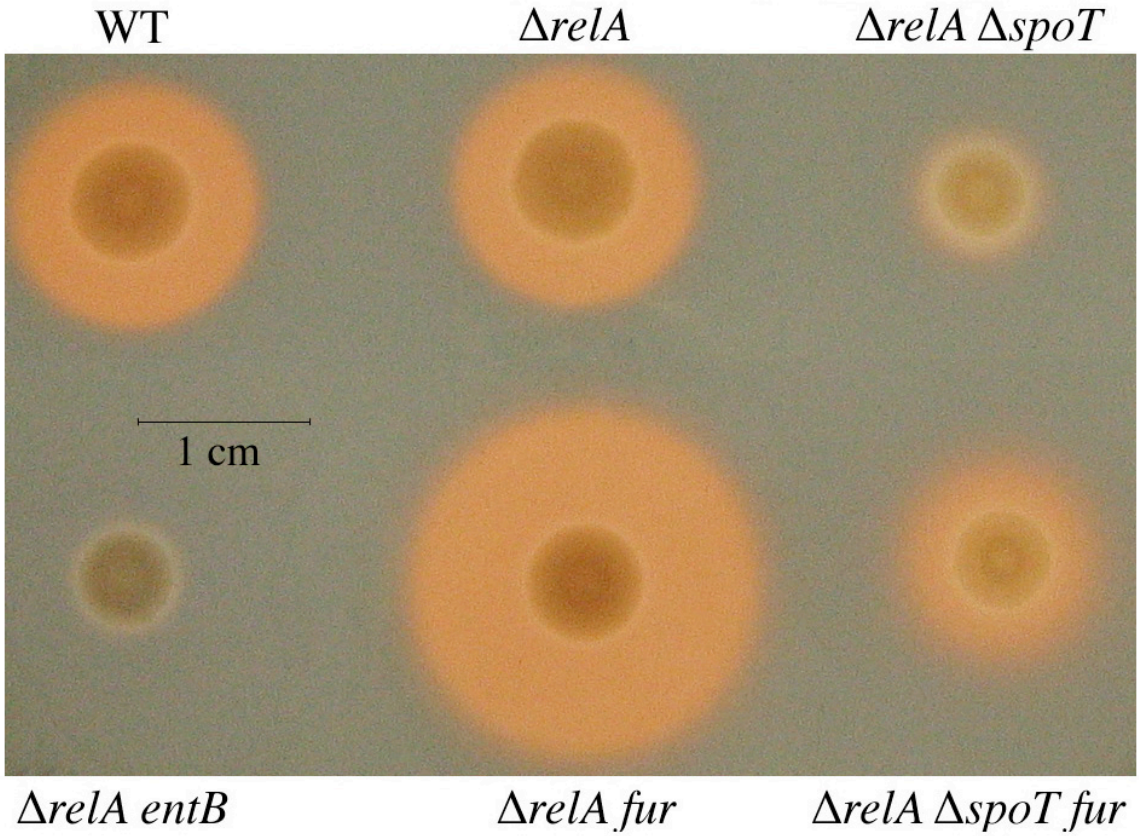

WT
ΔrelA
ΔrelA ΔspoT
1 cm
ΔrelA entB
ΔrelA fur
ΔrelA ΔspoT fur

FIG. 2

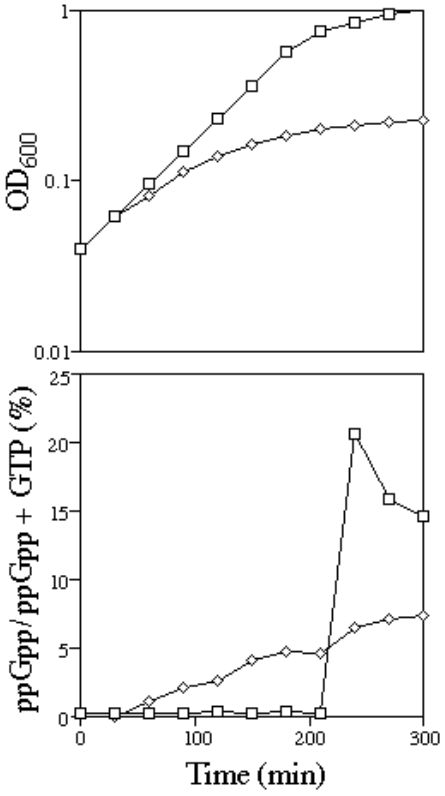
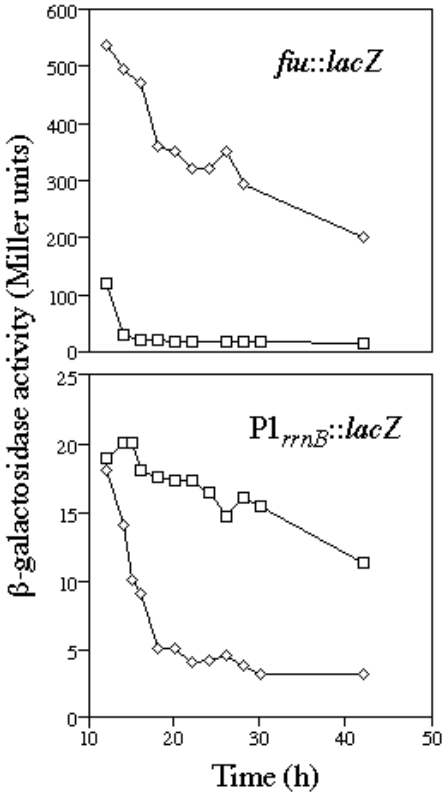



FIG. 4



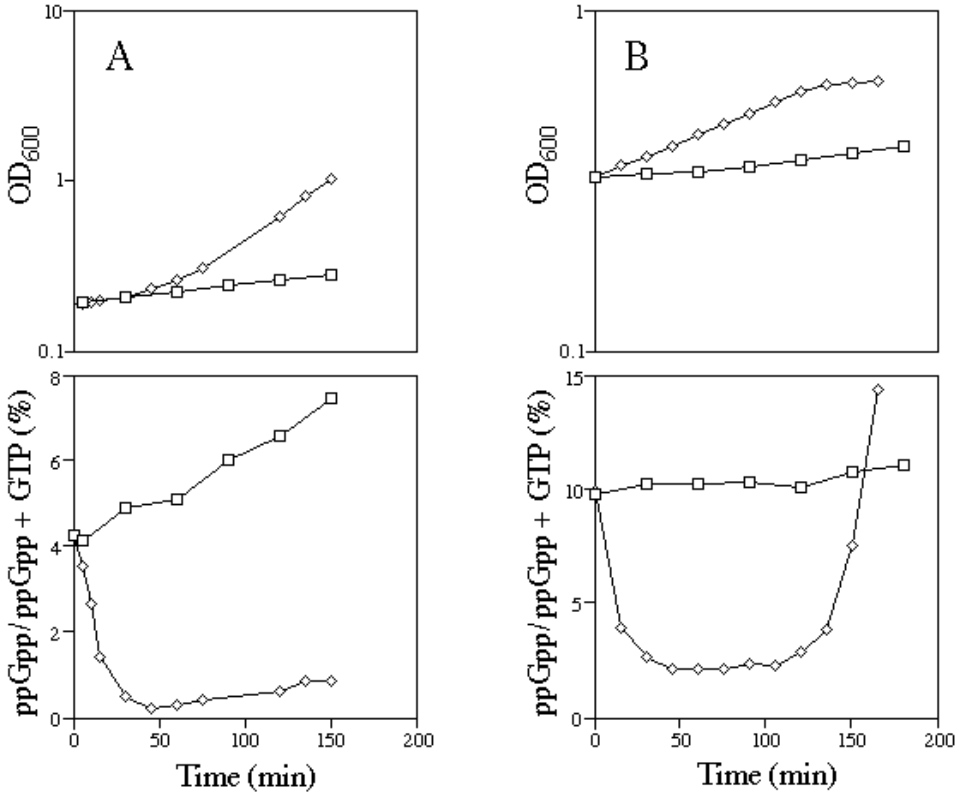


FIG. 3