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Mapping of a Mutation Affecting Regulation of Iron Uptake Systems in *Escherichia coli* K-12

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The site of a mutation resulting in constitutive derepression of iron uptake systems has been localized at 15.7 min on the genetic map of *Escherichia coli* K-12.

Escherichia coli K-12 can assimilate iron through a variety of high-affinity uptake mechanisms. These include transport systems for siderophores produced by other microbial species (e.g., ferrichrome, rhodotorulic acid), for ferric citrate, for siderophores indigenous to *E. coli* (e.g., enterobactin), and for aerobactin in bacteria harboring the ColV plasmid (11). The production of both siderophores and their cognate receptors is regulated by the availability of iron (9). Mutants defective in iron regulation (the mutation is designated *fur*) have been isolated in both *Salmonella typhimurium* (5) and *E. coli* (6). These mutants are constitutively derepressed for the synthesis of siderophores and their receptors. Hantke (7) has partially mapped a *fur* mutant by complementation with F'lac. In this paper we describe the isolation and mapping of a *fur* mutant at another locus, near *nagA*.

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To isolate a mutant derepressed for iron uptake, a *lacZ* operon fusion to the cloned aerobactin genes was constructed in vitro and subsequently transferred to the ColV plasmid. A 3.3-kilobase *Bam*HI fragment of pJA1, constructed by Judy Allen of this department, carries the complete *lacZ* structural gene and a fragment of the *lac* operator including the ribosome-binding site. The 5' end of this fragment was derived from the 1.1-kilobase *Bam*HI-to-*Cla*I fragment of pRZ5605 (8). The 3' end was derived from the 2.2-kilobase ClaI-to-*Bam*HI fragment of pKK137-8, constructed by Jurgen Brosius. Both fragments were cloned into the vector pUC8 (14). pKK137-8 has a *Bam*HI site added at a former *Hae*III site of *lacZ*, 80 base pairs 3' of the *Eco*RI site.

The *Bam*HI fragment carrying the *lacZ* gene was purified from pJA1 and inserted between the two closely spaced *Bam*HI sites in the *iucC* gene of the multicopy plasmid, pABN5 (3) (Fig. 1). Gene *iucC* (iron uptake chelate) specifies the third polypeptide distal to the promoter required for biosynthesis of aerobactin (A. Bindereif and J. B. Neilands, unpublished data).

In *E. coli* the pColV-encoded aerobactin genes are normally present in only a few copies per cell. Because regulation of aerobactin synthesis may be perturbed when the aerobactin operon is present at high copy number, the *iucC-lacZ* fusion was transferred to the ColV plasmid by homologous recombination (Fig. 2). Production of β-galactosidase by pColV-*lacZ* was inducible 10-fold by iron starvation (Fig. 3A).

Using this *iucC-lacZ* fusion, we were able to isolate mutants derepressed for β-galactosidase synthesis under iron-replete conditions (Fig. 3B). Strain BN407 [Δ(*lac*)U169 (pColV-*lacZ*)] harboring the fusion was mutagenized with Tn*5* by using λ::Tn*5*. Colonies resistant to 40 μg of kanamycin per ml were plated on MacConkey lactose medium containing 20 mM sodium citrate. On these indicator plates wild-type colonies carrying the *lacZ* fusion were repressed and therefore were white. A total of 30 red, derepressed colonies were picked. Most of these mutants had Tn*5* insertions in the enterobactin synthesis genes and required the siderophore for growth on iron-deficient medium. One, however, overproduced β-galactosidase even in the presence of an exogenously supplied siderophore, ferrichrome. This siderophore suppressed the overproduction of β-galactosidase in the *ent* mutants. The *fur*::Tn*5* mutant was able to grow on low-iron medium, unlike *tonB* mutants or mutants defective in enterobactin synthesis or utilization. Because the mutant appeared to be competent in iron transport, but deficient in regulation, the lesion must be in an iron transport regulatory gene, or *fur* gene.

The *fur*::Tn*5* mutant was found to be derepressed not only for the synthesis of β-galactosidase, but also for the synthesis of catechol and hydroxamate (Table 1). Outer membrane proteins normally derepressed only under low-iron conditions were also constitutively derepressed (Fig. 4). This phenotype is characteristic of previously described *fur* mutants.

To map the *fur*::Tn*5* mutation, it was transferred by P1 transduction to strain Hfr3000, selecting for Tn*5* (kanamycin resistance). Because Tn*5* was found to "hop" in 1 out of 10 transductants, any putative *fur*::Tn*5* transductants were checked for overproduction of catechol. Interrupted mating experiments, performed as described by Miller (10), revealed that the Hfr3000 *fur*::Tn*5* strain transferred kanamycin resistance to AB1515 about 2.5 min after the *purE* gene, thus placing the *fur*::Tn*5* mutation at about 14.5 min on the *E. coli* K-12 map.

P1 transduction experiments were performed to map the mutation more precisely. The strains W620 *gal7* (*gltA*$^-$), JRG33 (*lip*$^-$), and JP5053 (*nagA*$^-$) were transduced to kanamycin resistance with a P1 lysate grown on the *fur*::Tn*5* mutant and screened for both overproduction of catechol and maintenance of the nearby marker (*gltA*, *lip*, or *nagA*).

The resulting *fur*::Tn*5* strains (BN904, BN905, and BN900) were used to determine the cotransduction frequency of

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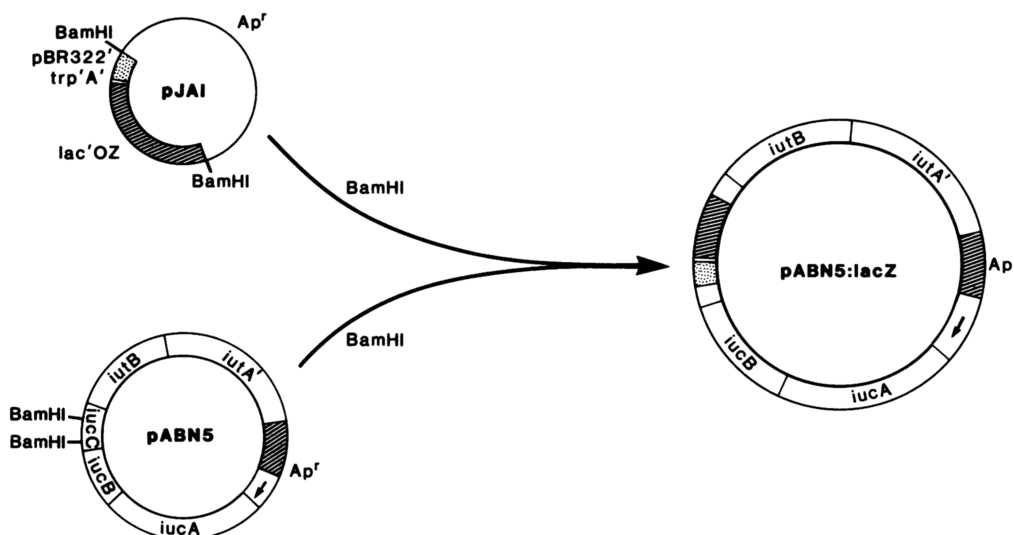

FIG. 1. Construction of the aerobactin-*lacZ* operon fusion. The *Bam*HI fragment of pJA1 containing the *lacZ* gene was cloned between two *Bam*HI sites in the *iucC* gene of pABN5. *iuc*, Iron uptake chelate; *iut*, iron uptake transport.

fur$^+$ with a variety of markers. $P1_{vir}$ grown on strain RE103 was used to introduce wild-type alleles of certain markers into these strains. A total of 200 wild-type transductants for each marker were screened for kanamycin sensitivity. The insertion was found to be 11% cotransducible with *lip*, 16% cotransducible with *gltA*, and 93% cotransducible with *nagA*. Using the formula $D = 2(1 - \sqrt[3]{f})$ (15) to convert cotransduction frequency into map distance, this places *fur*::Tn*5* at 0.05 min away from *nagA*.

This locus is well separated from any site known to be involved in iron transport and is different from Hantke's (6) mapping of *fur* in the vicinity of *lac*. The disparity between our results may be due to the existence of more than one locus that can be mutagenized to yield the *fur* phenotype. It

TABLE 1. Production of extracellular catechol and hydroxamate[a]

Strain	Fe	Hydroxamate[b]	Catechol[c]
BN8015 (*fur*$^+$)	+	0.22	0.05
	−	1.3	0.50
BN8021 (*fur*::Tn*5*)	+	1.2	7.9
	−	1.4	2.1

[a] Strains BN8015 [*fur*$^+$ *trp cir*(pColV-K30 Tet^r)] and BN8021 [*fur*::Tn*5* *trp cir*(pColV-K30 Tet^r)] were grown overnight in nutrient broth. They were diluted 1/100 into M9 minimal medium supplemented with tryptophan, B1, glucose, and ± 10 μM $FeSO_4$. Cultures were incubated for 30 h at 37°C. The cells were spun down, and the Arnow test (1) for catechol and the ferric perchlorate assay (2) for hydroxamate were performed on the supernatants. Production of enterobactin may exceed its aqueous solubility in the absence of iron (12).

[b] As aerobactin, in micromoles per milligram (dry weight) of cells.

[c] As 2,3-dihydroxybenzoic acid, in micromoles per milligram (dry weight) of cells.

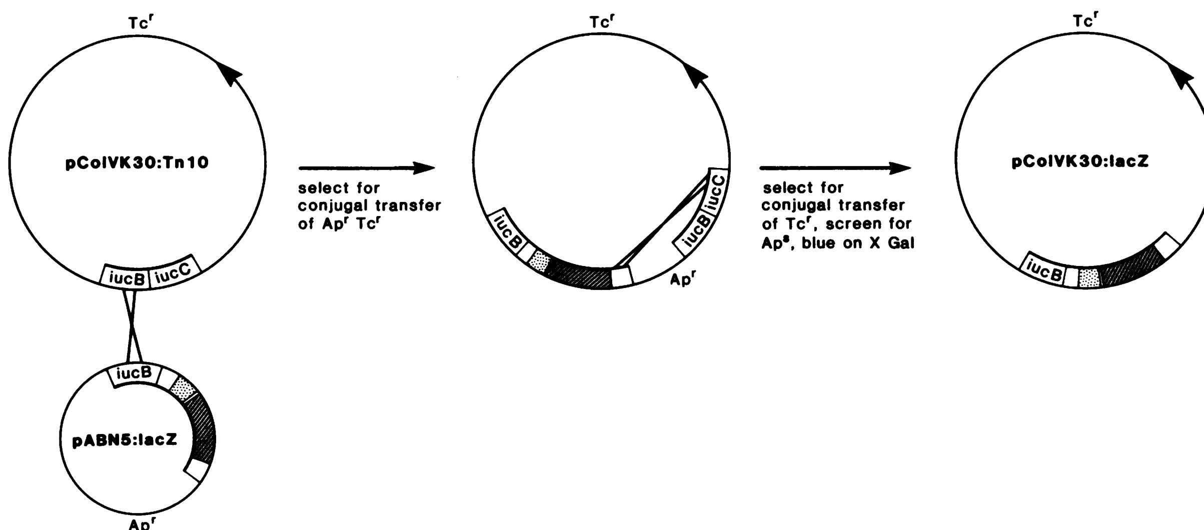

FIG. 2. Transfer of the *lacZ* fusion to the ColVK30 plasmid. The *lacZ* fusion was transferred from the multicopy pABN5 to single-copy pColVK30 by selecting for a series of homologous recombinations.

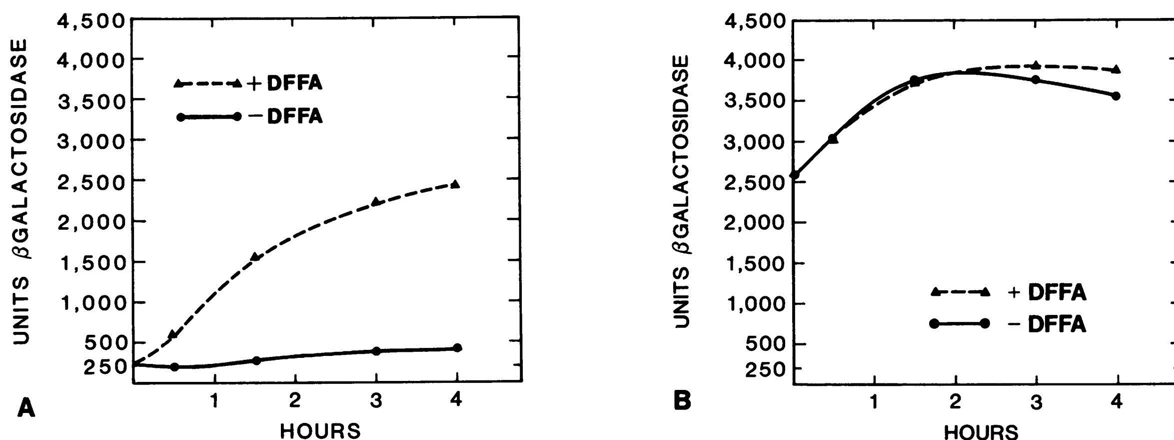

FIG. 3. (A) Synthesis of β-galactosidase is induced 10-fold by iron starvation. Deferriferrichrome A (DFFA), a non-utilizable iron chelator, was added to log-phase cultures of ABN407 in nutrient broth at t = 0. Cells were lysed by the method of Putnam and Koch (13). Synthesis of β-galactosidase was induced 10-fold in the iron-starved (+DFFA) culture. (B) Synthesis of β-galactosidase in the *iucC-lacZ* fusion is constitutively derepressed in the *fur*::Tn*5* mutant. The assay was performed as in A, with strain BN4020 (*fur*::Tn*5*) rather than BN407.

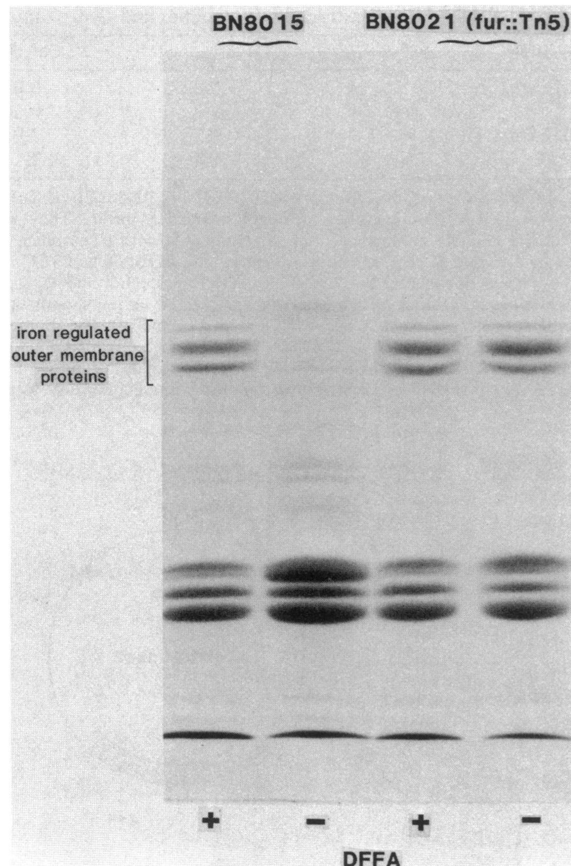

FIG. 4. Iron-regulated outer membrane proteins are constitutively expressed in the *fur*::Tn*5* mutant. Hfr3000 and Hfr3000 *fur*::Tn*5* were grown in nutrient broth to an optical density at 600 nm of 0.1. Bipyridyl (200 μM) was then added to half of each 200 μM culture, and the cultures grown for another 3.5 h. The cells were harvested, and the outer membranes were purified (7). A 20-μg sample of protein (assayed by the Bradford procedure [4]) was added to each lane of this 10% polyacrylamide-sodium dodecyl sulfate gel.

is obviously necessary to isolate several additional mutants before we can determine how many loci exist.

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