JOURNAL OF BACTERIOLOGY, Dec. 1983, p. 1171–1177
0021-9193/83/121171-06$02.00/0

Vol. 156, No. 3

Regulation of Enterobactin Iron Transport in *Escherichia coli*: Characterization of *ent*::Mu *d*(Ap^r *lac*) Operon Fusions

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Received 8 June 1983/Accepted 12 September 1983

The vector Mu *d*(Ap^r *lac*) was utilized to construct operon fusions in the *Escherichia coli* enterobactin (*ent*) biosynthetic and transport genes. Enzyme assays indicated a 5- to 15-fold increase in the expression of β-galactosidase when the fusion strains were grown under iron-deficient conditions. The polarity effects seen by Mu *d* insertions into *entA*, *entC*, and *entE* were consistent with a single operon, *entA(CGB)E*. The direction of transcription from iron-regulated promoters was determined by directional transfer of selected genetic markers after the insertion of F'ts114 *lac*$^+$. Regulatory mutants were isolated in the fusion strains by the selection for constitutive expression of β-galactosidase and the iron-regulated outer membrane proteins.

Iron is essential for bacterial growth. However, under normal environmental conditions iron exists as an insoluble ferric hydroxide polymer or is complexed by host iron-binding proteins such as lactoferrin or transferrin and is relatively unavailable for bacterial assimilation (25). In response to such iron-limiting conditions, bacteria synthesize specific iron-chelating compounds, siderophores, and the cognate iron transport components necessary for siderophore binding and uptake. The native siderophore of *Escherichia coli* is a cyclic trimer of 2,3-dihydroxybenzoylserine, referred to as enterobactin or enterochelin (26, 27).

The enterobactin iron uptake system consists of at least seven biosynthesis genes and three transport genes that map near min 13 (1). Two other transport genes are located elsewhere on the *E. coli* chromosome. From a common intermediate of aromatic amino acid synthesis, the enterobactin biosynthesis genes *entC*, *entB*, and *entA* convert chorismate to 2,3-hihydroxybenzoic acid (32). 2,3-Dihydroxybenzoic acid and L-serine are substrates for the production of enterobactin by enterobactin synthetase, which is composed of four enzymes, the products of *entD*, *entE*, *entF*, and *entG* (20, 29). Release of the siderophore into the medium occurs by an uncharacterized mechanism. Enterobactin avidly sequesters external iron, and the ferric enterobactin complex thus formed binds to a specific receptor, FepA, an outer membrane protein of M_r 81,000 (81K) (10, 28). Internalization of this complex involves the action of FepB in concert with two common high-affinity uptake proteins, TonB (7) and ExbB (13). The *fes* gene product controls the subsequent release of iron and degradation of the ligand (18).

Laird et al. (16) observed that the enterobactin cistrons span approximately 29 kilobases of DNA and are organized in the following order: *entD*, *fes*, *entF*, *fep*, and *ent(CA)GBE*. The *fep* mutations used in this study all fell into the same complementation group now defined as *fepB* (21). Insertion of Tn*5* into these genes was nonpolar on the other genes in the cluster, suggesting that all of the enterobactin cistrons (possibly excepting *entC* and *entA*) represent single transcriptional units (17). A recent investigation resulted in a slight modification of the gene order, with *fepA* located between *fes*, and *entF* and *fep* now replaced by *fepB* (C. L. Pickett, S. S. Chenault, J. R. Pierce, L. Hayes, and C. F. Earhart, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K180, p. 207).

FepA and two other outer membrane proteins (83K and 74K or Cir) are coordinately regulated by iron availability in *E. coli* (14, 23). The expression of enzymes used in enterobactin biosynthesis is also regulated by iron. Alternate iron transport systems present in *E. coli*, including ferrichrome uptake (11) and the aerobactin iron utilization system (9), all respond to the concentration of iron for control of their expression.

To investigate the regulation of enterobactin synthesis and membrane transport components at a transcriptional level, we have utilized the Mu *d*(Ap^r *lac*) operon fusion vector of Casadaban and Cohen (3). This report describes the isolation and characterization of operon fusions of Mu *d*(Ap^r *lac*) into enterobactin biosynthesis

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype[a]	Reference
E. coli		
MC4100	F⁻ *araD139* Δ (*lacIOPZYA*)*U169 rpsL thiA*	3
MC4100-46	Δ*purE* derivative of MC4100	This study
MAL103	F⁻ Mu*cts dl*(Ap^r *lac*) Mu*cts* (*proAB-lac IOPZYA*) *rpsL*	3
AB1515	*leu proC purE trp thi*	5
AN90	*leu proC trp thi entD*	5
AN193	*leu proC trp thi entA*	5
AN192	*leu proC trp thi entB*	5
AN191	*leu proC trp thi entC*	5
AN93	*leu proC trp thi entE*	5
AN117	*leu proC trp thi entF*	5
AN102	*leu proC trp thi fepB*	15
AN194	*entA*⁺ revertant of AN193	18
MF1170	MC4100-46 Φ(*entF-lac*)	This study
MF176	MC4100-46 Φ(*entF-lac*)	This study
MF104	MC4100-46 Φ(*entF-lac*)	This study
MF165	MC4100-46 Φ(*entA-lac*)	This study
MF242	MC4100-46 Φ(*entA-lac*)	This study
MF148	MC4100-46 Φ(*entC-lac*)	This study
MF252	MC4100-46 Φ(*entE-lac*)	This study
MF290	MC4100-46 Φ(*tonA-lac*)	This study
MF277	MC4100-46 Φ(*fepA-lac*)	This study
MF1367	MC4100-46 Φ(*cir-lac*)	This study
MF1155	MC4100-46 Φ(*tonB-lac*)	This study
MFR-5	Constitutive β-galactosidase expression derivative of MF1170	This study
MFR-6	Constitutive β-galactosidase expression derivative of MF1170	This study
AS4	AB1515 *entD*::Tn*5*	This study
CSH57B	F⁻ *ara leu lacY purE gal trp his argG malA strA xyl mtl ilv metB thi*	24
Plasmids		
F′ 254/CSH 57B[b]	Episome: *lac*⁺ *proC*⁺ *tsx*⁺*purE*⁺ *ent*⁺	19
F′ ts114 *lac*⁺	Episome: *lac*⁺, temperature sensitive	1

[a] Φ, Fusion; Δ, deletion.

[b] Mutant derivatives of F′ 254 constructed in this study include *entA*, *-B*, *-C*, *-D*, *-E*, and *-F*, and *fepA*, *fepB*, and *fes*.

and transport genes. Analysis of β-galactosidase production in the fusion strains isolated is consistent with the concept of coordinate regulation of *fepA* (81K), *cir* (74K), and enterobactin biosynthesis at the transcriptional level. The polarity effects exhibited by these insertions have led us to propose an operon of the biosynthesis genes *entA(CGB)E*, with *entD*, *entF*, and *fepA* controlled as separate transcriptional units. The fusion strains have also provided a scheme for isolating regulatory mutants in the system.

MATERIALS AND METHODS

Organisms and media. The *E. coli* strains used in this study are listed in Table 1. L-broth (LB) and M9 medium (24) and the iron-poor Tris-succinate minimal medium (22) have been described previously. M9 was made iron rich by the addition of 20 μM $FeSO_4$ and 10 mM sodium citrate. In minimal media, the bacteria were supplied with appropriate amino acids and adenine at 50 μg/ml, thiamine at 10 μg/ml, glucose at 0.4%, succinate at 30 mM, and ampicillin at 25 μg/ml.

Preparation of Mu *d*(Ap^r *lac*) lysate (from MAL103) and its use for mutant isolation. Preparation of the Mu lysate was as described previously (3). Mutants were produced by infection of MC4100-46 at a multiplicity of infection of 0.1 followed by plating on M9 plates supplemented with glucose, adenine, ampicillin, and 5-chloro-4-bromo-3-indolyl-β-D-galactoside. The blue colonies were purified and screened for enterobactin production by bioassay. Ent⁻ colonies were further characterized by genetic complementation with F′ 254. FepA⁻ and TonB⁻ mutants were selected as colicin B-resistant colonies. Cir⁻ strains were isolated by colicin I-resistance, and TonA⁻ strains were isolated by resistance to φ80.

Bioassay. The indicator strain AN90 (*entD*) was overlayered in plain top agar onto a Tris-succinate minimal plate. Colonies to be assayed were spotted onto the overlay, and the plates were incubated overnight at 30°C. Enterobactin-producing strains were detected by the appearance of a halo of growth of the indicator strains around the test strain. Ent⁻ strains were then tested for 2,3-dihydroxybenzoic acid production by using the indicator strain AN193 (*entA*).

Complementation experiments. Donor (F′ 254 or *ent* derivatives) and *recA* recipient strains were grown to the early log phase in LB. The cultures were diluted

TABLE 2. Genetic complementation of Mu *d*(Ap^r *lac*) insertion mutations in *ent* region genes

Strain	Complementation with donor episome[a]:							
	entD	*fepA*	*entF*	*fepB*	*entA*	*entC*	*entB*	*entE*
AS4	−	+	+	+	+	+	+	+
MF277	+	−	+	+	+	+	+	+
MF1170	+	+	−	+	+	+	+	+
MF176	+	+	−	+	+	+	+	+
MF104	+	+	−	+	+	+	+	+
MF165	+	+	+	+	−	−	−	−
MF242	+	+	+	+	−	−	−	−
MF148	+	+	+	+	+	−	−	−
MF252	+	+	+	+	+	+	+	−

[a] *ent* or *fep* derivatives of F′ 254 were introduced into *recA* derivatives of relevant operon fusion strains as discussed in the text. Complementation of genetic defects in the recipient fusion strains was scored as growth (+) or no growth (−) on iron-deficient medium after 4 days at 30°C.

1/50 into 10^{-2} M Tris (pH 7.0), and 20 μl of both donor and recipient were spot mated on Tris-succinate plates. Positive complementation yielded growth in 4 days at 30°C.

Homogenotization of *ent* markers to F′ 254. The method of Miller (24) was utilized to transfer *ent* point mutations onto the F′ 254 (19). The *ent* derivatives were stored in strain CSH57B (*recA*).

β-galactosidase assay. β-Galactosidase activity was assayed with chloroform-treated cells as described previously (24).

Selection of regulatory mutants. Strain MF1170 was mutagenized with ethyl methanesulfonate as described previously (24). The organisms were plated at a concentration of 10^6 cells per plate onto MacConkey-lactose medium with added iron (25 μM). After 48 h at 30°C red colonies were isolated and assayed for iron-mediated regulation of expression of β-galactosidase.

Direction of transcription. F′ts114 *lac*$^+$ (18) was introduced into MF1170 carrying *entF*::Mu *d*(Ap^r *lac*) (Lac$^+$, inducible), MF104, *entF*::Mu *d*(Ap^r *lac*) (Lac$^-$, uninducible), MF165, *entA*::Mu *d*(Ap^r *lac*) (Lac$^+$, inducible); and MF277, *fepA*::Mu *d*(Ap^r *lac*) (Lac$^+$, inducible). Cultures of these strains contain a population of Hfrs resulting from F′ integration into Mu *d*(Ap^r *lac*) via *lac* homology. The recipient strain AN194 contained the plasmid pMB9, into which the gene for Mu immunity had been cloned, to prevent killing due to zygotic induction of Mu *d*. Samples (0.5 ml) of early log cultures were mixed. At intervals (15, 30, 45, 60, 120 min), 0.1-ml samples were vortexed vigorously to interrupt matings and plated on minimal plates to detect *trp*$^+$ or *leu*$^+$ exconjugants.

Membrane preparation. Sarkosyl-insoluble outer membrane proteins were prepared as described previously (6).

Gel electrophoresis. Samples of outer membrane protein were applied to 0.75-mm-thick 9 to 14% gradient polyacrylamide gels prepared and electrophoresed (100 V, 9 h) by the method of Laemmli (15).

RESULTS

Selection of Mu *d*(Ap^r *lac*) fusions into enterobactin biosynthetic genes. The isolation of *ent*::Mu *d* fusion strains was accomplished by plating fusion derivatives on minimal 5-chloro-4-bromo-3-indolyl-β-D-galactoside medium that contained enough iron so that Ent$^-$ mutants were able to grow, but not so much iron that the expression from iron-regulated promoters was repressed. M9 minimal medium, which contained approximately 1 μM iron, satisfied both of these criteria. Approximately 3,000 blue colonies were screened, and 120 colonies were determined to be Ent$^-$ by bioassay. From this pool, only 13 mutants were complemented by F′ 254, which contains the wild-type enterobactin biosynthesis genes. To ensure that fusions to relevent genetic loci were not overlooked by this selection procedure, 500 white colonies were screened by bioassay. Seven were Ent$^-$, and one was complemented by F′ 254. This strain, MF104, proved to have Mu *d* inserted in a reverse orientation. Ent$^-$ fusion strains that were not complemented by F′ 254 were not further examined.

RecA derivatives of the fusion strains were characterized by genetic complementation assays. Known point mutations were transferred

TABLE 3. Production of β-galactosidase[a] in response to iron concentration in enterobactin operon fusions

Strain	Relevant marker	β-Galactosidase (units)	
		+Fe[b]	−Fe
MF1170	*entF*	28	329
MF176	*entF*	26	246
MF104	*entF*	53	31
MF165	*entA*	37	305
MF242	*entA*	30	280
MF148	*entC*	24	210
MF252	*entE*	28	310
MF277	*fepA*	23	260
MF290	*tonA*	76	97
MF1367	*cir*	65	420
MF1155	*tonB*	77	87

[a] The values represent the mean of three independent B-galactosidase assays for each strain.

[b] M9 glucose supplemented with 10 mM sodium citrate plus 20 μM $FeSO_4$.

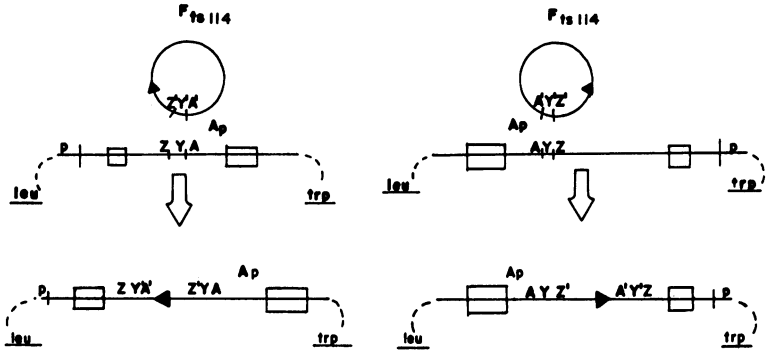

FIG. 1. Direction of transcription of *ent* genes. Hfrs formed via *lac* homology between F'ts114 *lac*$^+$ and Mu *d*(Ap^r *lac*) insertions. (A) High frequency of *trp* transfer when Mu *d*(Ap^r *lac*) was inserted in an orientation reflecting clockwise gene transcription. (B) Counterclockwise transcription as determined by high frequency of *leu* transfer.

onto F' 254 by homogenotization and utilized as donors in these experiments (Table 2). A strong polarity effect was observed when Mu *d* was inserted into the *entA* and *entC* cistrons (MF165 and MF148, respectively), resulting in the lack of expression of *entB* and *entE* (and presumably *entG*). These data suggest that *entA(CGB)E* comprise an operon transcribed from *entA* to *entE*; although the data indicate that *entC* is promoter proximal to both *entB* and *entE*, the exact location of *entG* cannot be defined at present. Two methods were employed to eliminate the possibility of Mu *d*-induced deletions as the explanation of the observed polarity. First, when RecA$^+$ fusion strains were used as recipients in the complementation experiments, Ent$^+$ recombinants were found in all genes downstream of the insertion site. No significant intracistronic recombination occurred. Secondly, X mutations (2) in Mu *d*, which allow the precise excision of Mu *d* inserts, were isolated for each fusion. In all cases, precise excision restored enterobactin biosynthesis. These tests verified that the polarity effects were not due to deletion formation.

Fusion strains with insertions into *entF* did not affect the expression of any other genes. Insertions into *fepA* had no effect on enterobactin biosynthesis, and these insertions complemented mutants AN102 (5) and AN270 (30), which we have defined as *fepB* (21).

Initial experiments using Tn*5* to probe the transcriptional organization of this system have resulted in two *entD*::Tn*5* mutations. No effect on *fes* is seen in the *entD*::Tn*5* mutants as these strains can grow normally when fed enterobactin (data not shown). However, the activation of adjacent genes that can occur with Tn*5* insertions (4) does not permit definitive interpretation of these data. The organization of *fes* as a single transcriptional unit or as a part of the *fepA* or *entD* transcripts is unclear at present.

β-galactosidase assay of Mu *d* insertion strains. The data in Table 3 demonstrate that β-galactosidase activity responds to the ambient iron concentration. Fusions of Mu *d* to the enterobactin biosynthesis genes, the outer membrane receptor *fepA* gene, and the iron-regulated outer membrane protein *cir* gene exhibited a similar 5- to 15-fold increase in enzyme activity under low-iron conditions. This degree of stimulation is in agreement with fusions isolated in other studies (31). *tonA*::Mu *d* inserts displayed a different regulatory pattern as previously noted (11, 31). Insertions into *tonB* did not exhibit iron regulation. Strain MF104 (*entF*::Mu *d*) does not produce β-galactosidase in response to iron concentration, indicating that the *lacZ* gene of Mu *d* is inserted in the opposite orientation behind the iron-regulated *entF* promoter.

Direction of transcription. The fusion strains enabled us to determine the direction of transcription of these genes. Marker transfer experiments were performed by utilizing the shared *lac* homology between F'ts114 and the fusion

TABLE 4. Direction of transcription

Strain	No. of exconjugants[a]	
	Leu$^+$	Trp$^+$
MF1170 (*entF*)	45	700
MF104 (*entF*)	650	30
MF165 (*entA*)	12	350
MF277 (*fepA*)	380	30

[a] Hfrs were formed via the *lac* homology on F'ts114 *lac*$^+$ and *ent*::*lac* or *fep*::*lac* operon fusions. Hfrs were mated with AN194 (*leu trp*); matings were disrupted after 45 min. Leu$^+$ or Trp$^+$ exconjugants were selected.

strains. Figure 1 shows the potential Hfr derivatives that would result from the insertion of F'ts114 in either orientation. The data (Table 4) demonstrate that *entF* and *entA* are transcribed clockwise with respect to the *E. coli* chromosome, whereas *fepA* is transcribed in the opposite direction. Strain MF104 donated markers counterclockwise as expected.

Isolation of regulatory mutations. Strain MF1170 was mutagenized with ethyl methanesulfonate. Colonies expressing increased β-galactosidase activity under iron-replete conditions were selected. This phenotype could result from a regulatory mutation or Mu *d* transposition to other promoter regions. Since *entF*, *fepA*, and *cir* (and perhaps the gene for 83K) map at different locations on the *E. coli* chromosome, concomitant expression of these iron-regulated outer membrane proteins with *entF* would differentiate between these possibilities.

Table 5 illustrates two such mutants isolated. Strains MFR-5 and MFR-6 expressed high levels of β-galactosidase when grown in LB. However, MFR-5 reproducibly expressed β-galactosidase at the induced level (with respect to the parent strain, MF1170) regardless of the availability of iron, whereas the uninduced level of β-galactosidase activity in MFR-6 was consistently lower. Outer membrane proteins isolated from LB-grown cultures revealed the elevated expression of 83K, 81K, and 74K (Fig. 2). There were distinct differences in the outer membrane protein profiles of these mutants. In comparison with MFR-5, MFR-6 contained increased levels of OmpF and membrane proteins with apparent molecular weights of 120K and 25K. The expression of the 25K protein has previously been shown to be iron regulated (14). The observed differences in the outer membrane protein profiles and the levels of β-galactosidase expression between MFR-5 and MFR-6 would suggest that the mutations in these strains are different. The map position of our regulatory mutations has not been defined. Attempts to restore wild-type regulation by introducing F' factors covering the *fur* region described by Hantke (12) have not been successful.

TABLE 5. β-Galactosidase[a] assay of regulatory mutants[a]

Strain	β-Galactosidase (units)	
	+Fe[b]	−Fe
MF1170	15	348
MFR-5	336	465
MFR-6	185	395

[a] The values represent the mean of three independent β-galactosidase assays for each strain.

[b] M9 glucose supplemented with 10 mM sodium citrate and 20 μM $FeSO_4$.

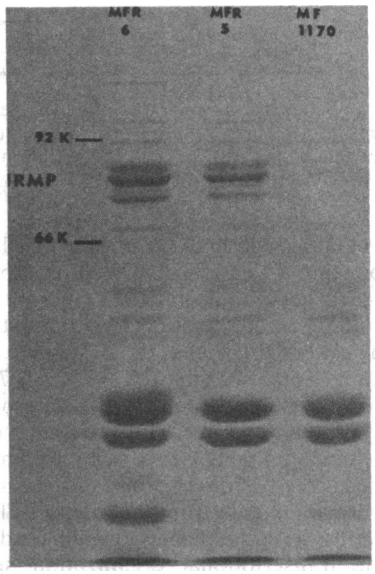

FIG. 2. Outer membrane protein analysis of strains MF1170, MFR-5, and MFR-6. Strains were grown overnight in LB. Proteins were resolved on a 9 to 14% gradient sodium dodecyl sulfate-polyacrylamide gel and stained with Coomassie blue. IRMP, Iron-regulated membrane proteins.

DISCUSSION

The insertion of Mu *d*(Ap[r] *lac*) into various enterobactin biosynthesis and transport genes allowed us to precisely monitor iron-regulated gene expression at a transcriptional level. This study provided further evidence of coordinate regulation between the iron-induced membrane proteins and enterobactin biosynthesis (14, 23). Common regulatory components must exist for the enterobactin system, its specific receptor FepA, the colicin Ia receptor Cir, and the outer membrane protein 83K.

Polarity effects observed with the insertions indicate that the enterobactin biosynthesis genes *entA(CGB)E* comprise a single operon that is transcribed from *entA* to *entE* in a clockwise direction on the *E. coli* chromosome. The precise order of the genes *entC*, *entG*, and *entB* has not been established. The operon organization from our data is in conflict with the work of Laird and Young (17), which suggests that these genes exist as individual transcriptional units. We cannot at present explain this discrepancy; however, it has been shown that Tn*5* may activate adjacent genes in an operon (4). The current study also indicates that *entF* exists as a single transcriptional unit and is transcribed clockwise relative to the standard *E. coli* map. Tn*5* insertions into *entD* do not affect enterobactin transport, which suggests a single transcriptional unit for *entD*. Mu *d* and Tn*5* insertions into *fepA* do

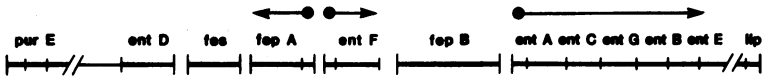

FIG. 3. Schematic overview of the *ent* gene organization. Genetic loci are not drawn to scale. Arrows indicate transcripts; (●) putative promoter sequences. *fepB* may represent more than one gene. The relationship of the *fes* gene to the *entD* or *fepA* transcriptional units is unknown. The direction of transcription of *entD*, *fes*, or *fepB* has not been determined.

not affect enterobactin biosynthesis. The lack of insertions into *fes* and *fepB* prevent the speculation of their gene organization.

This study, in conjunction with the data provided by others (16; Pickett et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, p. 207), upgrades our present concept of the enterobactin genetic system (Fig. 3). The proximity of the genetic loci *fepA* and *entF* (as determined by preliminary restriction enzyme analysis of isolated DNA fragments from this region; Fleming and McIntosh, unpublished results) and their opposing transcriptional organization suggest that they may share a common regulatory locus (e.g., an operator). This arrangement would allow a tight coordinate regulation of both biosynthesis and transport of enterobactin.

The Mu *d* fusion strains offer a means to select regulatory mutations (11, 31). We have isolated strains that express both high levels of β-galactosidase from *entF*::Mu *d* and the iron-regulated outer membrane proteins under iron replete conditions. This phenotype is expected of a specific mutation at a regulatory locus and not the transposition of Mu *d* behind a constitutively expressed promoter (8). These characteristics could result from mutation(s) in (i) a putative regulatory component, (ii) an iron storage protein(s), or (iii) an intracellular iron shuttling mechanism. In all of these cases, a low intracellular iron level would be detected by the regulation mechanism (even in the presence of high intracellular iron concentration), resulting in constitutive expression of iron-regulated cistrons. The map position of our regulatory mutants has not been determined. Attempts to restore wild-type regulation by introducing an F′ covering the *fur* region described by Hantke (12) have not been successful. We conclude that mutations at multiple sites result in a similar phenotype. The differences observed in β-galactosidase expression and outer membrane protein profiles in MFR-5 and MFR-6 also supports this contention.

ACKNOWLEDGMENTS

We are grateful to Karen Ehlert for preparation of this manuscript.

This work was supported in part by Department of Health and Human Service Biomedical Research Support grant DHHS BR5 5387 to the University of Missouri School of Medicine and by grant PCM 8210415 from the National Science Foundation.

ADDENDUM IN PROOF

Recent physical analysis of isolated enterobactin gene sequences revealed that the correct gene order of the left end of the gene cluster is *entD fepA fes entF*. The lack of Mu *d* or Tn*5* insertions into the *fes* gene prevents establishing its transcriptional organization with respect to *fepA* and *entF*.

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