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Anaerobic Biosynthesis of Enterobactin in *Escherichia coli*: Regulation of *entC* Gene Expression and Evidence against Its Involvement in Menaquinone (Vitamin K_2) Biosynthesis

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In *Escherichia coli*, isochorismate is a common precursor for the biosynthesis of the siderophore enterobactin and menaquinone (vitamin K_2). Isochorismate is formed by the shikimate pathway from chorismate by the enzyme isochorismate synthase encoded by the *entC* gene. Since enterobactin is involved in the aerobic assimilation of iron, and menaquinone is involved in anaerobic electron transport, we investigated the regulation of *entC* by iron and oxygen. An operon fusion between *entC* with its associated regulatory region and *lacZ*$^+$ was constructed and introduced into the chromosome in a single copy. Expression of *entC-lacZ* was found to be regulated by the concentration of iron both aerobically and anaerobically. An established *entC*::*kan* mutant deficient in enterobactin biosynthesis was found to grow normally and synthesize wild-type levels of menaquinone under anaerobic conditions in iron-sufficient media. These results led to the demonstration of an alternate isochorismate synthase specifically involved in menaquinone synthesis encoded by the *menF* gene. Consistent with these findings, the *entC*$^+$ strains were found to synthesize enterobactin anaerobically under iron-deficient conditions while the *ent* mutants failed to do so.

The formation of isochorismic acid from chorismic acid is the first committed step in the biosynthesis of both enterobactin (2, 38) and menaquinone (vitamin K_2) (4) (Fig. 1). The conversion of chorismate to isochorismate is mediated by the enzyme isochorismate synthase encoded by the *entC* gene (27). It was reported by Kaiser and Leistner that *entC* encoded the isochorismate synthase necessary to supply the isochorismate required for both enterobactin and menaquinone (16). It is well established that the isochorismate synthase is derepressed in the absence of iron and repressed by iron (11, 38) and that menaquinone concentration is regulated by the availability of oxygen. Furthermore, enterobactin synthesis is reportedly required only under aerobic conditions for iron transport, as under anaerobic conditions iron occurs in the highly soluble ferrous form (11, 26). The regulation of *entC* by both iron and oxygen raises some intriguing questions. How does the organism keep menaquinone biosynthesis repressed aerobically on low-iron media when isochorismate synthase is fully derepressed? How does the organism synthesize menaquinone anaerobically if enterobactin is not synthesized under these conditions? If synthesis of isochorismate synthase under anaerobic conditions is assumed, how does the organism synthesize menaquinone in the presence of high concentrations of iron when isochorismate synthase is expected to be fully repressed? To answer these questions, we constructed an *entC-lacZ* operon fusion and introduced it in a single copy into the *Escherichia coli* chromosome. Assays of β-galactosidase activity in cells grown aerobically or fermentatively on glucose and in the presence of the electron acceptors nitrate, fumarate, or trimethylamine *N*-oxide (TMAO) established that the *entC-lacZ* fusion is regulated transcriptionally by iron but not by oxygen. In addition, we establish for the first time that enterobactin is synthesized anaerobically in response to iron deficiency. Furthermore, evidence is provided in support of an alternate isochorismate synthase specifically involved in menaquinone biosynthesis. Preliminary reports of some of these findings have appeared elsewhere (9, 18).

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MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The wild-type and mutant strains (and their genotypes), bacteriophages, and plasmids used in this study are shown in Table 1.

Construction of *entC-lacZ* fusion strain. An operon fusion between the *entC* gene and the *lacZ*$^+$ gene was constructed with the plasmid pRS415. Procedures for endonuclease digestion, ligation, transformation, isolation of plasmids and analysis of restriction fragments were carried out as previously described (28). The operon fusion was transferred to the lambda transducing phage λRZ5 as described by Simons et al. (34) and designated λMO1. A high-titer lysate of the phage containing the fusion was used to lysogenize MC4100, and a single lysogen was selected as described by Cotter and Gunsalus (7) and designated MC4100(λMO1). For the introduction of the *fnr* mutation, λMO1 was used to lysogenize strain SM1. For the introduction of *arc*, phage P_1 was used to transduce the mutation into the fusion strain and selected for Kan resistance. For the introduction of *fur*::Tn*5* into the lysogen, phage P_1 was used.

Media. For routine growth of cultures, L agar and L broth were used. Cultures were stored in glycerated L broth at −80°C. Ampicillin and kanamycin were used at concentrations of 50 and 40 μg/ml, and tetracycline was used at 15 μg/ml. To detect the Lac$^+$ phenotype, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 40 μg/ml. In some experiments MacConkey agar plates were used.

All glassware used for the growth of cultures was thoroughly cleaned by soaking overnight in 2 N HCl and rinsing thoroughly in double-distilled water, or, alternatively, disposable plasticware was used.

The minimal medium used was as described previously (35), except that $FeSO_4 \cdot 7H_2O$ was omitted. For iron-deficient and iron-sufficient media, 50 μM dipyridyl or 50 μM $FeSO_4 \cdot 7H_2O$ were included unless otherwise specified. Glucose or glycerol at a concentration of 40 mM was used as a carbon source. The media were supplemented with 0.1% Casamino Acids and the required growth factors. For anaerobic growth in the presence of electron acceptors, nitrate, fumarate, or TMAO at a concentration of 40 mM was added.

Growth of cells and assay of β-galactosidase. For the assay of β-galactosidase, a single colony of the lysogen was inoculated into 5 ml of the appropriate medium with or without electron acceptors, contained in test tubes (16 by 150 mm), and incubated with shaking at 250 rpm. After overnight growth, a 1% inoculum was transferred into 5 ml of fresh medium of the same composition as the inoculum and growth continued until an optical density at 600 nm of 0.3 to 0.7 was reached. For the anaerobic growth of cells, a single colony was inoculated into 10 ml of the appropriate medium with or without electron acceptors, contained in completely filled screw-cap tubes (13 by 100 mm), and grown overnight. The media were boiled to remove oxygen. A 1% inoculum from the overnight culture was used to inoculate 10-ml volumes of medium of the same composition,

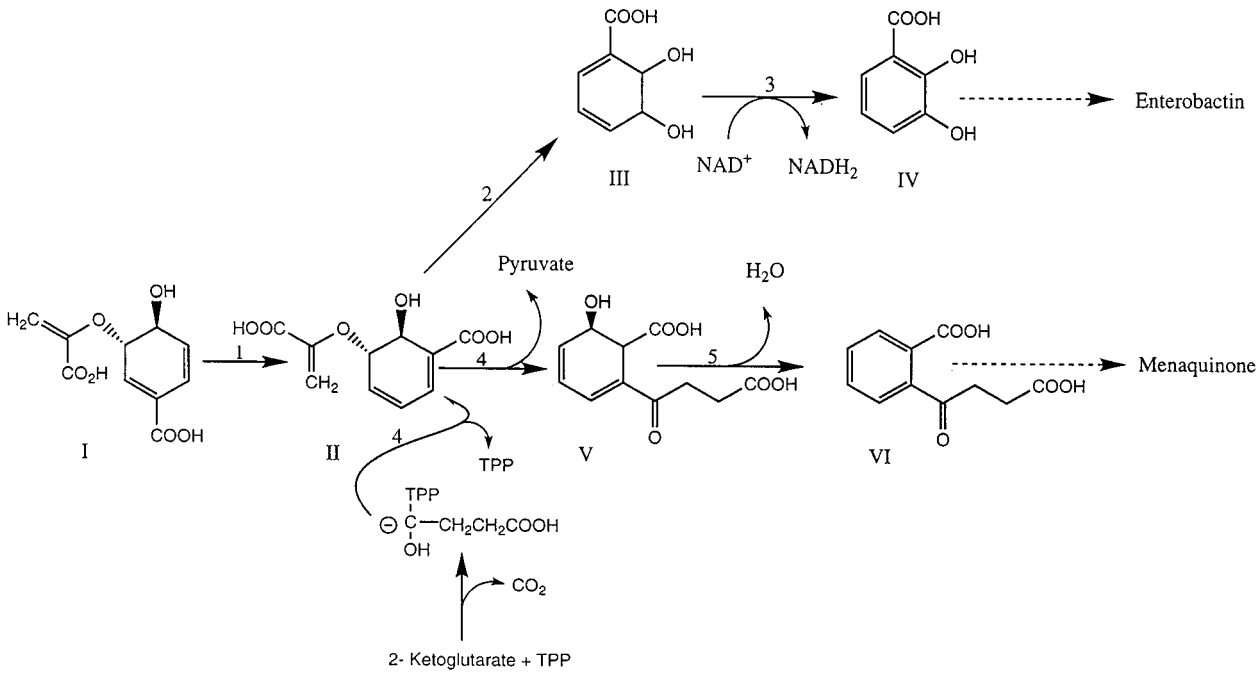

FIG. 1. Conversion of chorismate to enterobactin and menaquinone. Only the pertinent reactions are shown. Compounds are identified by roman numerals, and enzymes and the genes encoding them are identified by arabic numerals. Compounds: I, chorismate; II, isochorismate; III, 2,3-dihydro-DHBA; IV, DHBA; V, SHCHC; VI, OSB. Enzymes (genes): 1, isochorismate synthase (*entC*); 2, isochorismatase (*entB*); 3, 2,3-dihydro-DHBA dehydrogenase (*entA*); 4, SHCHC synthase–2-ketoglutarate decarboxylase (*menD*); 5, OSB synthase (*menC*). TPP, thiamine PP_i.

and growth continued until an optical density at 600 nm of 0.3 to 0.7 was reached. Cells grown as described above were assayed for β-galactosidase, and the units of activity were calculated as described by Miller (25). The β-galactosidase activities represent the average of at least five experiments with a variation of no more than 10% from the mean.

Growth of cells and assay of enterobactin. For the monitoring of growth and for enterobactin assays, overnight cultures were grown aerobically in 5 ml of medium with either glucose or glycerol as the carbon source. The cells were centrifuged at 2,500 rpm for 20 min in an IEC centrifuge, and the pellet was resuspended in 5 ml of medium without the carbon source. A 1% inoculum was used to inoculate 25 ml of medium in a 500-ml sidearm flask and grown with shaking at 250 rpm. For anaerobic cultures, 1% of the suspension was inoculated into 10 ml of medium in screw-cap tubes (13 by 100 mm). In all cases, glucose- or glycerol-grown overnight cultures were used to inoculate glucose and glycerol media, respectively. Growth was monitored in a Klett meter with a red filter (wavelength, 640 to 700 nm).

For the assay of enterobactin, cultures were grown in the presence and the absence of iron until cessation of growth. The cells were centrifuged at 12,000 rpm in a microcentrifuge for 3 min, and the clear supernatant was assayed for enterobactin. The concentration of enterobactin was determined by the method of Arnow (1), and the units of activity were calculated as described elsewhere (2, 10).

Assay of menaquinone. For the analysis of menaquinone, 8 g of cells was suspended in 12 ml of 0.02 M potassium phosphate, pH 7, and passed through a French pressure cell at 12,000 lb/in^2 and the extract was centrifuged at 3,000 × *g* for 10 min to remove whole cells. The supernatant was centrifuged at 15,000 × *g* for 30 min, and the pellet containing membranes was extracted with 20 ml of chloroform-methanol (2:1, vol/vol) for approximately 3 h with a magnetic stirrer in the dark. The chloroform-methanol extract was filtered through Whatman no. 1 filter paper, and the filtrate was dried in a rotary evaporator at 40°C. The lipid extract was dissolved in a small volume of acetone and applied to silica gel F254 thin-layer chromatography plates (Analabs). The plates were developed in petroleum ether (boiling point, 36.9 to 54.5°C)–diethyl ether (85:15, vol/vol). Authentic MK-8 was used as the standard (22).

Alternatively, 0.5 g of whole cells was extracted and analyzed by high-pressure liquid chromatography (HPLC) on a reversed-phase column with authentic MK-8 as standard (37).

Assay of isochorismate synthase. The fluorometric assay commonly used for the assay of isochorismate synthase (31, 39) failed to show enzymatic activity in the *entC* mutant. Since the fluorometric assay measures only 25% of the isochorismate formed after its thermal conversion to salicylic acid (39), it is not sensitive enough to detect the low levels of the activity expected. To overcome this difficulty we transformed the *entC* mutant with plasmid pJP101 (29, 30). This plasmid contains the *menD* gene which codes for both 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid (SHCHC) synthase and 2-ketoglutarate decarboxylase activities and allowed us to use the more sensitive HPLC assay as follows. The assay mixture contained the following (amounts in parentheses are in micromoles unless otherwise indicated): Tris HCl buffer, pH 7.9, (300); chorismate (2.7); thiamine PP_i (TPP) (2.6); 2-ketoglutarate (6.8); $MgCl_2$ (22.5); and extract (0.5 ml). The tubes were incubated for 30 min at 30°C. The SHCHC formed was converted to *o*-succinylbenzoic acid (OSB) by alkaline hydrolysis and assayed by HPLC (28). Authentic OSB was used as the standard.

RESULTS

Construction of *entC-lacZ* operon fusion plasmid. In order to study the regulation of the *entC* gene, an operon fusion between the gene containing its regulatory region and the *lacZ*$^+$ gene was constructed. A 0.991-kb *Eco*RI fragment from the *entC*$^+$ plasmid pITS557 was cloned into the unique *Eco*RI site of the *lacZ* operon fusion plasmid pRS415. To ensure that potential regulatory elements were not missed, the entire upstream region to the 5′ end of the *entC* gene from the *Ava*I site was included in the insert. The *Ava*I site CCCGAG is a single base pair downstream of the −35 region of the *fepB* gene located 5′ to the *entC* gene, which is transcribed in the opposite direction. Thus, this *Eco*RI insert contained 120 bp upstream of, and 851 bp downstream of, the translational initiation codon of the *entC* gene (5). The construct was verified by restriction analysis and designated pMO1.

Transfer and integration of *entC-lacZ* operon fusion into the chromosome of *E. coli*. The operon fusion contained in pMO1 was transferred to phage λRZ5 by homologous recombination as previously described (7, 34). The resulting phage, λMO1, was then integrated into the Lac$^-$ bacterial strain MC4100 at the λ attachment site. The lysogens were identified by the Lac$^+$ phenotype conferred on the host by the integrated fusion phage. Eight to ten blue colonies were selected, and β-galactosidase levels were assayed to distinguish single lysogens from

TABLE 1. *E. coli* K-12 strains, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Origin	Genotype or phenotype	Source or reference
Strains			
MC4100		F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	7
PC35	MC4100	Δ*arcA* Kan^r	8
SM1	MC4100	Δ*fnr* Tet^r	23
SBC24		Δ(*ara-leu*)*7697 araD139* Δ(*lac*)*X74 galE galK rpsL thi malF*Δ*3 phoA*Δ*Pvu*II *phoR zad*::Tn*10 pcnB fur*::Tn*5*/F′ *lac pro lacI*^{q}	6
AB1515		*thi trpE purE proC leuB lacY mtl xyl rpsL azi tonA tsx supA*	31
MT147	AB1515	*entC*::*kan*	27
AN92-MK3		*proA2 argE3 pheA1 tyrA4 trp-401 aroB351 rpsL entA*::*kan*; Mu immune	36
RM923	AB1515	*entA*::*kan* P_1 transductant	This study
MC4100(λMO1)	MC4100	MC4100(λMO1)	This study
MO400(λMO1)	SM1	SM1(λMO1)	This study
MO401(λMO1)	MC4100	*fur*::Tn*5*(λMO1)	This study
DH5α		F$^-$ ϕ80 *dlacZ*ΔM15 Δ(*lac ZYA-argF*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR supE44 thi-1* λ$^-$ *gyrA96 relA1*	Clontech
Phages			
λRZ5		*'bla 'lacZ lacY*$^+$	15
λMO1	λRZ5	Φ(*entC'-lacZ*) *lacY*$^+$ *bla*$^+$	This study
P1vir			25
Plasmids			
pITS557	pGEM3	*entC*$^+$	31
pRS415		*lacZ*$^+$ *lacY*$^+$ *bla*$^+$	34
pMO1	pRS415	Φ(*entC'-lacZ*) *lacY*$^+$ *bla*$^+$	This study
pJP101		*menD*$^+$	30

multiple lysogens as described by Cotter and Gunsalus (7). The single lysogen was designated as MC4100(λMO1).

Effects of iron, dipyridyl, oxygen, and carbon source on expression of *entC-lacZ*. In order to verify the expression of the gene, the fusion strain was grown aerobically in the minimal salts medium with Casamino Acids and glucose and was assayed for β-galactosidase activity. The enzyme was fully derepressed and showed 1,155 U of activity. In order to determine whether iron would repress the β-galactosidase activity, cells were grown in the presence of 1 to 100 μM $FeCl_3$. It was found that at 1 μM the enzyme activity was unaffected (1,158 U), while at 5 μM the activity was repressed (80 U). Increasing the iron concentration further did not repress the activity significantly further (data not shown). In spite of the fact that the enzyme was fully derepressed in iron-deficient media, we added dipyridyl at a concentration of 50 μM in all experiments to ensure consistent iron deficiency. Addition of dipyridyl did not appreciably change the level of activity (Fig. 2). As expected, this finding is in complete agreement with the known regulatory properties of the *entC* gene (5). To determine the effect of oxygen, cultures were incubated anaerobically in the absence of iron and the enzyme activity was assayed. Surprisingly, the anaerobic cultures showed twice the activity of the aerobic cultures (Fig. 2). When the aerobic cultures were grown in the presence of an oxidizable carbon source, the activity was similar to that of anaerobic glucose-grown cultures (Fig. 2).

Effects of alternate electron acceptors NO_3, fumarate, and TMAO on *entC-lacZ* expression. Since the enzyme was expressed under anaerobic conditions in the absence of iron, and since *E. coli* can use NO_3, fumarate, and TMAO as electron acceptors, it was of interest to determine the effect of these compounds on *entC* expression. Accordingly, cells were grown anaerobically on glucose media in the presence and absence of iron with each electron acceptor and then assayed for β-galactosidase activity. As seen in Fig. 3A, enzymatic activity was at least twofold higher when cells were grown anaerobically, both in the presence and absence of the electron acceptors, compared with that for cells grown aerobically on glucose medium (Fig. 2 and 3A). The highest level of activity was observed when cells were grown in the presence of NO_3.

To confirm the effect of electron acceptors, the fermentable carbon source glucose was replaced with the oxidizable carbon source glycerol and cells were assayed for enzymatic activity. Again, the level of enzymatic activity obtained when cells were grown in the presence of the electron acceptors (data not shown) was higher than that obtained when cells were grown aerobically on glucose.

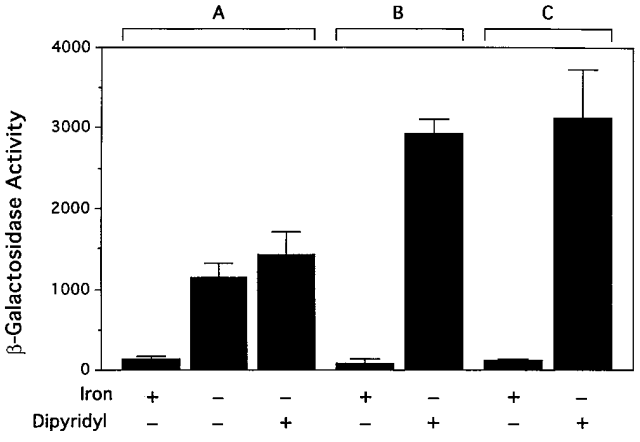

FIG. 2. Effects of iron, dipyridyl, and carbon source in the presence and absence of oxygen on *entC-lacZ* expression. (A) Aerobic glucose medium in the presence of 50 μM iron, the absence of iron, and the presence of 50 μM dipyridyl in the absence of iron; (B) anaerobic glucose medium in the presence of iron and the absence of iron with dipyridyl; (C) aerobic glycerol medium in the presence of iron and the absence of iron with dipyridyl. Error bars, standard deviations.

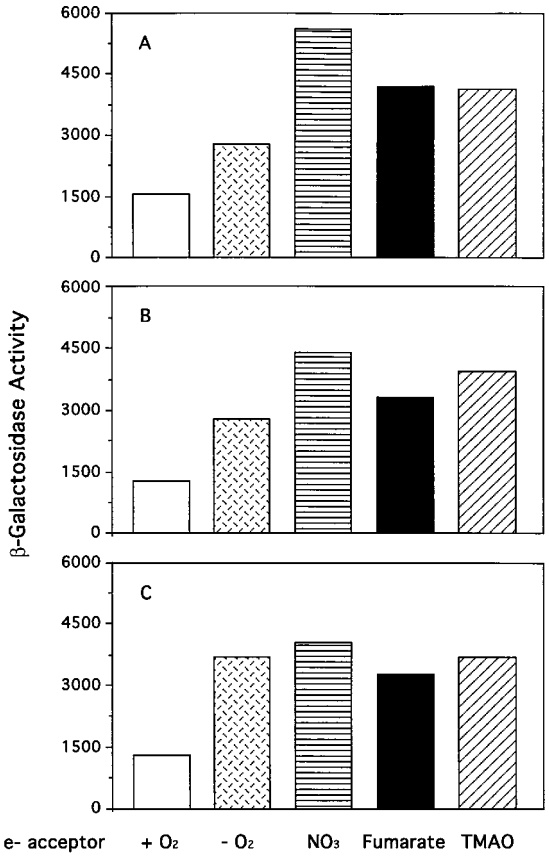

FIG. 3. Effects of various electron (e-) acceptors in glucose media on *entC-lacZ* expression under conditions of iron deficiency. (A) Wild type; (B) *arcA*; (C) *fnr*.

Effects of *arcA*, *fnr*, and *fur* on *entC-lacZ* expression. A number of regulatory mutations known to affect the expression of other operons was tested to see whether they affected the expression of *entC-lacZ*. Accordingly, fusion strains carrying these mutations were grown in glucose media in the presence and absence of iron with various electron acceptors. As seen from Fig. 3B and C, the level of expression remained about the same as that of the wild-type strain in the case of *arcA* and *fnr* mutants. In contrast, the *fur* mutation, which is known to cause constitutive expression of the *entC* gene (2), showed high levels of enzymatic activity with the various electron acceptors, irrespective of whether the medium was iron sufficient or deficient, both in the presence and absence of oxygen (Fig. 4).

Aerobic growth of *ent*$^+$ and *entC* and *entA* mutants in glucose medium under iron-sufficient and -deficient conditions. The aerobic growth of the *ent*$^+$ strain AB1515 was determined under conditions of iron deficiency and sufficiency. It was found that the strain grew at the same rate with a slightly diminished growth yield in the absence of iron compared with that in the presence of iron (Fig. 5A). The fusion strain MC4100 showed identical results (data not shown). In contrast, the *entC* mutant under conditions of iron deficiency showed a decrease in growth yield and an increase in lag (2 h) compared with growth under iron-sufficient conditions (Fig. 5A). For comparative purposes an isogenic *entA* mutant was used. The *entA* mutant showed an even greater lag (12 h) under the same conditions.

Absence of menaquinone requirement for anaerobic growth of *entC* mutant in glucose medium under iron-sufficient and -deficient conditions. As shown above, isochorismate synthase

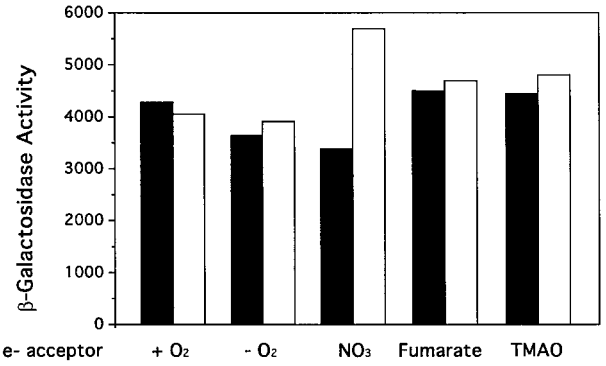

FIG. 4. Effects of *fur* mutation on *entC-lacZ* expression in glucose media in the presence of various electron (e-) acceptors in the presence (solid bars) and absence (open bars) of iron.

encoded by the *entC* gene is derepressed under iron-deficient and repressed under iron-sufficient conditions, irrespective of whether the cells were grown in the presence or absence of oxygen. Unlike the established menaquinone deficient mutants

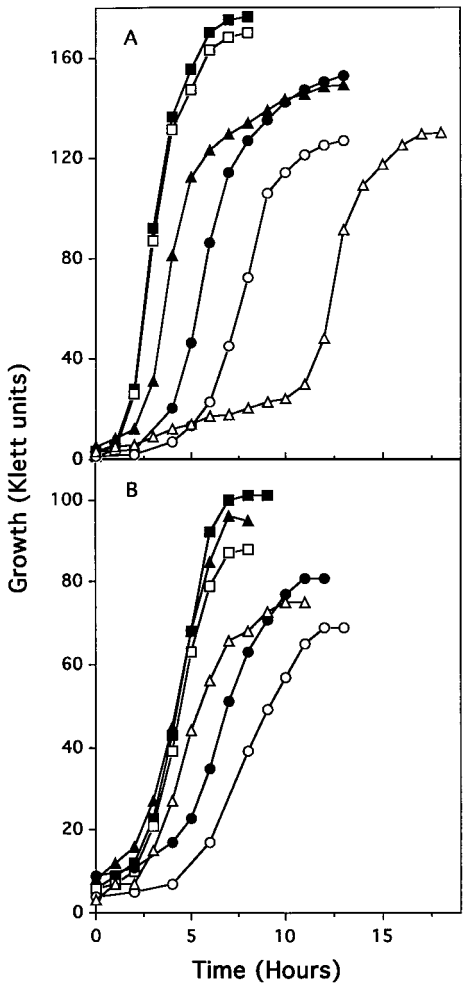

FIG. 5. Growth of wild-type and *ent* mutant strains in glucose media under aerobic (A) and anaerobic (B) conditions in the presence (filled symbols) and absence (open symbols) of iron. Squares, AB1515 *ent*$^+$; circles, MT147 *entC*; triangles, RM923 *entA*.

(*men*), the *entC* mutant does not require either uracil or the menaquinone precursors OSB or 1,4-dihydroxy-2-naphthoic acid for best anaerobic growth on glucose media. As expected (Fig. 5B), the *entC*$^+$ strain, AB1515, grew well anaerobically both in the presence and absence of iron. The *entC* and *entA* mutants each showed an increased lag of about an hour and a decreased growth yield under conditions of iron deficiency compared with results under conditions of iron sufficiency (Fig. 5B). However, the lag was significantly less than that observed under aerobic conditions.

Absence of menaquinone requirement for anaerobic growth with alternate electron acceptors. It is well established that for the reduction of nitrate, ubiquinone is required (17), while menaquinone is obligatory for growth on fumarate and TMAO as electron acceptors (17, 20). Hence, the *entC*$^+$ strain was inoculated into media containing the oxidizable carbon source glycerol and nitrate, fumarate, or TMAO as electron acceptors under anaerobic conditions. With nitrate as an electron acceptor, the *ent*$^+$ strain showed a decrease in growth yield in iron-deficient medium compared with results in iron-sufficient medium (Fig. 6A). As expected, in the absence of nitrate no growth was observed either in the presence or absence of iron (data not shown).

The *entC* mutant showed an increase in lag of about 2 h in addition to a decrease in growth yield under conditions of iron deficiency compared with results under conditions of iron sufficiency (Fig. 6A). The *entA* mutant showed an even greater lag (about 9 h) (Fig. 6A).

In glycerol medium with fumarate as the electron acceptor, the *ent*$^+$ strain showed a decrease in growth rate and yield under conditions of iron deficiency (Fig. 6B). In iron-sufficient media, the *entC* and *entA* mutants showed the same growth rate as the parent *ent*$^+$ strain with an increase in lag. However, the *entC* mutant showed a great reduction in growth rate under conditions of iron deficiency compared with the *ent*$^+$ strain (Fig. 6B). The *entA* mutant showed an even greater reduction in growth rate than did the *entC* mutant under conditions of iron deficiency (Fig. 6B).

The anaerobic growth of the *ent*$^+$, *entC*, and *entA* strains in glycerol media with TMAO as an electron acceptor under iron-sufficient and -deficient conditions was similar to that obtained in glycerol-fumarate media described above (data not shown).

Presence of menaquinone and isochorismate synthase activity in the *entC* mutant. As demonstrated above, the *entC* mutant was able to grow anaerobically on iron-sufficient media with fumarate or TMAO as electron acceptors. It is also well established that for the utilization of fumarate or TMAO as electron acceptors, the presence of menaquinone is obligatory (12, 20). To verify the presence of menaquinone, we analyzed anaerobically grown cells of the *entC* mutant and those of its parent, AB1515, by established procedures (Materials and Methods). It was found that the mutant cells had as much menaquinone as did the parent cells (data not shown). Since for the biosynthesis of menaquinone the presence of isochorismate synthase is obligatory, a cell extract was assayed for the presence of isochorismate synthase activity. The enzyme activity was found to be 0.6 nmol/h/mg of protein.

Biosynthesis of enterobactin by *entC*$^+$ strains and mutants under aerobic and anaerobic conditions. Since the *entC-lacZ* fusion was expressed anaerobically on glucose-containing media and on glycerol-NO_3, glycerol-fumarate, and glycerol-TMAO media (Fig. 3A), we wanted to determine whether this expression is reflected in the biosynthesis of enterobactin. Consequently, we undertook an analysis of enterobactin accumulation in supernatants from cultures grown in various media.

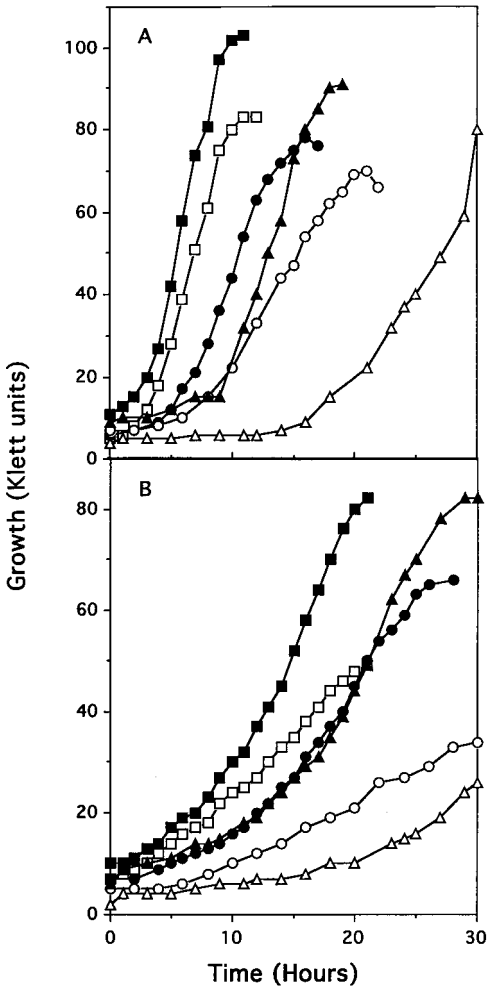

FIG. 6. Anaerobic growth of wild-type and *ent* mutant strains in glycerol media with nitrate (A) or fumarate (B) as electron acceptors. Filled symbols, growth with iron; open symbols, growth without iron. Squares, AB1515 *ent*$^+$; circles, MT147 *entC*; triangles, RM923 *entA*.

Cultures were inoculated into the media, and the growth of each culture was monitored. When the cultures reached stationary phase, cells were removed by centrifugation and the supernatant was analyzed for enterobactin as described in Materials and Methods. As seen in Table 2, the *entC*$^+$ strains produced low or extremely low concentrations of enterobactin in the presence of iron when the cells were grown aerobically, anaerobically on glucose media, or anaerobically on glycerol media with NO_3, fumarate, or TMAO as electron acceptors. In contrast, in the absence of iron, enterobactin biosynthesis was fully derepressed under all conditions. On the other hand, the *entA* mutant produced no enterobactin, irrespective of whether iron was present. In the case of the *entC* mutant in the presence of iron, no enterobactin was produced. In the absence of iron, low levels of enterobactin were produced only when an alternate electron acceptor was present.

DISCUSSION

The pathways involved in the biosynthesis of enterobactin (3, 13, 38) and menaquinone (4) have been reviewed and indicate that isochorismate is a common intermediate for the biosynthesis of both enterobactin (27) and menaquinone (4). It

TABLE 2. Effects of iron, carbon source, and electron acceptors on enterobactin formation by various strains

Strain	Iron[a]	Enterobactin formation[b] on carbon source (electron acceptor)				
		Glucose ($+O_2$)[c]	Glucose ($-O_2$)[d]	Glycerol (NO_3)	Glycerol (fumarate)	Glycerol (TMAO)
AB1515 *ent*$^+$	+	11	0	8	15	7
	−	78	33	107	142	138
MC4100 *ent*$^+$	+	7	0	0	0	0
	−	292	71	97	134	103
MT147 *entC*	+	0	0	0	0	0
	−	0	0	12	20	13

[a] +, growth with iron; −, growth without iron.
[b] The enterobactin was assayed, and units of activity were calculated as described previously (2, 10) and are given in micromoles per milligram (dry weight) of cells (10). The strain RM923 *entA* showed no enterobactin formation in the presence or absence of iron on any of the carbon sources with the given electron acceptor.
[c] Aerobically grown.
[d] Anaerobically grown.

has been reported that the isochorismate synthase encoded by the *entC* gene supplies the isochorismate required for both pathways (16). However, the established conditions under which enterobactin and menaquinone are synthesized and perform their functions are contradictory.

Enterobactin is required for the transport of iron when the availability of this metal is limiting (11, 26). The poor availability of this metal under aerobic conditions is due to the poor solubility of Fe^{3+} in water (11, 26). Since under anaerobic conditions iron is present in the highly soluble Fe^{2+} form, it is generally believed that the synthesis of enterobactin is unnecessary for the growth and metabolism of the cell (11, 26).

In contrast, menaquinone functions under anaerobic conditions in *E. coli* (4). When the organism is grown with fumarate, TMAO, or dimethyl sulfoxide as the electron acceptor, the presence of menaquinone is obligatory (12, 20, 24). When oxygen or nitrate is the electron acceptor, *E. coli* uses the aerobic quinone, ubiquinone (17). It can be stated that while the conditions which favor ubiquinone biosynthesis and function are compatible with the biosynthesis and function of enterobactin, they are incompatible with those of menaquinone.

This apparent contradiction raises intriguing questions. How does *E. coli*, while growing aerobically under iron-deficient conditions, when the *entC* gene-encoded isochorismate synthase is fully derepressed, prevent the synthesis of menaquinone? Similarly, under anaerobic conditions, how does *E. coli* prevent the synthesis of enterobactin when menaquinone synthesis is fully induced?

At first we thought that this paradox might be resolved if the *entC* gene were to be regulated by iron in the presence of O_2 and independently by the requirement for menaquinone in the absence of O_2. To study this regulation, we generated an operon fusion of the *entC* gene (*entC-lacZ*) and introduced it in a single copy into the chromosome. This fusion strain was used to study the regulation. In media containing glucose as the carbon source, the enzyme was derepressed by iron deficiency and repressed by iron, both in the presence and absence of oxygen. In fact, the expression in anaerobically grown cells was twofold higher than that in aerobically grown cells. Since the enzyme was derepressed on the fermentable carbon source, glucose, we wanted to determine the effect of alternate electron acceptors in the presence of the fermentable carbon source glucose and the oxidizable carbon source glycerol. As electron acceptors, NO_3, fumarate, and TMAO were selected. The rationale for this selection was that it included electron acceptors whose reduction depends on either ubiquinone or menaquinone so that possible differences in regulation could become evident. Although both quinones are made under both aerobic and anaerobic conditions, more ubiquinone than menaquinone is made aerobically and more menaquinone than ubiquinone is made anaerobically. It has been clearly shown that the utilization of O_2 and NO_3 is dependent on the presence of ubiquinone (17, 20). Consequently, growth of menaquinone-deficient (*men*) mutants with NO_3 as the electron acceptor is unaffected (20). In contrast, for the utilization of fumarate and TMAO, the presence of menaquinone is obligatory and, consequently, *men* mutants are unable to grow utilizing these compounds as electron acceptors (12, 20).

It was found that *entC-lacZ* was expressed to the same extent on NO_3, fumarate, and TMAO as were cells grown fermentatively. With glycerol as a carbon source, expression was similar. A number of regulatory mutants were tested to determine their effects on the expression of *entC-lacZ*. It was found that *arcA* and *fnr* had no effect on expression. In contrast, the *fur* mutation resulted in constitutive expression of the enzyme.

Since *entC-lacZ* was expressed under both aerobic and anaerobic conditions, we determined the effect of the *entC* mutation on aerobic and anaerobic growth. Hence, the *entC* mutant and its parent strain were grown aerobically in glucose minimal medium with and without iron. The wild-type strain AB1515 grew at the same rate, with a slightly diminished yield, under conditions of iron deficiency compared with conditions of iron sufficiency. The growth under conditions of iron deficiency is due to the biosynthesis of enterobactin and the consequent scavenging of traces of iron in the medium. As expected, the *entC* mutant grew at the same rate as did the parent in the presence of iron. Surprisingly, under conditions of iron deficiency the growth rate was unaltered although the lag period was increased, suggesting the possibility of some enterobactin biosynthesis. In order to explore this possibility, an *entA* mutant which is blocked in the enterobactin pathway further down (Fig. 1) was tested. Unlike that of the *entC* mutant, the growth of the *entA* mutant was severely impaired under iron-deficient conditions. However, the growth of neither mutant was as severely affected during iron deficiency under fermentative conditions on glucose. This is consistent with the diminished need for iron under fermentative conditions.

The fact that the growth of the *entC* mutant under iron-sufficient conditions (under which the *entC*$^+$ gene is completely repressed) anaerobically on fumarate and TMAO occurred at the same rate as those for the *entC*$^+$ strain and the *entA* mutant suggested that the *entC* mutant is able to synthesize sufficient isochorismate to meet the demand for menaquinone biosynthesis. It is well established that the presence of menaquinone is obligatory for the reduction of fumarate and TMAO (12, 20). Hence, we undertook an analysis of menaquinone in the *entC* mutant and its parent *entC*$^+$ strain AB1515. It was found that the parent strain produced as much menaquinone as the mutant strain. Enzymatic analysis by conversion of the small amount of isochorismate formed in the presence of the bifunctional enzyme SHCHC synthase–2-ketoglutarate decarboxylase followed by thermal conversion of the SHCHC to OSB (29, 31), revealed the presence of an isochorismate synthase activity of 0.6 nmol/h/mg of protein. This observation provided evidence for the existence of an alternate isochorismate synthase activity. Hence, we undertook a search for the gene encoding this alternate isochorismate synthase. Such a gene was identified and localized at 51 min on the *E.*

coli chromosome, 5′ to the *menD* gene in the *men* cluster, and designated *menF* (9, 21).

Consistent with the presence of two isochorismate synthases is the fact that neither *entC* mutants nor *menF* mutants have been isolated during screenings for mutants blocked in enterobactin and menaquinone, although mutants blocked in other reactions of the pathways have been described previously (4, 12, 38). Mutants previously described as *entC* turned out to be *entA* (for a discussion, see the publications of McIntosh and colleagues [27, 36]). Subsequently, after sequence analysis and identification of an open reading frame showing similarities to a family of chorismate utilizing enzymes, a *kan* cassette was engineered into this open reading frame. The *kan* gene was inserted in the opposite orientation, which prevented transcription of the entire operon because of polarity effects on all the downstream genes (27). In this mutant, although the entire *ent* operon was repressed, supplementation of the medium with 2,3-dihydroxybenzoic acid (DHBA) (an intermediate after isochorismate) resulted in a low level of enterobactin production (27). This low level of expression of the *ent* operon might explain the near-normal growth of the *entC* mutant with O_2 or the anaerobic growth with NO_3 as an electron acceptor. It has been previously established that the reduction of O_2 and NO_3 are ubiquinone dependent (17). This is consistent with the fact that the growth of the *men* mutants with O_2 and NO_3 as electron acceptors is not affected (20). Since menaquinone is not required for O_2 and NO_3 reduction, the *menF*-encoded isochorismate synthase probably provides sufficient isochorismate for a low level of enterobactin biosynthesis, thus bypassing the block imposed by the *entC* mutation. Consistent with this is the fact that the *entA* mutant which has a block in the enterobactin biosynthetic pathway subsequent to isochorismate shows extremely poor growth with O_2 and NO_3 as electron acceptors.

This argument is further strengthened by imposing a requirement for menaquinone by growing the organism on fumarate or TMAO as an electron acceptor. As already mentioned, for the utilization of these two electron acceptors, menaquinone is required (12, 20, 35). When fumarate is used as the electron acceptor, the *menF*-encoded isochorismate synthase is required for the biosynthesis of menaquinone. Hence, in iron-deficient fumarate medium, growth of the *entC* mutant was extremely poor and resembled that of the *entA* mutant.

The anaerobic expression of the *entC* gene led to an examination of the biosynthesis of enterobactin under anaerobic conditions. This study clearly establishes that enterobactin is synthesized under anaerobic conditions under conditions of iron deficiency. The anaerobic biosynthesis of enterobactin demonstrated in this study complements the previous demonstration of anaerobic uptake of ferrienterobactin and the demonstration of the siderophore outer membrane proteins FepA and FhuA in anaerobic iron-deficient cells (19). It is generally assumed that the solubility of ferrous iron, being greater than that of ferric iron, makes the requirement for enterobactin unnecessary (11, 26). However, this assumption has been questioned on the ground that it is very unlikely that the highly hydrated ferrous ion can pass freely through the cell membrane (19). Recently, a ferrous ion uptake system in anaerobically grown *E. coli* has been reported (11). Further, as pointed out by Lodge and Emery (19) specific high-affinity transport systems for both magnesium and manganous ions are present in anaerobically grown *E. coli* (14, 33), although both ions are more soluble than ferrous ion.

It appears that there are no detailed studies on the biosynthesis of enterobactin in anaerobically grown *E. coli*. It has been stated in a review citing unpublished experiments that *E. coli* K-12 does not produce enterobactin anaerobically (32). In contrast, Lodge and Emery (19) have reported that spent supernatants of cultures from iron-deficient anaerobically grown cells gave a positive Arnow reaction for catechols, suggesting that anaerobically grown cells do synthesize enterobactin. Cells grown anaerobically in the presence of iron gave a negative Arnow reaction. However, no data were provided (19).

Studies reported in the present paper clearly establish for the first time that enterobactin is synthesized under anaerobic conditions. However, the exact role of enterobactin in anaerobically growing cells remains to be determined. As pointed out by Guerinot (11) in a recent review, "one of the next challenges will be to understand the interrelationships of the various regulatory systems involved, either directly or indirectly, in monitoring iron levels in microbial cells."

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REFERENCES

1. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **118:**531–537.
2. **Bagg, A., and J. B. Neilands.** 1985. Mapping of a mutation affecting regulation of iron uptake systems in *Escherichia coli* K-12. J. Bacteriol. **161:**450–453.
3. **Bentley, R.** 1990. The shikimate pathway—a metabolic tree with many branches. Crit. Rev. Biochem. Mol. Biol. **25:**307–384.
4. **Bentley, R., and R. Meganathan.** 1987. The biosynthesis of the isoprenoid quinones ubiquinone and menaquinone. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, p. 512–520. American Society for Microbiology, Washington, D.C.
5. **Brickman, T. J., B. A. Ozenberger, and M. A. McIntosh.** 1990. Regulation of divergent transcription from the iron-responsive *fepB-entC* promoter-operator regions in *Escherichia coli*. J. Mol. Biol. **212:**669–682.
6. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759–4764.
7. **Cotter, P. A., and R. P. Gunsalus.** 1989. Oxygen, nitrate, and molybdenum regulation of *dmsABC* gene expression in *Escherichia coli*. J. Bacteriol. **171:** 3817–3823.
8. **Cotter, P. A., and R. P. Gunsalus.** 1992. Contribution of the *fnr* and *arcA* gene products in coordinate regulation of cytochrome *o* and *d* oxidase (*cyo ABCDE* and *cydAB*) genes in *Escherichia coli*. FEMS Microbiol. Lett. **91:** 31–36.
9. **Daruwala, R., O. Kwon, M. E. S. Hudspeth, and R. Meganathan.** 1994. Menaquinone (vitamin K_2) biosynthesis: evidence for a new gene (*menF*) encoding an alternate isochorismate synthase, abstr. K-158, p. 303. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology, 1994. American Society for Microbiology, Washington, D.C.
10. **Foster, M. S., J. N. Carroll, and E. C. Neiderhoffer.** 1994. Phenylalanine- and tyrosine-dependent production of enterobactin in *Escherichia coli*. FEMS Microbiol. Lett. **117:**79–84.
11. **Guerinot, M. L.** 1994. Microbial iron transport. Annu. Rev. Microbiol. **48:** 743–772.
12. **Guest, J. R.** 1979. Anaerobic growth of *Escherichia coli* K-12 with fumarate as terminal electron acceptor. Genetic studies with menaquinone and fluoroacetate-resistant mutants. J. Gen. Microbiol. **115:**259–271.
13. **Haslam, E.** 1993. Shikimic acid. Metabolism and metabolites. John Wiley & Sons, Inc., New York.
14. **Jasper, P., and S. Silver.** 1977. Magnesium transport in microorganisms, p. 7–47. *In* E. D. Weinberg (ed.), Microorganisms and minerals. Marcel Dekker, New York.
15. **Jones, H. M., and R. P. Gunsalus.** 1987. Regulation of *Escherichia coli* fumarate reductase (*frdABCD*) operon expression by respiratory electron acceptors and the *fnr* gene product. J. Bacteriol. **169:**3340–3349.
16. **Kaiser, A., and E. Leistner.** 1990. Role of *entC* gene in enterobactin and menaquinone biosynthesis in *Escherichia coli*. Arch. Biochem. Biophys. **276:** 331–335.
17. **Kröger, A.** 1977. Phosphorylative electron transport with fumarate and nitrate as terminal electron acceptors. Symp. Soc. Gen. Microbiol. **27:**61–93.

18. **Kwon, O., M. E. S. Hudspeth, and R. Meganathan.** 1993. Anaerobic regulation of *entC* gene expression by iron and evidence against its involvement in vitamin-K biosynthesis in *Escherichia coli*, abstr. K-69, p. 272. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.
19. **Lodge, J. S., and T. Emery.** 1984. Anaerobic iron uptake by *Escherichia coli*. J. Bacteriol. **160:**801–804.
20. **Meganathan, R.** 1984. Inability of *men* mutants of *Escherichia coli* to use trimethylamine-*N*-oxide as an electron acceptor. FEMS Microbiol. Lett. **24:**57–62.
21. **Meganathan, R.** 1996. Biosynthesis of the isoprenoid quinones menaquinone (vitamin K_2) and ubiquinone (coenzyme Q), p. 642–656. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
22. **Meganathan, R., and R. Coffel.** 1985. Identity of the quinone in *Bacillus alcalophilus*. J. Bacteriol. **164:**911–913.
23. **Melville, S. B., and R. P. Gunsalus.** 1990. Mutations in *fnr* that alter anaerobic regulation of electron transport-associated genes in *Escherichia coli*. J. Biol. Chem. **265:**18733–18736.
24. **Miguel, L., and R. Meganathan.** 1991. Electron donors and the quinone involved in dimethyl sulfoxide reduction in *Escherichia coli*. Curr. Microbiol. **22:**109–115.
25. **Miller, J. H.** 1992. A short course in bacterial genetics. Laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Neilands, J. B.** 1984. Siderophores of bacteria and fungi. Microbiol. Sci. **1:**9–14.
27. **Ozenberger, B. A., T. J. Brickman, and M. A. McIntosh.** 1989. Nucleotide sequence of *Escherichia coli* isochorismate synthase gene *entC* and evolutionary relationship of isochorismate synthase and other chorismate-utilizing enzymes. J. Bacteriol. **171:**775–783.
28. **Palaniappan, C., V. Sharma, M. E. S. Hudspeth, and R. Meganathan.** 1992. Menaquinone (vitamin K_2) biosynthesis: evidence that the *Escherichia coli menD* gene encodes both 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid synthase and α-ketoglutarate decarboxylase activities. J. Bacteriol. **174:**8111–8118.
29. **Palaniappan, C., H. Taber, and R. Meganathan.** 1994. Biosynthesis of *o*-succinylbenzoic acid in *Bacillus subtilis*: identification of *menD* mutants and evidence against the involvement of the α-ketoglutarate dehydrogenase complex. J. Bacteriol. **176:**2648–2653.
30. **Popp, J. L.** 1989. Sequence and overexpression of the *menD* gene from *Escherichia coli*. J. Bacteriol. **171:**4349–4354.
31. **Popp, J. L., C. Berliner, and R. Bentley.** 1989. Vitamin K (menaquinone) biosynthesis in bacteria: high performance liquid chromatographic assay of overall synthesis of *o*-succinylbenzoic acid and of 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid synthase. Anal. Biochem. **178:**306–310.
32. **Rosenberg, H., and I. G. Young.** 1974. Iron transport in enteric bacteria, p. 67–82. *In* J. B. Neilands (ed.), Microbial iron metabolism. Academic Press, New York.
33. **Silver, S., and P. Jasper.** 1977. Manganese transport in microorganisms, p. 105–149. *In* E. D. Weinberg (ed.), Microorganisms and minerals. Marcel Dekker, New York.
34. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
35. **Spencer, M. E., and J. R. Guest.** 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bacteriol. **114:**563–570.
36. **Tummuru, M. K. R., T. J. Brickman, and M. A. McIntosh.** 1989. The *in vitro* conversion of chorismate to isochorismate catalyzed by the *Escherichia coli entC* gene product. J. Biol. Chem. **264:**20547–20551.
37. **Unden, G.** 1988. Differential roles for menaquinone and demethylmenaquinone in anaerobic electron transport of *E. coli* and their *fnr*-independent expression. Arch. Microbiol. **150:**499–503.
38. **Walsh, C. T., J. Liu, F. Rusnak, and M. Sakaitani.** 1990. Molecular studies on enzymes in chorismate metabolism and the enterobactin biosynthetic pathway. Chem. Rev. **90:**1105–1129.
39. **Young, I. G., T. J. Batterham, and F. Gibson.** 1969. The isolation, identification and properties of isochorismic acid, an intermediate in the biosynthesis of 2,3-dihydroxybenzoic acid. Biochim. Biophys. Acta **177:**389–400.