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Derepression of Certain Aromatic Amino Acid Biosynthetic Enzymes of *Escherichia coli* K-12 by Growth in Fe^{3+}-Deficient Medium[1]

JOSEPH W. McCRAY, JR.,[2] AND KLAUS M. HERRMANN*
Department of Biochemistry, Purdue University, Lafayette, Indiana 47907

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3-Deoxy-*arabino*-heptulosonic acid 7-phosphate synthase, prephenate dehydratase, tryptophan synthase, and 2,3-dihydroxybenzoylserine synthase enzyme activities are derepressed in wild-type *Escherichia coli* K-12 cells grown on Fe^{3+}-deficient medium. This derepression is reversed when $FeSO_4$ is added to the growth medium. Addition of shikimic acid to the Fe^{3+}-deficient growth medium causes repression of the first three enzyme activities but not of 2,3-dihydroxybenzoylserine synthase activity. Addition of 2,3-dihydroxybenzoic acid to the Fe^{3+}-deficient growth medium has no effect on any of the above-mentioned enzyme activities. The Fe^{3+} deficiency-mediated derepression of 3-deoxy-*arabino*-heptulosonic acid 7-phosphate synthase activity is due to an elevation of the tyrosine-sensitive isoenzyme; the phenylalanine-sensitive isoenzyme is not derepressed under these conditions.

Growth of *Escherichia coli* in an Fe^{3+}-deficient medium elicits numerous physiological responses (24, 26, 31-33), including derepression of the biosynthetic pathway to enterochelin, an Fe^{3+} chelator excreted by *E. coli* (2, 21). Enterochelin is a cyclic trimer of 2,3-dihydroxybenzoylserine (DHBS) (22). The 2,3-dihydroxybenzoic acid (DHBA) moieties of enterochelin are synthesized from chorismic acid via a pathway repressed by Fe^{3+} (33).

Chorismic acid is also the branch point intermediate from which all of the aromatic amino acids and aromatic vitamins are derived (23). Synthesis of chorismic acid is regulated by feedback inhibition and repression of the enzymes catalyzing the first reaction unique to the biosynthesis of aromatic compounds. In *E. coli* this reaction, the conversion of phosphoenolpyruvate and erythrose-4-phosphate to 3-deoxy-*arabino*-heptulosonic acid 7-phosphate (DAHP), is catalyzed by three isoenzymes (EC 4.1.2.15), each of which is regulated by one of the aromatic amino acids. DAHP synthase TYR is inhibited and repressed by tyrosine, DAHP synthase PHE is inhibited by phenylalanine and repressed by phenylalaine plus tryptophan, and DAHP synthase TRP is inhibited and repressed by tryptophan (3). Utilization of chorismic acid for aromatic amino acid biosynthesis is also regulated by feedback inhibition and repression of each of the three terminal pathways by one of the three aromatic amino acids (23).

In this paper we report that certain aromatic amino acid biosynthetic enzymes usually repressed by aromatic amino acids are derepressed by growth of *E. coli* in an Fe^{3+}-deficient medium. This derepression is reversed by the addition of either Fe^{3+} or shikimic acid to the Fe^{3+}-deficient growth medium. Possible mechanisms underlying this observation are discussed.

MATERIALS AND METHODS

Bacteria. All experiments were done with the *E. coli* K-12 strains listed in Table 1.

Chemicals. Erythrose-4-phosphate (27), DAHP (14), and phosphoenolpyruvate (7) were synthesized in our laboratory. Erythrose-4-phosphate solutions adjusted to pH 4.5 were assayed with glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (25). Solutions of the monocyclohexylammonium salt of phosphoenolpyruvate were adjusted to pH 6.5 and assayed by the coupled pyruvate kinase (EC 2.7.1.40)-lactate dehydrogenase (EC 1.1.1.27) reaction. Chorismic acid was isolated from the culture medium of *Aerobacter aerogenes* by the method of Gibson (12). The barium salt of prephenate was prepared by alkaline treatment of chorismate (11). Potassium prephenate was prepared by treating barium prephenate with an excess of K_2SO_4. Prephenate was assayed by acid hydrolysis to phenylpyruvate (8). L[U-^{14}C]serine (>100 mCi/mmol) was obtained from New England Nuclear

[1] Paper no. 5082 of the Purdue University Agricultural Experiment Station.

[2] Present address: Sandoz Forschungs-Institut, A-1235 Wien, Austria.

Corp., Boston. All other chemicals were of the purest grade obtainable commercially.

Media. All glassware used in the cultivation of bacteria was washed with aqua regia and autoclaved twice with glass-distilled water (30). The rich medium used was L broth (17). The E salts minimal medium of Vogel and Bonner (29) was extracted with 8-hydroxyquinoline-$CHCl_3$ to remove Fe^{3+} (30). Because 8-hydroxyquinoline-$CHCl_3$ extraction removes trace metals other than Fe^{3+} required for maximal growth, 1 ml of a micronutrient salts solution (20) per liter of medium was added. Waring and Werkman (30) estimated that this medium contained 0.013 to 0.054 μM Fe^{3+}. Glucose extracted with 8-hydroxyquinoline-$CHCl_3$ was added to a final concentration of 0.4% (wt/vol). Growth medium prepared in this way is called medium A. Solutions of $FeSO_4$ were prepared just before use and were used without sterilization. It was assumed that Fe^{2+} was quantitatively oxidized to Fe^{3+} by oxygen in the air (13). Shikimic acid, amino acids, and DHBA solutions were also extracted with 8-hydroxyquinoline-$CHCl_3$ before addition to the growth medium.

Growth of bacteria. Bacteria from a fresh overnight L broth culture were washed twice with sterile saline and diluted 50-fold into medium A. After incubation at 37 C with shaking for 10 or 11 h, 2-ml volumes from this culture were used to inoculate a series of flasks for the experiment. These flasks were incubated at 37 C with shaking for about 11 h. The cells were harvested by centrifugation and washed once with ice-cold saline.

Preparation of extracts. The bacteria from a 100-ml culture were suspended in 5 ml of 0.05 M potassium phosphate (pH 6.5) containing 10 mM phosphoenolpyruvate and 1 mM dithiothreitol. These suspensions were forced through a French pressure cell at 20,000 lb/in^2 to break the cells. After centrifugation at 40,000 rpm for 90 min in a Spinco type 40 rotor, the supernatants were collected and assayed for enzyme activities and protein.

Enzyme assays. DAHP synthase was assayed essentially by the procedure of Doy and Brown (10). One unit of DAHP synthase activity is defined as 1 μmol of DAHP formed at 37 C in 10 min. The contributions of the individual DAHP synthase TYR and DAHP synthase PHE isoenzymes were determined by a modification of the method of Brown and Doy (4). DAHP synthase PHE activity was assumed to be equivalent to the activity inhibited by 1 mM phenylalanine. DAHP synthase TYR was assumed to be equivalent to the activity inhibited by 1 mM tyrosine.

Dehydroquinate synthase (no Enzyme Commission number yet) was assayed after the disappearance of DAHP as measured by the thiobarbituric acid assay (10). One enzyme unit is defined as 1 nmol of DAHP disappearing at 37 C in 1 min.

Tryptophan synthase (EC 4.2.1.20) was assayed by the method of Smith and Yanofsky (28). One unit of tryptophan synthase catalyzes the conversion of 0.1 μmol of indole to tryptophan at 37 C in 20 min.

Prephenate dehydratase (EC 4.2.1.51) was assayed essentially by the procedure of Cotton and Gibson (8). One enzyme unit catalyzes the formation of 1.0 μmol of phenylpyruvate at 37 C in 10 min.

TABLE 1. *List of E. coli K-12 derivatives used*

Strain	Genotype	Source
W1485	Wild type	C. Yanofsky
AN191	*entC401* and 4 other markers	F. Gibson
AB1515	*purE42* and 14 other markers	E. A. Adelberg CGSC 1515
HE400	*purE42*	Constructed by phage P1 transduction and penicillin selection: donor, AB1515; acceptor, W1485
HE401	*entC401*	Constructed by phage P1 transduction: donor, AN191; acceptor, HE400

DHBS synthase (EC 6.3.2.14) was assayed by the procedure of Brot and Goodwin (1). One enzyme unit catalyzes, at 37 C in 15 min, the conversion of 1 nmol of L-[^{14}C]serine into products that are extracted from acidic solutions by ethyl acetate. This assay measures the incorporation of L-serine into enterochelin and its degradation products (5, 6).

Isochorismate synthase (EC 5.4.99.6) was assayed according to Young and Gibson (33) by fluorometric determination of salicylate formed from isochorismate upon heating.

Glutamate dehydrogenase (EC 1.4.1.4) and glutamate synthase (EC 2.6.1.53) activities were measured by following the rate of reduced nicotinamide adenine dinucleotide phosphate oxidation (18). One enzyme unit is defined as 1 μmol of substrate oxidized at 25 C per min.

Protein determination. Protein was determined by the method of Lowry et al. (16), with ovalbumin as a standard.

RESULTS

Enzyme activities in extracts of wild-type E. coli cells grown in Fe^{3+}-deficient medium. When wild-type *E. coli* cells are grown in medium A, the growth is stimulated by the addition of $FeSO_4$ (Fig. 1). Maximal growth is obtained when approximately 0.6 μM $FeSO_4$ is added to the growth medium.

Furthermore, the activities of certain aromatic biosynthetic enzymes vary with the iron content of the growth medium. DHBS synthase, prephenate dehydratase, tryptophan synthase, and total DAHP synthase activities are derepressed in extracts of cells grown in Fe^{3+}-deficient medium (Fig. 2). The increase of DHBS synthase is expected since Brot et al. (2) reported that this activity is repressed by Fe^{3+}. However, the dependence upon Fe^{3+} of the activities of the other three aromatic biosynthetic enzymes is unexpected and has not been

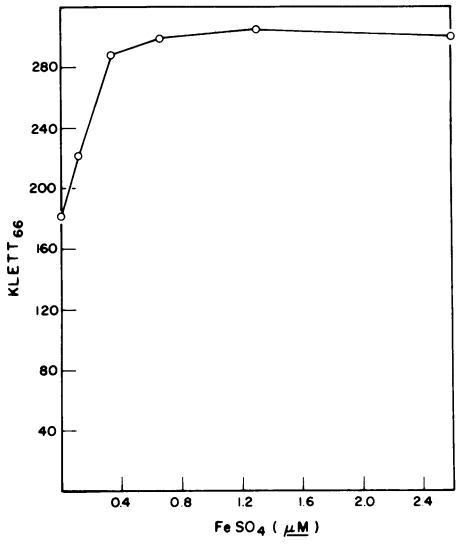

FIG. 1. *Dependence of growth yield of E. coli W1485 on iron supplementation of the growth medium. Wild-type E. coli cells were grown in medium A supplemented with various concentrations of $FeSO_4$. The turbidity (Klett 66) of the cultures after incubation for 11 h is plotted versus the concentration of $FeSO_4$ added to the growth medium.*

observed previously. Prephenate dehydratase is an enzyme in the terminal pathway to phenylalanine and is normally repressed by phenylalanine; tryptophan synthase is the last enzyme in the terminal pathway to tryptophan and is normally repressed by tryptophan; and DAHP synthase is the first enzyme in the common aromatic pathway. It consists of three isoenzymes and, as shown below, the isoenzyme normally repressed by tyrosine is the one that is dependent upon the Fe^{3+} content of the growth medium. The Fe^{3+}-mediated derepression of all these aromatic biosynthetic enzymes is reversed by addition of about 0.6 μM $FeSO_4$ to the growth medium. The patterns of derepression of these enzymes are qualitatively very similar (Fig. 2); only DHBS synthase requires larger amounts of $FeSO_4$ for repression. At 2.5 μM $FeSO_4$, this activity is no longer detectable.

The Fe^{3+}-mediated derepression seems to be a characteristic of some regulatory enzymes in aromatic biosynthesis. Dehydroquinate synthase, the product of the *aroB* gene, is not affected (Table 2). Also, glutamate dehydrogenase and glutamate synthase, two enzymes from unrelated pathways, do not show the Fe^{3+}-mediated derepression (Table 2). The activity of the iron-sulfur protein glutamate synthase (19) appears to be reduced (Table 2) when wild-type *E. coli* W1485 is grown in medium A supplemented with 0.1 μM $FeSO_4$. This is also a good indication that under these conditions the cells are starving for iron.

Effect of shikimic acid on the Fe^{3+} deficiency-mediated derepression of aromatic biosynthetic enzymes. Since our observations indicate that the Fe^{3+} deficiency is simultaneously affecting all enzymes repressed by any of the aromatic amino acids, we added shikimic acid, a precursor of all aromatic amino acids, to the Fe^{3+}-deficient growth medium. The addition of up to 500 μM shikimic acid to the growth medium causes the repression of DAHP synthase (Fig. 3), prephenate dehydratase, and tryptophan synthase (data not shown), but DHBS synthase is not significantly repressed (Fig. 3). Shikimic acid appears to be able to overcome the putative deficiency causing derepression of the aromatic amino acid-regulated enzymes in the Fe^{3+}-deficient medium. That the DHBS synthase levels are not affected by the addition of shikimic acid to the growth medium is consistent with the observation by Young and Gibson (33) that the enterochelin pathway is regulated solely by Fe^{3+} even in a mutant with a complete block in the common pathway.

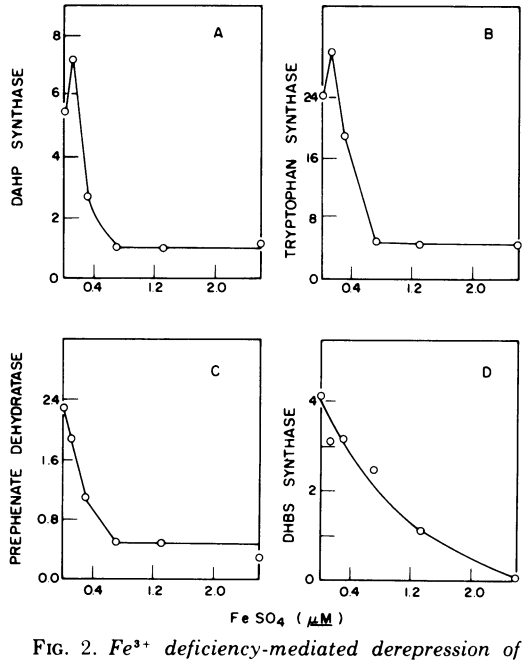

FIG. 2. *Fe^{3+} deficiency-mediated derepression of certain aromatic biosynthetic enzymes. Specific enzyme activities (units per milligram of protein) are plotted versus concentrations of $FeSO_4$ added to the growth medium: (A) DAHP synthase, (B) tryptophan synthase, (C) prephenate dehydratase, and (D) DHBS synthase.*

TABLE 2. *Specific enzyme activities in cell extracts of wild-type E. coli W1485*[a]

Medium A supplements		Sp act[b]					
$FeSO_4$ (μM)	AAA (μg/ml)	DAHP synthase TYR	DAHP synthase PHE	DHQS	TRPS	GDH	GLUS
0.1	0	3.4	2.1	3.3	12.0	0.398	0.053
2.5	0	0.2	2.1	3.8	4.1	0.278	0.185
0.1	100	0.2	0.8	3.7	1.2	0.458	0.081
2.5	100	0.1	0.6	3.3	0.7	0.262	0.199

[a] Abbreviations: AAA, aromatic amino acids phenylalanine, tyrosine, and tryptophan; DHQS, dehydroquinate synthase; TRPS, tryptophan synthase A; GDH, glutamate dehydrogenase; GLUS, glutamate synthase.
[b] For a description of the units, see Materials and Methods.

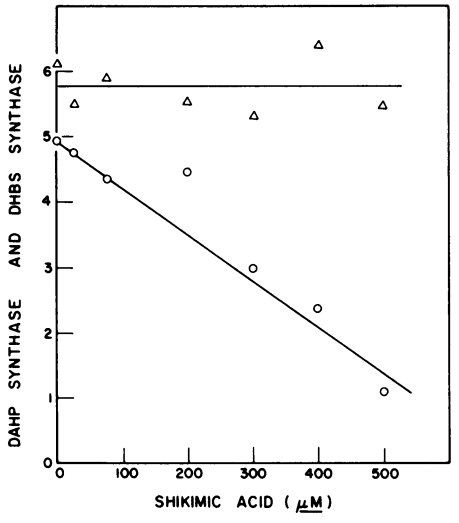

FIG. 3. *Effect of shikimic acid on Fe^{3+} deficiency-mediated derepression of aromatic biosynthetic enzymes. Specific enzyme activities are plotted versus concentrations of shikimic acid added to the growth medium:* (O) *DAHP synthase,* (Δ) *DHBS synthase.*

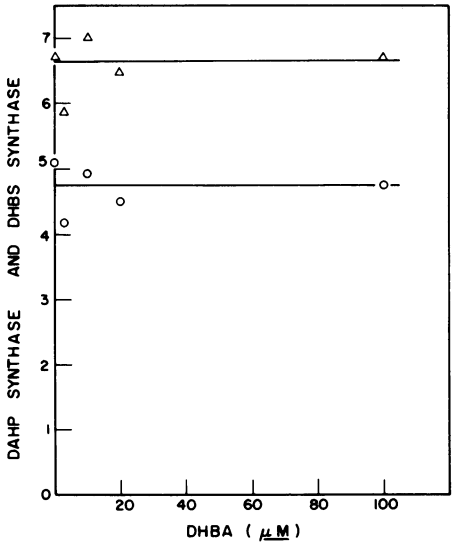

FIG. 4. *Effect of DHBA on Fe^{3+} deficiency-mediated derepression of aromatic biosynthetic enzymes. Specific enzyme activities are plotted versus concentrations of DHBA added to the growth medium:* (O) *DAHP synthase,* (Δ) *DHBS synthase.*

The results of these experiments suggest that addition of excess aromatic amino acids to the growth medium would reverse the Fe^{3+}-mediated derepression of aromatic amino acid biosynthetic enzymes. This is indeed the case (Table 2).

Effect of DHBA on the Fe^{3+} deficiency-mediated derepression of aromatic biosynthetic enzymes. When up to 100 μM DHBA, an intermediate in the pathway from chorismate to enterochelin, is added to the growth medium, no effects on the levels of DAHP synthase or DHBS synthase (Fig. 4) are found. Therefore, DHBA cannot overcome the effect of Fe^{3+} deficiency-mediated derepression of aromatic amino acid-regulated enzymes. That DHBS synthase activity is not affected by the addition of DHBA to the growth medium is consistent with the observation that DHBA does not appear to be involved in the regulation of the enterochelin pathway (33).

Selective derepression of DAHP synthase TYR in Fe^{3+}-deficient medium. In wild-type *E. coli* cell extracts, about 80% of the total DAHP synthase activity is contributed by the phenylalanine-sensitive isoenzyme, about 20% is contributed by the tyrosine-sensitive isoenzyme, and only traces of the tryptophan sensitive isoenzyme can be found. We repeated the experiment depicted in Fig. 2A, but this time measured the amounts of the phenylalanine- and tyrosine-sensitive portions of the total DAHP synthase activity. The results (Fig. 5) show that Fe^{3+} deficiency has no effect on the phenylalanine-sensitive isoenzyme but that the

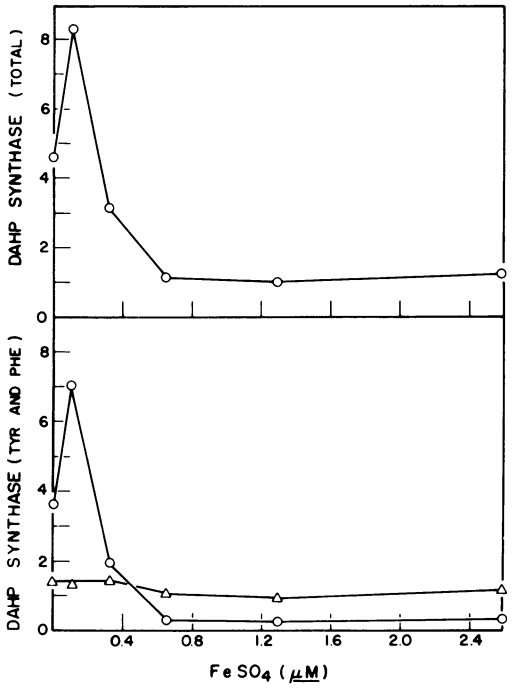

FIG. 5. *Fe³⁺ deficiency-mediated derepression of DAHP synthase. Specific enzyme activities are plotted versus concentrations of $FeSO_4$ added to the growth medium. Top, total DAHP synthase activity; bottom, DAHP synthase TYR (O) and DAHP synthase PHE (Δ) plotted separately.*

tyrosine-sensitive isoenzyme is derepressed about 30-fold when wild-type cells are grown in Fe^{3+}-deficient medium. This extent of derepression of the tyrosine-sensitive isoenzyme is also found in cells of an *aroD* mutant strain grown on limiting aromatic amino acids and aromatic vitamins (data not shown). An assessment of the tryptophan-sensitive isoenzyme was not attempted. The observation that the tyrosine-sensitive, but not the phenylalanine-sensitive, isoenzyme is derepressed is noteworthy because prephenate dehydratase, another enzyme repressed by phenylalanine, is derepressed about 10-fold in cells grown on Fe^{3+}-deficient medium.

Fe^{3+}-mediated derepression of aromatic biosynthetic enzymes in entC mutant cells. Fe^{3+} uptake in *E. coli* involves the Fe^{3+} chelator enterochelin. The first step in the terminal pathway to enterochelin, the conversion of chorismate to isochorismate, is catalyzed by isochorismate synthase. The structural gene for isochorismate synthase is *entC*. Thus, *entC* mutant cells cannot use chorismate for enterochelin biosynthesis. Furthermore, in *entC* mutant cells chorismate cannot be channeled into the enterochelin pathway. To obtain a pair of isogenic *entC*/*entC*$^+$ strains for comparison of aromatic biosynthetic enzyme levels in such cells grown in Fe^{3+}-deficient medium, the *entC401* allele of strain AN191 was transduced into the W1485 genetic background. The transductant HE401 grows in minimal salts medium supplemented with succinate and citrate or with succinate and DHBA. The transductant does not grow in minimal salts medium supplemented with succinate alone. These growth requirements are characteristic for *ent* mutants. To positively identify strain HE401 as an *entC* mutant, an extract of cells grown in Fe^{3+}-deficient medium was prepared and applied to a Bio-Gel diethylaminoethyl column. Column fractions were analyzed for isochorismate synthase as described by Young and Gibson (33). The results are plotted in Fig. 6 together with data obtained from a parallel experiment performed with an extract of cells from wild-type strain W1485. It is clear that the cell extract of strain HE401 does not contain isochorismate synthase activity. Much to our surprise, however, Fe^{3+} deficiency-mediated derepression of aromatic biosynthetic enzymes in strain HE401

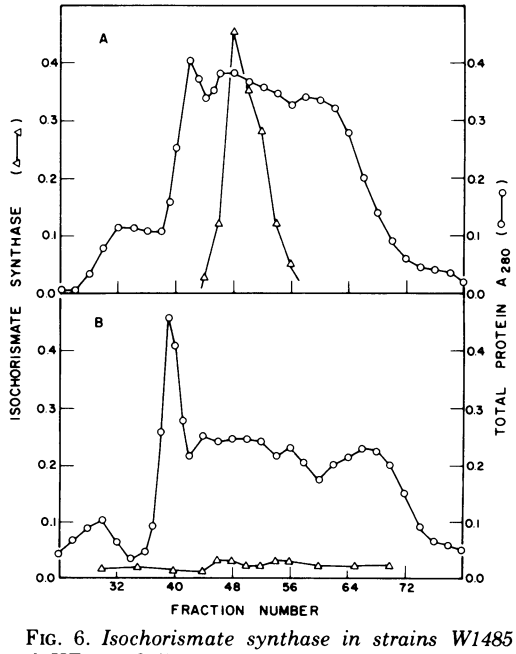

FIG. 6. *Isochorismate synthase in strains W1485 and HE401. Cell extracts of strain W1485 (A) and strain HE401 (B) were fractionated on Bio-Gel diethylaminoethyl, and the fractions were assayed for isochorismate synthase by the method of Young and Gibson (33). Total protein (O) and enzyme activity (Δ) are plotted versus the fraction number.*

is qualitatively very similar to the derepression seen in the wild-type strain W1485 (Fig. 7). The significance of the small quantitative differences between the responses of the aromatic biosynthetic enzymes to Fe^{3+} deficiency in strains W1485 and HE401 has not been ascertained.

DISCUSSION

Growth of wild-type *E. coli* K-12 in Fe^{3+}-deficient medium causes derepression of aromatic amino acid biosynthetic enzymes that are normally repressed by the aromatic amino acids. This derepression elicited by the Fe^{3+} deficiency is reversed through the addition of Fe^{3+} or shikimic acid, but not DHBA, to the growth medium. A related observation (Y. K. Bakshi and R. P. Williams, Bacteriol. Proc., p. 145, 1969) states that the activity of DAHP synthase in *Bacillus anthracis* varies with the concentration of iron in the growth medium and that cells grown in an iron-deficient medium excrete 3,4-dihydroxybenzoic acid when resuspended in phosphate buffer containing glucose. Downer et al. (9) suggest that this may be caused by reduced levels of aromatic amino acids due to diversion of chorismate to iron-chelating compounds. This suggestion is based on the observation that *Bacillus subtilis* (9) and *A. aerogenes* (33) grown on Fe^{3+}-deficient medium excrete DHBA and that the synthesis of DHBA is not regulated by DHBA in *A. aerogenes* (33).

Most results given in this paper and in the report of elevated levels of certain aromatic amino acid-regulated biosynthetic enzymes in extracts of a *tonB* mutant strain of *E. coli* (J. McCray, Jr., R. Schoner, and K. M. Herrmann, Fed. Proc. **32**:1357, 1973) are consistent with the suggestion of Downer et al. (9). Briefly, under Fe^{3+}-deficient growth conditions, DHBA synthesis is derepressed and depletes the intracellular pool of chorismic acid, especially since there is no feedback inhibition of DHBA synthesis in *E. coli* (33). Exogenously supplied shikimic acid would replenish the chorismic acid pool. Enough chorismic acid would be available to maintain sufficient levels of aromatic amino acids to prevent the derepression of their pathways. Repression of the enterochelin pathway by increasing the Fe^{3+} concentration of the growth medium would have the same effect: increased availability of chorismic acid for aromatic amino acid biosynthesis and, thus, repression of the terminal aromatic amino acid pathways. Supplementation of the Fe^{3+}-deficient medium with DHBA would not be expected to affect the derepression of aromatic

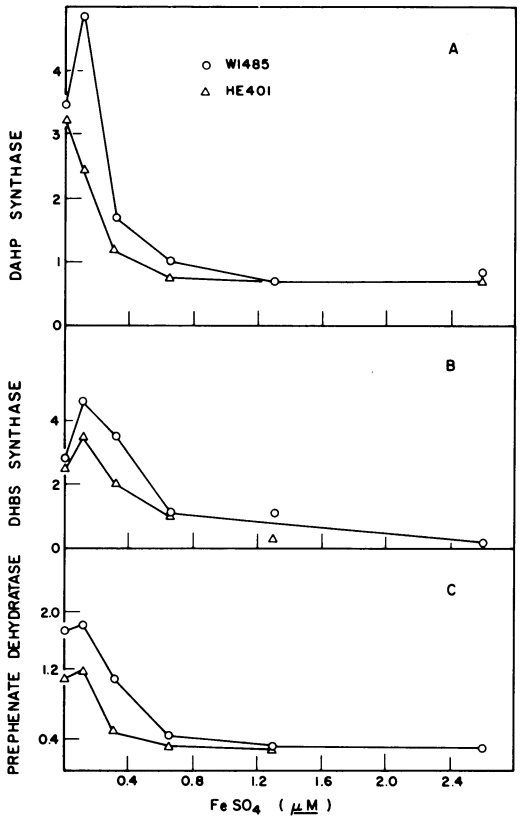

FIG. 7. *Fe^{3+} deficiency-mediated derepression of aromatic biosynthetic enzymes in cells of a pair of isogenic entC/entC$^+$ strains. Specific enzyme activities of strain W1485 (○) and strain HE401 (△) are plotted versus concentrations of $FeSO_4$ added to the growth medium: (A) DAHP synthase, (B) DHBS synthase, (C) prephenate dehydratase.*

amino acid-regulated enzymes since DHBA has no regulatory effect on the enterochelin pathway and is not able to feed into the chorismic acid pool.

However, the results obtained with strain HE401 seem to conflict with this interpretation of the data. One possible alternative explanation of our results would involve the putative repressor of the enterochelin pathway in the regulation of the aromatic amino acid biosynthetic operons. Although this kind of multiple regulator control has been reported in the galactose system (14), there is no direct evidence for or against it in the aromatic biosynthetic system.

Data given in Table 2 and Fig. 3 exclude the fact that Fe^{3+} is necessary for the synthesis of repressor or co-repressor of aromatic amino acid operons. Therefore, it appears that another possible interpretation of our results involves

Fe^{3+} in the expression of a different, hitherto unrecognized regulatory signal. It is tempting to speculate that this signal involves cognate transfer ribonucleic acids since it has been reported (26, 31) that a number of transfer ribonucleic acids, including some species recognizing tyrosine, phenylalanine, and tryptophan, lack certain modifications in *E. coli* B cells grown on Fe^{3+}-deficient medium. It is possible that this lack of modification could cause the type of derepression we have observed.

The fact that Fe^{3+} deficiency-mediated derepression discriminates between DAHP synthase TYR and DAHP synthase PHE suggests two different mechanisms for the regulation of the biosynthesis of these two isoenzymes. More experimentation is necessary to elucidate the mechanism underlying Fe^{3+} deficiency-mediated derepression of the aromatic amino acid biosynthetic enzymes.

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