JOURNAL OF BACTERIOLOGY, July 1977, p. 331-339

Vol. 131, No. 1
Printed in U.S.A.

Coordinate Regulation by Iron of the Synthesis of Phenolate Compounds and Three Outer Membrane Proteins in *Escherichia coli*

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Received for publication 15 February 1977

The biosynthesis of the low-molecular-weight iron carrier enterochelin and of three outer membrane polypeptides appears to be coordinately regulated by the amount of cell-associated iron in *Escherichia coli* K-12. Measurements of iron acquisition made throughout the growth cycle in iron-deficient media indicate that a very rapid accumulation of iron occurs in the first 2 h of growth; there is comparatively little iron uptake during exponential growth, which results in a gradual decrease in the cellular iron content with each generation. When this level falls below 400 ng of iron per mg (dry weight) of cells, there is a simultaneous onset of synthesis of the three outer membrane polypeptides and of enterochelin. This coordinate regulation was also observed in cells able to transport iron actively using only citrate as an iron-carrier.

Three independent high-affinity systems for iron transport have been described for *Escherichia coli* B and K-12 strains (24). The major transport system involves the solubilization of iron from a medium by the bacterial siderophore enterochelin (enterobactin) and transport into the cell as a ferric-enterochelin complex (16). In addition, iron can be transported by the fungal siderophore ferrichrome (19), and in the presence of citrate an auxiliary system is induced that brings complexes of ferric-citrate into the cell (9). *E. coli* cells also have a low-affinity iron uptake system, which may function by passive diffusion (28).

Transport of ferric-siderophore complexes involves system-specific receptors in the outer membrane of the *E. coli* envelope. It has been known for several years that the product of the *tonA* gene is an outer membrane polypeptide that, among other functions (8), serves as a receptor site for ferric-ferrichrome complexes (13, 20). Recently, outer membrane receptors that function as components of the other two high-affinity systems have been tentatively identified. Several groups (11, 22, 23, 26) have observed outer membrane proteins whose synthesis is affected by the amount of iron in the medium. In *E. coli* K-12, at least three polypeptides appear to be regulated in this manner; one, an 81,000-dalton protein (81K) specified by the *feuB* gene, may be the receptor for ferric-enterochelin complexes (12). The precise function(s) in iron uptake of the other two polypeptides (83K and 74K) is unknown. A citrate-inducible protein involved in iron transport has also been reported (12).

Regulation of high-affinity iron transport may involve a system in which intracellular iron serves as a corepressor of the synthesis of the components of the transport mechanisms (4, 5, 24). This implies that once the intracellular iron concentration falls below a specific level, synthesis of iron transport components becomes derepressed. Evidence exists that enzymes involved in enterochelin biosynthesis are so regulated (5, 29). The experiments reported here were conducted to study the relationship between the cell-associated iron concentration and the synthesis of components of these iron uptake systems. We have determined the minimal cell-associated concentration of iron necessary to repress the synthesis of at least three outer membrane polypeptides and suggest that synthesis of these proteins is regulated coordinately with the biosynthesis of enterochelin. The precise regulation of the synthesis of specific outer membrane polypeptides by iron and their coordinate control with enterochelin biosynthesis provide additional evidence that these proteins play an important role in the assimilation of iron. It is also shown that when *E. coli* is grown in iron-deficient medium, a very rapid and efficient accumulation of iron occurs in the earliest portion of the growth cycle. A steady decrease in the amount of cell-associated iron with each generation indicates that little additional iron is taken up during the remainder of the growth cycle.

MATERIALS AND METHODS

Strains. *E. coli* K-12 strains were used exclusively. CR63 (F^+ *supD60*) was from the culture collection of this laboratory. AX116 and its *lon* derivative, AX117 (27), were provided by J. R. Walker; AB1515 (F^- *proC14 leu-6 trpE38 purE42 tonA23*) (16) was obtained from the Coli Genetic Stock Center; and the AB1515 derivatives AN102 (*fep*, unable to transport ferric-enterochelin) (6) and RW193 (*entA*, unable to synthesize enterochelin) (17) were obtained from C. E. Lankford and J. B. Neilands, respectively.

Media and reagents. M9 and T media have been described (22). Minimal media were supplemented with 0.4% glucose and 5 μg of thiamine per ml. Where needed, L-leucine, L-proline, and L-tryptophan were added to a final concentration of 50 μg/ml, and deoxyadenosine was added to 150 μM; $FeCl_3$ and sodium citrate were added at the concentrations indicated. The cation exchange resin Chelex 100 was obtained from Bio-Rad Laboratories; 2,3-dihydroxybenzoic acid (Aldrich Chemical Co.) was a gift of C. E. Lankford; the disodium salt of 3-(2-pyridyl)-5,6-bis-(4-phenylsulfonic acid)-1,2,4-triazine, available commercially as ferrozine solution, was obtained from Hach Chemical Co.; and 8-quinolinol (8-hydroxyquinoline) was purchased from Eastman Chemical Products.

Preparation of iron-depleted media. M9 medium was depleted of available iron by two procedures. In one procedure, contaminating trace metals were removed from a 5× concentrated solution by three passages through the cation exchange resin Chelex 100. After each passage, the resin was washed and regenerated. The extracted medium was filter-sterilized and stored at room temperature; it was diluted into sterile demineralized water and supplemented as described for growth of cells. The second procedure was a modification of that of Pugsley and Reeves (23). A 10× concentrate of M9 medium was extracted with 1% 8-hydroxyquinoline and repeatedly back-extracted with chloroform until no hydroxyquinoline could be detected in the chloroform phase by spectrophotometry (244 nm). Both procedures decreased the iron concentration of M9 medium by approximately 1 μM, to a final concentration of 0.4 μM.

Determination of iron. Iron was measured by flameless atomic absorption spectroscopy in a Perkin-Elmer model 306 spectrophotometer and colorimetrically by using the iron chelator ferrozine (25). The two iron assay methods yielded identical results for measurements on both cell-associated iron and the iron content of media. In the latter determinations, however, it was necessary to use a deuterium background corrector to eliminate salt effects from the spectrophotometric measurements. Iron-free glassware for these experiments was prepared by soaking in 1 N HCl for 8 h, followed by rinsing with demineralized water. Glassware was sterilized in a hot-air oven.

Isolation of membrane. Total membrane was isolated by the procedure of Inouye and Guthrie (14).

Polyacrylamide gel electrophoresis. Membranes were solubilized at 100°C in the digestion mix described by Laemmli (15). Electrophoresis of samples containing 40 μg of protein was carried out on 10% sodium dodecyl sulfate-polyacrylamide slab gels in a discontinuous buffer system (15) as described previously (22) or on 11% slab gels in the system described by Lugtenberg et al. (21), as modified by Hancock et al. (12). Gels were stained by the procedure of Fairbanks et al. (7). Molecular weight standards included phosphorylase A (94,000), lactoperoxidase (92,000), urease (80,000), bovine serum albumin (68,000), and ovalbumin (43,000).

Chemical analyses. Protein was assayed by the procedure of Lowry et al. (18). Phenolate production was determined by the Arnow reaction (1) and used as an assay for enterochelin and enterochelin-related compounds. For purposes of brevity, all phenolates measured will be referred to in the text as enterochelin.

RESULTS

The synthesis of several large outer membrane polypeptides is derepressed when *E. coli* is grown under iron-limiting conditions. The number of such proteins that can be detected depends upon both the strain being examined and the conditions of electrophoresis. Thus, we identified two iron-regulated polypeptides in *E. coli* B (22), and two (23, 26) or three (12) similarly regulated polypeptides in K-12 strains have been reported. Using the gel system of Lugtenberg et al. (21), we have been able to demonstrate the iron-dependent regulation of three outer membrane polypeptides corresponding to 83K, 81K, and 74K (3) in K-12 strains, but we have not found a protein corresponding to 83K in *E. coli* B strains. (In our hands, the 83K, 81K, and 74K of Braun et al. [3] have molecular weights of 88K, 83K, and 75K, respectively; for clarity, the nomenclature of Braun et al. will be used.) The effect of iron on the membrane protein composition of several *E. coli* K-12 strains is shown in Fig. 1. It is important to note that the electrophoresis procedure of Laemmli (15) was used; in this system, 83K and 81K cannot be resolved readily. When CR63, AX116, and AX117 were grown in iron-deficient medium (T), 83-81K and 74K were prevalent; their synthesis was repressed, however, when these strains were grown in M9 or in T medium supplemented with 10 μM $FeCl_3$. This result strongly suggested that iron was the component responsible for the medium-dependent alterations in AX116 and AX117 noted previously (D. E. Lentzen and E. E. M. Moody, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P74, p. 157). In contrast, AN102, a mutant unable to transport ferric-enterochelin complexes (6), had derepressed levels of 83-81K and 74K when grown in M9, which contains

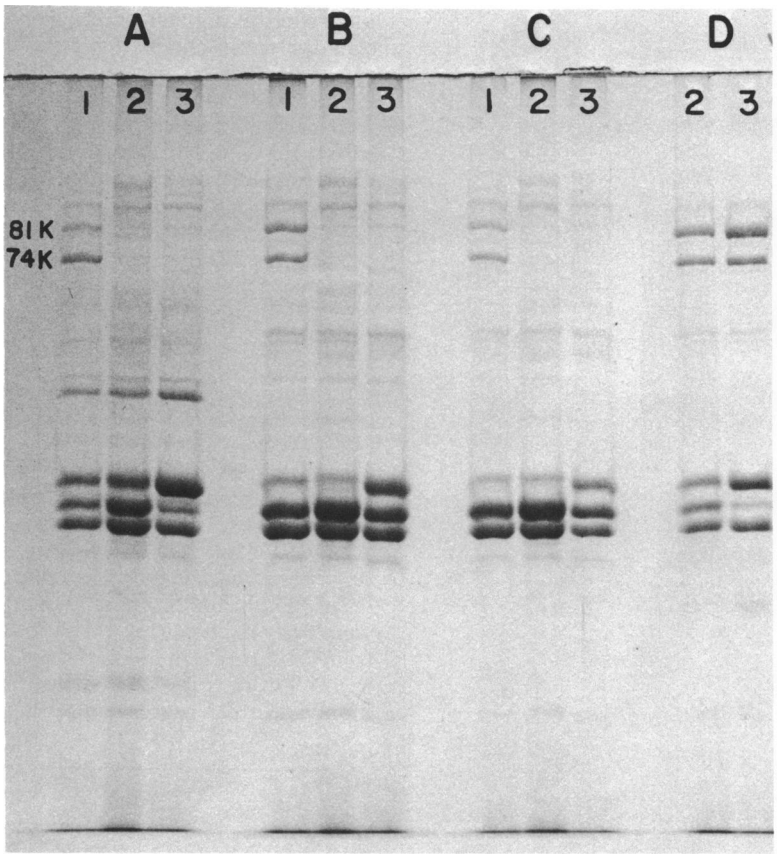

FIG. 1. *Effect of media on the membrane protein composition of E. coli. K-12 strains were grown to a concentration of 2 × 10⁸ cells per ml in T medium (1), T medium supplemented with 10 μM $FeCl_3$ (2), or M9 medium (3). Total membranes were isolated and their protein complements were analyzed by polyacrylamide gel electrophoresis as described by Laemmli (15). Strains studied were CR63 (A), AX116 (B), AX117 (C), and AN102 (D).*

adequate iron (22), or in iron-supplemented T medium and failed to grow in unsupplemented T medium. Derepression of these proteins was also observed when a strain (RW193) unable to synthesize enterochelin was grown in iron-supplemented T medium (data not shown).

Citrate enhances the growth of AN102 in iron-deficient medium by inducing the citrate-dependent iron uptake system (6). Figure 2 illustrates that the membrane protein complements of midexponential-phase AN102 cells contained reduced amounts of 83-81K and 74K when they were prepared from cells grown in the presence of 100 μM or 1 mM citrate.

These results indicated that it was the intracellular iron concentration, and not the specific iron transport system being used, that was important in affecting the synthesis of 83-81K and 74K. The same conclusion has recently been reported by Braun et al. (3). A similar control mechanism exists for enterochelin biosynthesis; AN102 growing in the presence of 10 mM citrate produced less than one-tenth the amount of enterochelin and enterochelin-related compounds than when growing in its absence (6). The effect of 1 mM citrate on enterochelin production was followed throughout the AN102 growth cycle (Fig. 3). In the absence of citrate, enterochelin synthesis paralleled cellular growth. In the presence of this citrate concentration, the level of enterochelin in the culture medium was low until late in the growth cycle, when enterochelin biosynthesis became derepressed; the final concentration was similar to that produced by AN102 cells grown in the absence of citrate.

The production of 83-81K and 74K was similar to the pattern of enterochelin synthesis when total membrane preparations isolated from AN102 cells at various stages of the

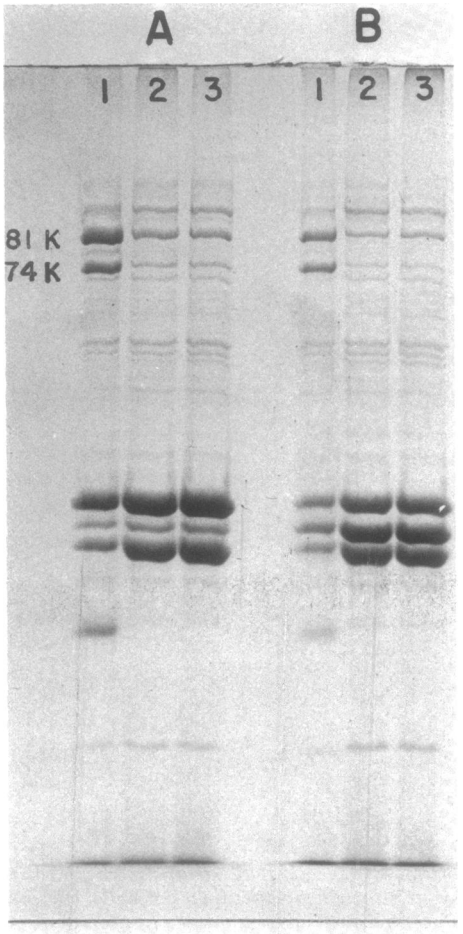

FIG. 2. *Effect of citrate on the protein composition of total membranes from AN102. Total membrane preparations from fep cells grown to a concentration of 2 × 10^8 cells per ml in M9 medium (A) or in T medium supplemented with 10 μM $FeCl_3$ (B) were analyzed as in Fig. 1. The media were supplemented with sodium citrate as follows: no citrate (1); 100 μM citrate (2); and 1 mM citrate (3).*

growth cycle were examined (Fig. 4). Large quantities of both proteins were synthesized throughout the growth cycle when no citrate was added. In medium supplemented with a citrate concentration (1 mM) unable to maintain repression of enterochelin synthesis throughout the growth cycle, the synthesis of these proteins was repressed during the early and midexponential growth phases; however, large quantities of 83-81K and 74K were present in late-exponential- and stationary-phase cells.

These results suggested that the synthesis of specific outer membrane components is regulated coordinately with the synthesis of the iron chelator enterochelin and that this control is exerted by the intracellular iron content. Additional experiments designed to test this idea were carried out by growing AB1515, the parental strain of AN102, in M9 minimal salts medium rendered iron deficient by Chelex 100 or 8-hydroxyquinoline treatment. To determine the amount of iron associated with cells grown under different iron conditions, AB1515 cells were grown in Chelex 100-treated M9 medium supplemented with concentrations of iron ranging from 0 to 5 μM. The cells were harvested in stationary phase, and total membrane preparations were isolated. In addition, dry-weight determinations and iron assays by atomic absorption spectroscopy were performed on each culture (Table 1). It can be seen that the levels of cell-associated iron in stationary-phase cells increased with increasing iron supplements. Analysis of the membrane composition of these cells (Fig. 5A) demonstrated that the three outer membrane proteins (83K, 81K, and 74K) were fully expressed in all samples except that from cells grown in medium supplemented with 5 μM $FeCl_3$ (Fig. 5A6). The amount of iron involved in the repression of these proteins is,

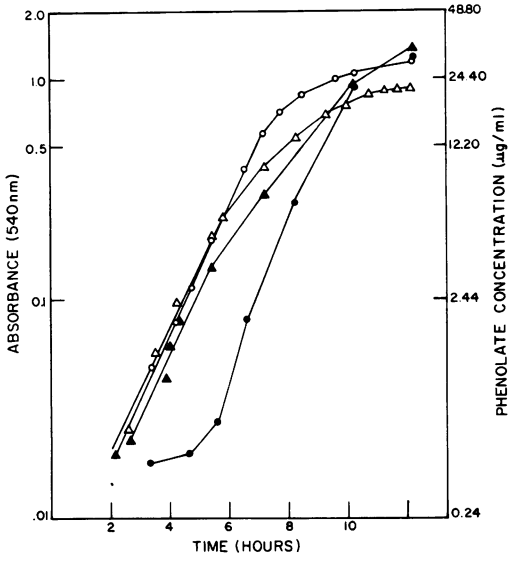

FIG. 3. *Effect of citrate on the production of enterochelin during the growth cycle of AN102. Cells were grown in M9 medium in the absence or presence of 1 mM sodium citrate. Samples (10 ml) were taken at various intervals and centrifuged, and the supernatant fluids were assayed for phenolates. Absorbance measurements are shown in open symbols, and phenolate concentrations are given by closed symbols. M9 medium (△,▲); M9 + 1 mM citrate (○,●).*

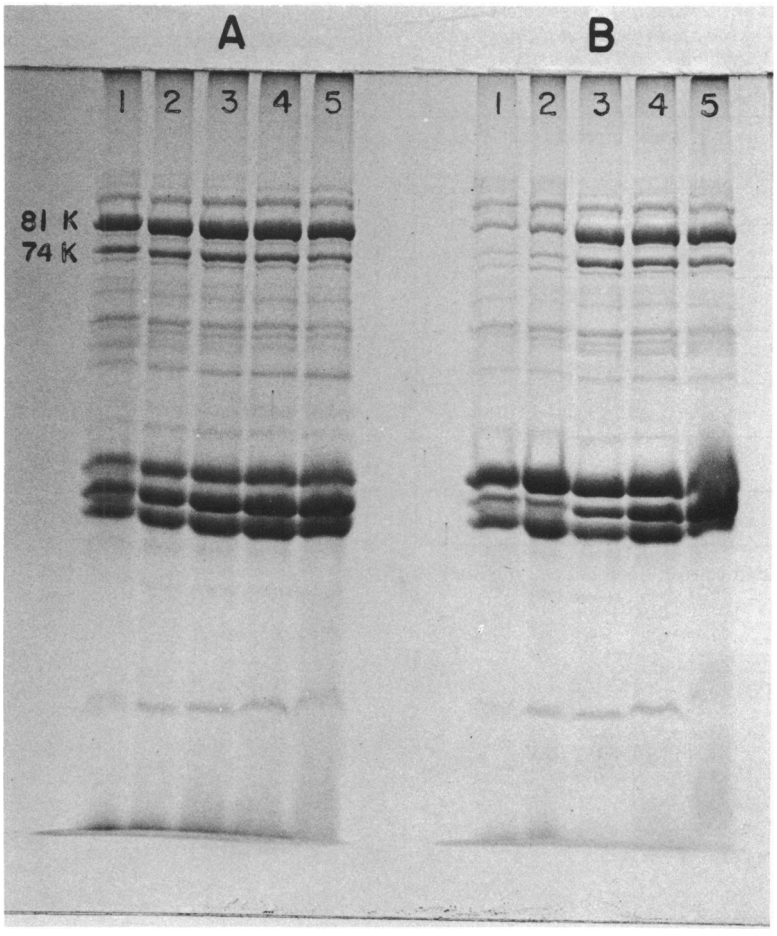

FIG. 4. *Effect of citrate on the abundance of the iron-regulated polypeptides throughout the growth cycle of AN102. Total membrane preparations from AN102 grown in M9 (A) or in M9 supplemented with 1 mM citrate (B) were analyzed as in Fig. 1. Cells were harvested at optical densities at 540 nm of 0.1 (1), 0.2 (2), 0.4 (3), 0.8 (4), or in stationary phase (5).*

therefore, probably between 400 and 500 ng of iron per mg (dry weight) of cells.

This experiment was repeated with exponentially growing cells; under these conditions, the cell-associated iron concentrations were much higher than in the corresponding stationary-phase cells (Table 1). In addition, the appearance of the three polypeptides in the total membrane complement was repressed in the cultures that were supplemented with iron (Fig. 5B2-5), all of which had iron levels greater than 500 ng/mg of cells. (A fourth polypeptide, which has a molecular weight of approximately 76,000 and whose synthesis is also apparently regulated by iron, is evident in Fig. 5B1. It has not been possible to establish a definite relationship between this polypeptide and the available iron supply, however, because it was usually not detectable in membrane preparations isolated from cells grown in iron-depleted medium.) These experiments thus provided additional evidence that the iron concentration necessary for the repression of these outer membrane polypeptides is less than 500 ng/mg (dry weight) of cells.

These experiments also suggested that *E. coli* cells growing in minimal media were able to accumulate large reserves of iron soon after being subcultured, which were utilized by the cells later in the growth cycle. Consequently, the iron concentration of AB1515 cells grown in Chelex 100-treated M9 medium supplemented with either 3 or 1 μM $FeCl_3$ was followed throughout the growth cycle. At intervals during growth at 37°C, samples were harvested, dry-weight and atomic absorption spectroscopy

TABLE 1. *Effect of increasing iron supplements on the levels of cell-associated iron in stationary-phase and exponentially growing (optical density at 540 nm of 0.3) AB1515 cells*

IRON SUPPLEMENT [a] (μM)	IRON CONTENT OF CELLS (ng/mg dry weight)	
	STATIONARY	EXPONENTIAL
0	71	170
1	222	536
2	346	672
3	356	1155
4	414	1560
5	531	1635

[a] Iron was added to iron-depleted M9 medium as $FeCl_3$.

determinations were performed, and total membrane preparations were isolated. In addition, the culture supernatants were measured for phenolate and iron content. Almost all available iron had been removed from the medium by the time the culture reached an optical density of 0.05 (data not shown). As expected, the cell-associated iron concentrations increased markedly in the early exponential growth of the culture and then slowly decreased throughout the remainder of the growth cycle (Table 2). The initial enterochelin concentrations in the supernatant were very low and remained constant until the iron concentration in the cells dropped below 400 ng/mg. On a per-cell basis, the enterochelin concentrations were highest in early logarithmic growth but decreased as the cells continued to grow and accumulate iron. The level remained low until the iron concentration dropped below the necessary level for repression, at which point enterochelin biosynthesis became derepressed. Once again, the profiles of membrane protein indicated co-

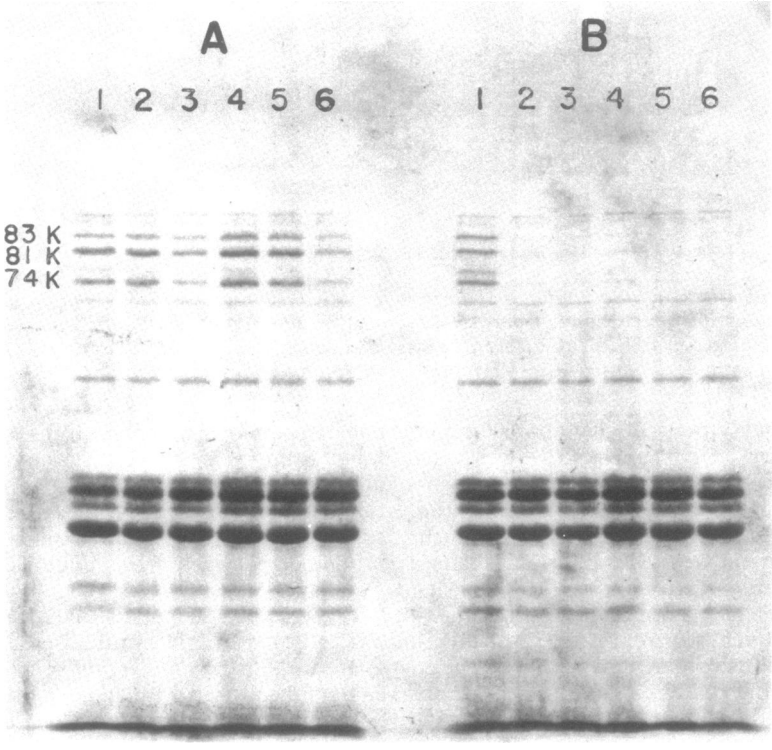

FIG. 5. *Effect of increasing iron concentrations on the abundance of 83K, 81K, and 74K in total membranes from AB1515 cells. Total membrane preparations isolated from stationary- (A) or midexponential-phase (3×10^8 cells per ml) (B) AB1515 cells grown in iron-depleted M9 medium were analyzed by the SDS-polyacrylamide gel system of Lugtenberg et al. The media were supplemented with $FeCl_3$ as follows: 0 μM (1); 1 μM (2); 2 μM (3); 3 μM (4); 4 μM (5); and 5 μM (6).*

TABLE 2. *Amount of cell-associated iron and level of phenolates present throughout the AB1515 cellular growth cycle in iron-deficient medium supplemented with 3 or 1 μM $FeCl_3$*

OPTICAL DENSITY (540 nm)	IRON CONTENT (ng/mg dry wt.)		PHENOLATE CONTENT (μg/ml)		PHENOLATE CONTENT (pg/cell)	
	3 μM	1 μM	3 μM	1 μM	3 μM	1 μM
1.4[a]	402	158	16.50	36.60	.007	.015
0.05	6318	1916	0.78	1.00	.017	.022
0.10	2860	900	0.75	1.00	.007	.010
0.20	1870	703	0.90	1.15	.003	.004
0.40	1025	373	0.94	1.95	.002	.005
0.60	513	146	1.00	2.25		
0.80	473	114	1.14	4.65		
0.91	380		1.90			
0.98	396	138	3.10	14.90	.002	.011
1.10		131		24.45		

[a] AB1515 cells were grown overnight, in iron-deficient M9 medium supplemented with either 3 or 1 μM $FeCl_3$, to an optical density at 540 nm of 1.4. The cells were subcultured by a 100-fold dilution into fresh medium.

ordinate regulation with enterochelin synthesis; 83K, 81K, and 74K were repressed in samples taken from cells containing greater than 400 ng of iron per mg of cells. However, when iron concentrations fell below this level, these outer membrane polypeptides began to appear in the membrane complements in greater quantities (Fig. 6B4-6). (Samples subjected to electrophoresis in wells A5 and A6 were from cultures with iron contents just below 400 ng/mg [dry weight]; the iron-regulated polypeptides were difficult to observe, presumably because [i] their synthesis was not completely derepressed and [ii] insufficient time had elapsed for readily detectable amounts to accumulate in total membrane preparations.)

DISCUSSION

The synthesis of the low-molecular-weight iron carrier enterochelin appears to be regulated coordinately with the synthesis of three outer membrane polypeptides in *E. coli* K-12 strains. In *fep* cells growing with suboptimal concentrations of citrate, these proteins become detectable in electropherograms of membrane polypeptides at the point in the growth cycle that enterochelin levels in the culture medium begin to increase. A similar simultaneous onset of synthesis of enterochelin and these polypeptides occurs in AB1515 cells growing in iron-deficient medium. This coordinate regulation provides further evidence that 74K, 81K, and 83K are important in the cellular uptake of iron.

It is the intracellular iron concentration, and not any one specific iron transport system, that is involved in the regulation of synthesis of enterochelin and the three outer membrane polypeptides. These components are not synthesized in large quantities when cells with a defect in the enterochelin-mediated uptake system are in an environment where an alternative iron transport system can operate. Thus, RW193 (*entA*) cells do not overproduce the outer membrane polypeptides when grown in media containing citrate or high concentrations of iron, and, similarly, when the *fep* mutant is grown in the presence of citrate, only low levels of enterochelin and the iron-regulated membrane polypeptides are synthesized. We noted, however, that the low-affinity uptake system was unable to prevent derepressed synthesis by AN102 (*fep*) of enterochelin and 74K, 81K, and 83K; these molecules were produced in large quantities by AN102 growing in M9 medium supplemented with 400 μM $FeCl_3$. Apparently, the amount of enterochelin released into the medium by *fep* cells is sufficient to bind (and thereby render unavailable for uptake by the low-affinity system) the majority of the iron supplement. In support of this explanation, supernatants from these cultures were the pink color characteristic of ferric-enterochelin complexes.

Atomic absorption analyses of stationary-phase and exponentially growing AB1515 cells grown in minimal media supplemented with varying iron concentrations indicate that the regulatory level of cell-associated iron is approximately 400 ng/mg (dry weight) of cells. The relative standard deviation in these measurements was less than 5%. Under the conditions used in these experiments, at an optical density at 540 nm of 0.1, there are approximately 10^8 cells per ml, and each cell has a dry weight of approximately 0.35 pg. The regulatory level of cell-associated iron is, therefore, 2.55×10^{-18} mol per cell. Assuming that the cellular volume is 1 μm^3 (10) and that all the cell-associated iron is intracellular, the regulatory level is 2.55 mM. This figure is similar to that obtained by indirect measurements for the regulation of the

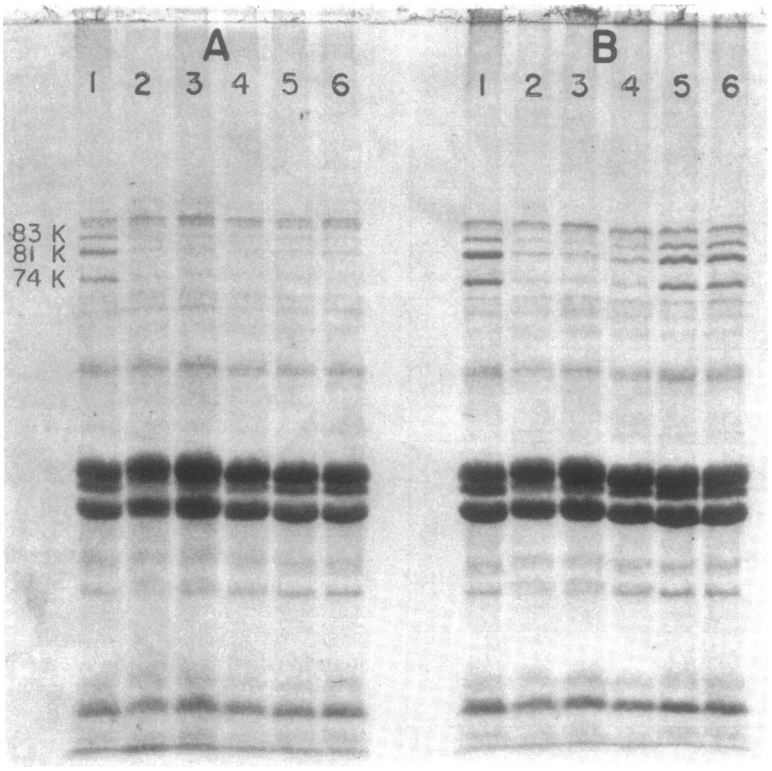

FIG. 6. *The relative abundance of 83K, 81K, and 74K in the total membrane complement throughout the growth cycle of AB1515. Total membrane preparations from AB1515 cells grown in iron-depleted M9 medium supplemented with 3 μM (A) or 1 μM (B) $FeCl_3$ were analyzed as in Fig. 5. Cells were harvested from overnight cultures, which served as the inoculum (1), and then at optical densities of 0.1 (2), 0.2 (3), 0.4 (4), 0.8 (5), and in stationary phase (6).*

enterochelin-biosynthetic enzymes (5).

The atomic absorption spectroscopy determinations of cell-associated iron, which were performed on samples taken throughout the bacterial growth cycle and on cultures growing in varying concentrations of iron, demonstrate (i) the ability of *E. coli* to rapidly accumulate high levels of iron, (ii) that the extent of this accumulation is dependent on the amount of iron available in the medium, and (iii) that almost all available iron quickly becomes cell-associated. Therefore, during the exponential growth phase, the amount of iron per cell decreases with each generation. Rapid transport and accumulation of iron by *E. coli* have previously been demonstrated by uptake experiments with ^{59}Fe or ^{55}Fe (5, 30); recent studies indicate that any of the three active transport systems for iron may be able to carry out the extensive uptake (3, 23). In this work, the amount of cell-associated iron is shown to attain levels five to fifteen times greater than that necessary to shut off synthesis of the protein components of the enterochelin system; presumably, the synthesis of enterochelin-biosynthetic enzymes and the iron-regulated outer membrane polypeptides is halted at approximately 400 ng of iron per mg (dry weight) of cells. The mechanism by which this extensive uptake occurs is unclear. If this accumulation is dependent solely upon the enterochelin system, then it may be essentially an "overshoot" phenomenon, resulting from the continued functioning of the enterochelin system components made before the onset of repression. The basal levels of these components that are synthesized when the intracellular iron concentration is high may also contribute to this uptake. In addition, some of this uptake may occur by means of an uncharacterized high-affinity iron transport system as well as by the low-affinity system.

Models for the regulation of enterochelin synthesis have been proposed (4, 24); these schemes use negative regulation, although there is no evidence that excludes positive control. To extend these models to include regulation of 74K,

81K, and 83K, the repressor (aporepressor plus bound iron) must be able to recognize at least three widely scattered sites on the *E. coli* chromosome. The *ent* gene cluster is located at 13 min (2), the structural gene (*feuB*) for 81K is presumably at 72.5 min (12), and 74K is probably coded for by *cir*, which has been mapped at 43 min (2). (No mutants in the structural gene for 83K have been isolated.) An understanding of regulation in this sytem will require the isolation of mutants unable to synthesize a normal regulatory polypeptide; such mutants should overproduce enterochelin even in an environment where all four iron transport systems can function.

ACKNOWLEDGMENTS

This research was supported by grant BMS75-07693 from the National Science Foundation. M. M. is a Public Health Service predoctoral trainee (1 T32 GM-07126 from the National Institute of General Medical Sciences).

We thank C. E. Lankford and J. R. Walker for their interest and advice and S. Chenault for her excellent technical assistance.

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