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Iron Transport in *Escherichia coli*: Relationship Between Chromium Sensitivity and High Iron Requirement in Mutants of *Escherichia coli*

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Utilization of iron (Fe^{3+}) by *Escherichia coli* depends upon a system which is determined by at least two genetic loci. Mutants which carry a deletion of the *tonB-trp* region of the chromosome grow only when very high concentrations of iron are present in the medium. These strains are sensitive to chromic ion (Cr^{3+}) and, unlike the parent strain, fail to grow on $MnSO_4$ when $FeSO_4$ is not added to the medium. A second type of mutant, Chr2, which was isolated on the basis of its sensitivity to chromic ion, also requires a high concentration of iron for growth. This mutant can be distinguished phenotypically from the deletion mutants since it grows normally on low concentrations of iron, provided citrate is added to the medium. The chromium sensitivity of both types of mutants can be reversed by high concentrations of exogenous iron. The data are interpreted to indicate that the *E. coli* mutants studied are defective in iron transport and that residual iron transport is in some way inhibited by chromic ion.

Mutants of several different microorganisms show increased sensitivity to metal ions. Suskind and Kurek(10) found that one tryptophan auxotroph of *Neurospora crassa* is sensitive to the normal endogenous concentration of zinc ion. In *Salmonella typhimurium*, Corwin et al. (3) observed that a genetic locus (*chr*) responsible for sensitivity to chromic ion is closely linked to the *trp* operon and very near the site corresponding to *tonB* (receptor site for phage T1) on the *Escherichia coli* K-12 map. These authors showed that the requirement of these strains for citrate for growth on solid minimal media is attributable to the chromic ion (75 μM) that was present in the 1.5% agar plates.

We recently observed that two mutants of *E. coli* B, B/rlt and B/lt7, each of which carries a deletion covering *tonB* and *trp* loci, are sensitive to chromic ion and, at the same time, require abnormally high concentrations of ferric ion for optimal growth on minimal media. The experiments reported here were designed to define the basis of the chromium sensitivity of the two mutants and to determine whether the sensitivity is related to the unusual iron requirement. We also report the isolation and characterization of a new type of chromium-sensitive mutant of *E. coli* B/r which also shows a close correspondence between chromium sensitivity and iron requirement but which remains sensitive to phage T1.

MATERIALS AND METHODS

Bacteria. *E. coli* B/r and its derivative E. coli B/rlt, which carries a deletion covering both the *trp* operon and the gene governing the phage T1 receptor site, *tonB*, were kindly provided by E. C. Cox. *E. coli* B/lt7, also a *trp* operon-deleted mutant resistant to both T1 and T7, was originally obtained from C. Yanofsky, who isolated both of the deletion mutants (13, 14). *E. coli* K-12 2276, a methionine-cyano B_{12} auxotroph, was originally isolated by A. L. Taylor and was obtained from R. T. Taylor.

Growth conditions. All glassware (Pyrex) used in preparing media and cell growth was cleaned of adhering iron by rinsing with aqua regia and autoclaving twice in double glass-distilled water (12). Minimal medium M63 (7) plus 2 mg of glycerol per ml and 0.1 mg of the required amino acid per ml was extracted with 8-hydroxyquinoline and glass-distilled chloroform to prepare the iron-deficient medium (12). This medium is referred to as *extracted medium*. Bacterial cell cultures to be used as inocula were grown first in the rich ML medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose), and then transferred to extracted medium in an inoculum size of 1%. These cultures, grown up to a final cell density of about 10^9 cells per ml, were used as a standard source of inocula at a concentration of 1%, unless otherwise stated. Cultures (10 ml) were grown in 50-ml Erlenmeyer flasks, and the incubation was usually for 20 hr at 37 C with shaking in a gyratory shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.). Turbidities of the cell cultures were read in a Bausch & Lomb Spectronic 20 colorimeter (Bausch & Lomb,

Inc., New York, N.Y.) at 650 nm and cell densities were calculated from a standard curve relating optical density to dry weight.

Spectrographically pure metal salts, $FeSO_4$, $CrCl_3$, $MnSO_4$, $CoSO_4$, $NiSO_4$, $ZnSO_4$, and $CuSO_4$ were obtained from Johnson, Mathey & Co. The salts were dissolved in glass-distilled water and added to the extracted medium. Solutions of $CrCl_3$ must be autoclaved separately from the medium to prevent the formation of $CrPO_4$ precipitate. However, at a concentration of 0.5 mM, the chromium salt begins to precipitate even at room temperature. Therefore, a control was necessary for measuring the turbidity of cell cultures grown at concentrations of $CrCl_3$ higher than 0.25 mM. $FeSO_4$ in the medium is quantitatively auto-oxidized to Fe^{3+} by autoclaving.

Transduction. Transduction of strain B/rlt, was carried out by the use of phage P1 (vir) according to the method of Ikeda and Tomizawa (5). Phenotypically *trp*$^+$ transductants were selected by plating onto minimal agar plates supplemented with 0.2% glycerol (7).

Selection of chromium-sensitive mutants of E. coli B/r. *E. coli* B/r was mutagenized by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich) according to the procedure of Adelberg (1). After the mutagenized cells had been grown for four generations in the extracted medium supplemented with 10 μM $FeSO_4$ and 0.5 mM $CrCl_3$ to exhaust endogenous iron, the culture was treated with 3,000 units of Penicillin G per ml (Calbiochem, Los Angeles, Calif.) (4). The surviving cells were plated on 1.5% agar plates made with extracted medium. After incubation at 37 C for 48 hr, the plates were replicated to plates of the same composition which contained 0.5 mM $CrCl_3$ to identify chromium-sensitive colonies. The agar (Difco) used in the plates had been previously washed in 20 mM EDTA and rinsed three times in double glass-distilled water to remove the excess chromic ion. After this treatment, the plates should contain about 3 μM Cr^{3+} and 16 μM Fe^{3+} (3).

RESULTS

Growth response to ferric ion and manganous ion. Figure 1 shows the relationship between the concentration of $FeSO_4$ added to the extracted medium and growth of strains B/r and 2276, which show the wild-type response to chromic ion, and of strains B/rlt and B/lt7, *tonB-trp* deletion mutants which are sensitive to chromic ion. Although residual iron in the unsupplemented extracted medium allowed some growth of the wild-type strains, the stimulation of growth by added $FeSO_4$ is similar to that reported previously (6, 12). The growth responses of the individual strains differ greatly, however. The wild-type strains (B/r and 2276) required approximately 1 μM $FeSO_4$ for the maximal level of growth, whereas the chromium-sensitive mutants (B/rlt and B/lt7) required almost 5 μM $FeSO_4$ to reach a comparable cell density. Addition of 10 mM sodium citrate, a chelating agent of ferric ion (8, 9), did not affect the extent of growth for either mutant strain. The inability of the deletion strains to grow normally on a low concentration of iron is confirmed by the results shown in Fig. 2.

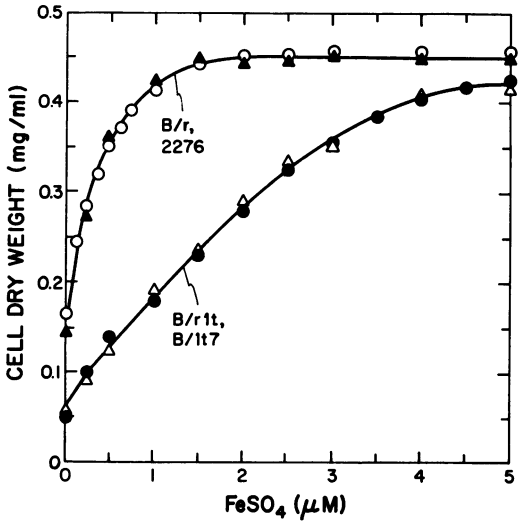

FIG. 1. *Relationship between growth of E. coli strains and $FeSO_4$ added to culture medium. Cells were grown in extracted medium supplemented with the concentrations of $FeSO_4$ indicated. After incubation for 20 hr at 37 C, the cell density of each culture was determined and expressed as dry weight per milliliter. Symbols:* ○, *B/r;* ▲, *2276;* ●, *B/rlt;* △, *B/lt7.*

Examination of the four strains for response to $MnSO_4$ in the extracted medium demonstrates that manganous ion can, in some way, substitute for the iron requirement of strains B/r and 2276, but not of strains B/rlt and B/lt7 (Fig. 3). Table 1 shows, however, that when higher concentrations of $FeSO_4$ (0.50 μM) were present in the growth medium, addition of $MnSO_4$ stimulated growth of strain B/rlt. This latter finding again reflects the requirement of the deletion mutants for high exogenous concentration of iron. Thus, although the exact role of manganous ion in "sparing" the requirement for iron is not clear, the effect is common to all of the strains studied.

Inhibition of growth by chromic ion and other heavy-metal ions. Figure 4 shows the effect of different concentration of $CrCl_3$ on the growth of the four strains of *E. coli* in the extracted medium containing 10 μM $FeSO_4$. The cell densities reached by strains B/r and 2276 after 20 hr of incubation were reduced by only 30% even in the presence of 0.5 mM chromic ion. The final level of growth reached by mutant strains B/lt7 and B/rlt was depressed by more than 90% at concentration of $CrCl_3$ above 50 μM. The presence of 10 mM citrate in the chromic ion-containing medium has no effect on strain B/r or strain 2276

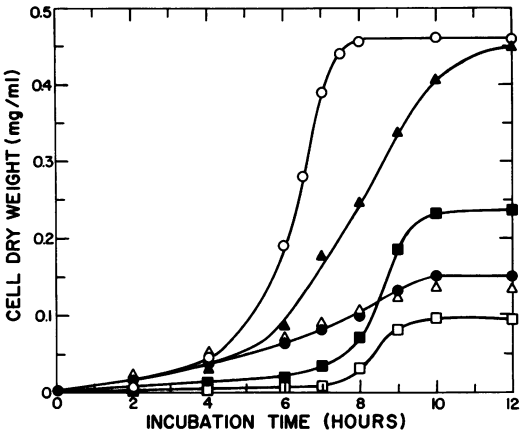

FIG. 2. *Rates of growth of E. coli strains in medium containing limited iron. Cells were grown in extracted medium containing 1 μM $FeSO_4$. Increases in cell densities were measured after the periods of incubation indicated. Symbols: ○, B/r; ▲, 2276; ●, B/rlt; △, B/lt7; □, Chr2; ■, Chr5.*

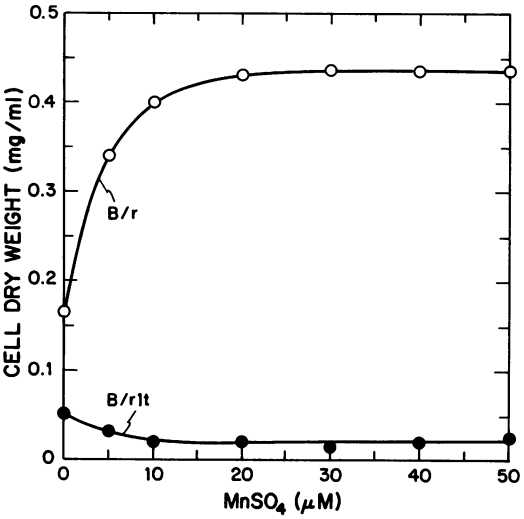

FIG. 3. *Effects of manganous ion on the growth of E. coli. The extracted medium was supplemented with $MnSO_4$ at the concentrations indicated. Cell densities were measured after incubation for 20 hr at 37 C. Symbols: ○, B/r; ●, B/rlt.*

but partially reverses the inhibition by chromic ion on the growth of the two deletion mutants at concentration of $CrCl_3$ below 100 μM. This effect of chromic ion has also been observed on the comparable deletion mutants of *S. typhimurium* growing on solid media containing 22 μM Fe^{3+} and 75 μM Cr^{3+} (3).

The effects of other heavy metals on the growth of the four *E. coli* strains were also examined. In

TABLE 1. *Influence of manganous ion on growth of E. coli B/rlt in iron-deficient medium*[a]

Concn of $FeSO_4$ (μM)	Cell dry wt (mg/ml)	
	Without $MnSO_4$	With 20 μM $MnSO_4$
None	0.050	0.020
0.25	0.100	0.330
0.50	0.140	0.450
1.00	0.190	0.445
2.00	0.290	0.455
3.00	0.365	0.450
4.00	0.410	0.442
5.00	0.435	0.455

[a] *E. coli* B/rlt cells were grown in extracted medium supplemented with concentrations of $FeSO_4$ and $MnSO_4$ as indicated. At the end of 20 hr of incubation at 37 C, the cell density of each culture was determined and expressed as dry weight.

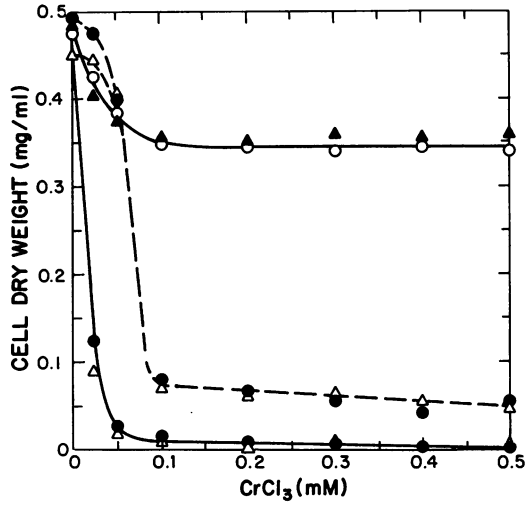

FIG. 4. *Effects of chromic ion on the growth of E. coli. The extracted medium was supplemented with 10 μM $FeSO_4$, and $CrCl_3$ in the concentrations indicated. Extents of cell growth were measured after incubation for 20 hr at 37 C. Dotted lines show final cell densities when 10 mM sodium citrate was included in the medium. Symbols: ○, B/r; ▲, 2276; ●, B/rlt; △, B/lt7.*

the extracted medium containing 10 μM $FeSO_4$, 20 μM $CoSO_4$ inhibited the growth of all the strains, whereas $CuSO_4$, $NiSO_4$, and $ZnSO_4$ at concentrations of 0.1 mM did not. None of these responses to metal ions was altered either by growing the cells at 30 or 42 C or by prolonging the incubation time to 40 hr.

Reversal of chromium inhibition by ferric ion. Since strains B/rlt and B/lt7 presumably arose by single mutational events, the results presented

above for these mutants suggest that chromium sensitivity may be related to the increased requirement for exogenous iron. Figure 5 shows that this is the case. As already observed in Fig. 4, the addition of 50 μM $CrCl_3$ severely limited the growth of strain B/rlt when low concentrations (< 10 μM) of $FeSO_4$ were present in the medium. Higher concentrations of $FeSO_4$ relieved this inhibition, however, and when 100 μM $FeSO_4$ was included in the medium the final level of growth was unaffected by the presence of 50 μM $CrCl_3$ (Fig. 5).

Nature of chromium sensitivity in E. coli B/rlt. Figure 6 shows inhibition of a growing culture of strain B/rlt by the addition of $CrCl_3$ and the reversal of this inhibition by further addition of excess $FeSO_4$. Inhibition of strain B/rlt by chromic ion was not immediate; cell mass, as well as the number of viable cells, continued to increase at a somewhat slower rate for another 4 to 5 hr after $CrCl_3$ was added. This result also indicates that chromium inhibition is bacteriostatic; iron reversed the inhibition 6 hr after addition of $CrCl_3$. This bacteriostatic nature of chromium inhibition on *E. coli* B/rlt is further supported by the good agreement between cell mass and viable cell counts in Fig. 6. There was no significant decrease in the number of viable B/rlt cells even 5 hr after growth had been stopped by the addition of excess $CrCl_3$.

Considering the manner in which ferric ion

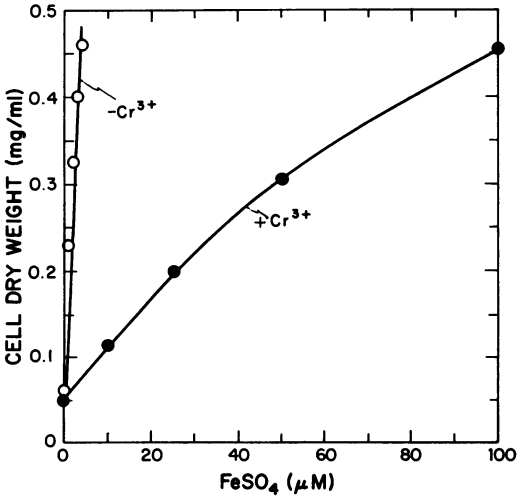

FIG. 5. *Reversal of chromium inhibition on the growth of E. coli B/rlt by addition of ferric ion. The extracted medium with no addition* (○) *or with 50 μM added $CrCl_3$* (●) *was supplemented with $FeSO_4$ at the concentrations indicated. The conditions of growth were those described for Fig. 1.*

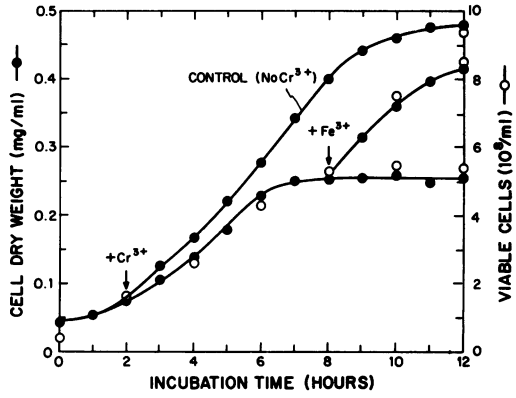

FIG. 6. *Bacteriostatic nature of chromium inhibition on the growth of E. coli B/rlt. The extracted medium was supplemented with 10 μM $FeSO_4$. At 2 hr, $CrCl_3$ (0.50 mM) was added to the experimental culture; at 8 hr, $FeSO_4$ (0.11 mM) was added to the same culture. No additions were made to the control culture. Cell densities* (●) *and viable cell counts* (○) *were determined at the times indicated.*

reverses the bacteriostatic effect of chromic ion on sensitive cells, two possible explanations for the action of chromic ion can be proposed: (i) interference with the uptake of exogenous iron or (ii) inhibition of cell growth by interference with utilization of endogenous iron. If chromic ion interferes only with uptake of ferric ion by the mutants, prior growth of the cells in iron-supplemented medium should make them temporarily insensitive to chromic ion, i.e., chromic ion should not interfere with immediate utilization of endogenous iron. Cells grown in iron-supplemented medium behaved in the same manner whether they were transferred to iron-deficient medium or to a medium containing iron along with an inhibiting concentration of $CrCl_3$; the cell number increased an average of 16-fold, or four cell generations (Table 2). This finding is consistent with explanation (i) above; endogenous iron enables the cells of strain B/rlt to grow for four generations either in medium lacking iron or in medium from which the uptake of iron is blocked by the presence of excess chromic ion. This interpretation is supported by the direct measurement on iron uptake by strains B/rlt and B/lt7 described in the accompanying paper (11).

Chromosomal location responsible for chromium sensitivity. To confirm that the altered responses of strains B/rlt and B/lt7 toward ferric, chromic, and manganous ions are related genetically to the segment of the chromosome deleted with the *tonB-trp* loci, strain B/rlt was transduced to trp^+ by the use of P1-mediated transduction. Two of

TABLE 2. *Influence of inoculum size on growth of E. coli B/rlt in chromic ion-supplemented and iron-deficient media*[a]

Supplement	Viable cells per ml		
	Initial	Final	Final/initial
(A) 10 μM $FeSO_4$ + 0.5 mM $CrCl_3$	1.4×10^7	23.0×10^7	16.2×10^7
	3.6×10^7	53.0×10^7	15.0×10^7
	7.1×10^7	128.0×10^7	18.0×10^7
	14.2×10^7	248.0×10^7	17.5×10^7
	21.3×10^7	350.0×10^7	16.4×10^7
	28.4×10^7	472.0×10^7	16.6×10^7
(B) None (iron-deficient)	0.54×10^7	10.0×10^7	18.5×10^7
	1.08×10^7	17.0×10^7	16.0×10^7
	2.16×10^7	27.0×10^7	12.5×10^7

[a] Inoculum was grown in extracted medium containing 10 μM $FeSO_4$ and washed in sterile extracted medium. These cells were then transferred to extracted medium containing supplements (A) or (B) at the cell densities indicated under "Initial." Viable cell counts were determined immediately after inoculation (initial) and after 20 hr of growth at 37 C (final).

the *trp*+ recombinants, which showed the ultraviolet-resistant phenotype of the original parent, strain B/r, were selected for study. Both of the *trp*+ transductants are sensitive to T1 phage and respond to heavy metals exactly like *E. coli* B/r (*see* Fig. 1, 3, 4). Thus, the genetic locus controlling cellular response to heavy metals must be adjacent to the *trp* operon and *tonB*.

Isolation of chromium-sensitive mutants of E. coli B/r. To investigate the relationship between chromium sensitivity and iron requirement more closely, chromium-sensitive strains of *E. coli* B/r were isolated directly as described in Methods and Materials. Twelve chromium-sensitive mutant strains were isolated and two of them, designated as Chr2 and Chr5, have been studied in detail. Both mutants are *trp*+ and sensitive to phage T1. Although the strains were selected for their sensitivity to chromium, they simultaneously acquired a growth requirement for high concentrations of iron (Fig. 7). This again indicates a close relationship between chromium sensitivity and the requirement for iron.

A comparison between these two strains and the *ton-B-trp* deletion mutant, strain B/rlt, shows that each of the mutants is different from the other two. (i) The chromium sensitivity of strain Chr2 is very similar to that of strain B/rlt, whereas strain Chr5 is less sensitive than the deletion mutant (Fig. 7). (ii) All of the strains require more than 5 μM $FeSO_4$ for maximal growth, but strain Chr5, like strain B/rlt, grows better than Chr2 at lower concentrations of iron (cf. Fig. 1, 8). (iii) The growth response of strain Chr2 to added $FeSO_4$ becomes identical to that of the parent strain when 10 mM citrate is added to the medium; citrate does not affect the growth of either strain B/rlt or strain Chr5 on a similar medium (Fig. 8). (iv) When $MnSO_4$ is used to replace $FeSO_4$ in the extracted medium, both strains Chr2 and Chr5 grow as well as the parent strain, B/r (*see* Fig. 3).

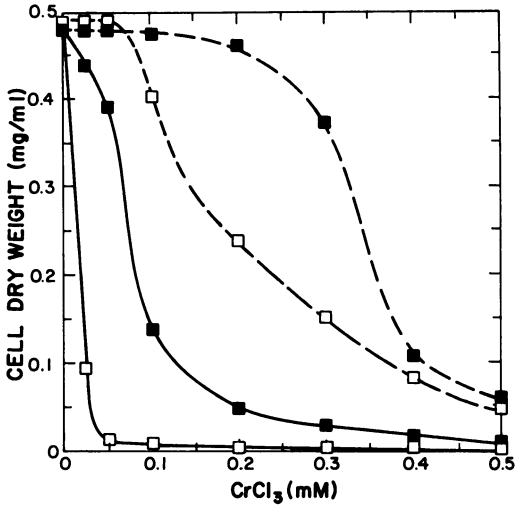

FIG. 7. *Effects of chromium on the growth of E. coli strains Chr2 and Chr5. Experimental conditions are described in Fig. 4. Dotted lines represent the extents of cell growth when 10 mM sodium citrate was also present in the medium. Symbols:* □, *Chr2;* ■, *Chr5.*

DISCUSSION

The observations reported above lead us to hypothesize that the chromium sensitivity of the *trp-ton*B deletion mutants of *E. coli* B results directly from damage to the iron transport systems in these strains. Thus, in extracted medium,

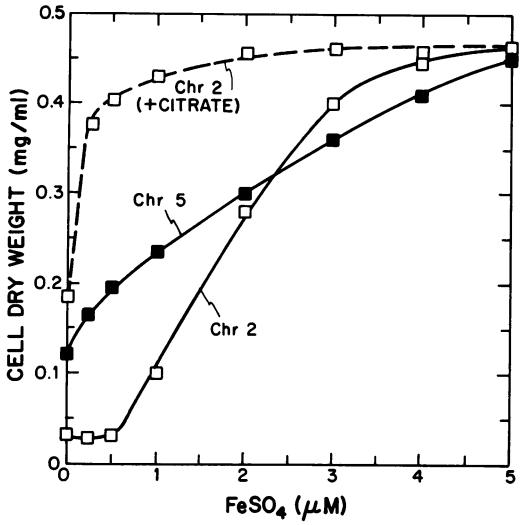

FIG. 8. *Effects of iron on the growth of E. coli strains Chr2 and Chr5. Experimental conditions are described in Fig. 1. The dotted line shows the final levels of cell growth when 10 mM sodium citrate was also present in the medium. Symbols:* □, *Chr2;* ■, *Chr5.*

both strain B/lt7 and strain B/rlt show the following behavior. (i) Full growth occurs only when iron is present in concentrations that are fivefold the level required for maximal growth of the wild-type strain (Fig. 1). (ii) Chromium sensitivity, which is shown at intermediate levels of iron, is reversed by addition of excess iron to the medium (Fig. 5). (iii) Chromic ion appears to interfere with utilization of exogenous, but not endogenous, iron (Fig. 6 and Table 2). (iv) The growth response to manganous ion also seems to be related to the iron requirements of these strains (Fig. 3). These findings are not limited to mutants of *E. coli* B. A *tonB-trp* deletion of *E. coli* K-12 (XAT 713, obtained from G. Degnen) shows the same responses to ferric ion and chromic ion (*unpublished data*).

The above hypothesis is supported by experiments which show that the *tonB-trp* mutants do not, in fact, possess the normal active transport system for iron and that residual uptake of the ion by the strains is inhibited by chromic ion (11).

Since mutations in the *trp* operon alone do not lead to chromium sensitivity of bacterial strains (3; and *unpublished data*) it is reasonable to assume that it is the *tonB* or a closely associated locus which is related both to this effect and to iron uptake. Using deletion mapping, Corwin, et al. (3) found that a chromium sensitivity locus (*chr*) can be located in *S. typhimurium* near the corresponding *tonB* locus of *E. coli* K-12. The close association of the chromium sensitivity locus with a phage T1 receptor locus in *E. coli* has now been confirmed by the examination of point mutants of the *tonB* locus. These *E. coli* K-12 strains (supplied by E. R. Signer) are also chromium-sensitive. Their responses to Fe^{3+}, Cr^{3+}, and Mn^{2+} are identical to those seen for the deletion mutants (Fig. 1–4). Also, all chromium-resistant revertants of these *tonB* point mutants were found to be sensitive to phage T1 (*unpublished data*).

The isolation of two new chromium-sensitive mutants of *E. coli* B/r, strains Chr2 and Chr5, which also require abnormally high levels of iron for growth (Fig. 8), further strengthens the correlation between chromium sensitivity and altered ability to utilize exogenous iron observed for strains B/rlt and B/lt7. The quantitative response of these new strains to heavy metals and their sensitivity to phage T1 distinguish these mutants from strains B/rlt and B/lt7. It is shown in the following paper (11) that strains Chr2 and Chr5 have active uptake system for iron but that strain Chr2 lacks the complete system required for synthesis both in vitro and in vivo of 2,3-dihydroxybenzoylserine, a strong chelator of Fe^{3+} (2). The latter finding, and the fact that citrate reverses the dependency of the strain on high concentrations of iron for normal growth (Fig. 8) indicate that in the wild-type strain a chelating agent may play some role in iron transport. This idea is more fully discussed elsewhere (11).

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