

Manganese-Resistant Mutants of *Escherichia coli*: Physiological and Genetic Studies

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Manganese is growth inhibitory for *Escherichia coli*. The manganese concentration required for inhibition is dependent upon the magnesium concentration of the medium. Mutants have been isolated which are partially resistant to manganese inhibition in both liquid and solid media. From conjugation experiments, the genetic locus for manganese-resistance, *mng*, appears to be between 34 and 37 min on the *E. coli* genetic map. Experiments with radioactive ^{28}Mg lead to the tentative conclusion that the *mng* mutants are altered in the inhibition constant for manganese as a competitive inhibitor for the magnesium accumulation system. Once high manganese enters the cells, it displaces internal magnesium and leads to a net cellular loss and hence growth inhibition. The *mng* mutants are somewhat less subject to manganese-induced magnesium loss under comparable conditions than are manganese-sensitive wild-type cells.

We have recently described separate active transport systems in *Escherichia coli* for the accumulation of high intracellular concentrations of magnesium and manganese (20-23). Whereas magnesium has no effect on the high affinity ($K_m = 2 \times 10^{-7}$ M Mn^{2+}) manganese accumulation system (22), manganese is a competitive inhibitor of magnesium uptake with a K_i of about 2×10^{-3} M in dilute broth (21).

To further our understanding of these unique transport systems, we have attempted to isolate and study mutants with altered transport properties. Experience with ion transport systems suggests two approaches: (i) isolation of mutants requiring high concentrations of substrate for growth (6, 9-11; Fed. Proc. 23:944-1001, 1964) and (ii) isolation of resistant mutants with decreased affinities for inhibitory analogues of the substrate (2, 17). We report here the isolation, preliminary mapping, and characterization of one of the second type of mutant: manganese-resistant mutants with apparently reduced affinities for manganese as a competitive inhibitor and substrate for the magnesium transport system.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strain K-12 derivatives were used throughout. Most of the manganese-resistant mutants were isolated from the Hfr strain K-10. The origin and genotype of this strain and the others used in this study are given in Table 1.

Media. The basic nutrient medium was a dilute tryptone broth containing 4.0 g of tryptone (Difco) and 2.5 g NaCl per liter. When made with deionized water, this medium contains 3.5×10^{-5} M Mg^{2+} and an undetectable (less than 10^{-7} M) level of Mn^{2+} as measured by atomic absorption spectroscopy and isotopic dilution techniques (22). Solid agar medium was made by adding 15 g of agar (Difco) per liter which brings the magnesium concentration up to the millimolar range since agar may contain several per cent Mg^{2+} according to USP standards.

Genetic procedures. Standard techniques for bacterial conjugation and transduction with P1 phage were used. Those variations currently in use in this laboratory were described by Whitney (29). Conjugation experiments involved uninterrupted matings for 1 to 2 hr with 1:10 mixtures of log-phase Hfr and F^- cells. P1 transduction utilized ultraviolet-irradiated phage which had been grown for at least two cycles on the donor strain.

Growth experiments. The growth of bacterial cells and the inhibition of growth by Mn^{2+} was followed by turbidity in a Klett-Summerson colorimeter with a no. 54 (green) filter. Overnight (15 hr) cultures grown in tryptone broth were diluted 100-fold into fresh broth in Erlenmeyer flasks with Klett side-arm tubes (Bellco Glass, Inc., Vineland, N.J.). Periodically aeration by shaking was interrupted momentarily to read the turbidity in the colorimeter. Both the shaker and the colorimeter were kept in a 37 C room.

^{28}Mg experiments. ^{28}Mg experiments were carried out as previously described (20, 21) using ^{28}Mg obtained from the Brookhaven National Laboratory and counting the radioactivity retained after washing on Millipore HA filters in a gas-flow plan-

TABLE 1. *Bacterial strains*

Strain	Relevant genotype ^a	Source	Reference
K-10	Hfr Cavalli proto-troph <i>str^r</i>	A. Garen	29
AB1157	F ⁻ <i>str^r thr⁻ leu⁻ arg⁻ his⁻</i>	A. J. Clark	12
X36	F ⁻ <i>str^r purC⁻ his⁻ trp⁻</i>	B. Wolf	29
AB1360	F ⁻ <i>str^r his⁻ aroD⁻</i>	J. Pittard	18

^a The nomenclature used conforms with the proposals of Demerec et al. (7) as modified by Curtiss (5). Only those growth requirements used in our crosses are included; *str^r* and *str^s* refer to resistance and sensitivity to streptomycin.

chet counter. Accumulation or uptake experiments (at room temperature) were begun by adding trace ²⁸Mg to cells grown at 37 C in dilute tryptone. Efflux (or loss) experiments were begun by centrifuging cells grown at 37 C for 2 to 3 hr in the presence of ²⁸Mg and resuspending the cells in fresh nonradioactive medium.

Atomic absorption spectroscopy. Atomic absorption spectroscopy measurements of magnesium were made with a Perkin-Elmer model 305 atomic absorption spectrometer. Duplicate 50-ml samples of cells (initially at 3.6×10^8 per ml) were centrifuged at $6,000 \times g$ for 10 min at room temperature, washed once with 0.9% (w/v) saline, and suspended in 5 ml of deionized water. Dilutions of washed cells were measured for turbidity in the Klett colorimeter and for magnesium content by atomic absorption spectroscopy. Cell magnesium is calculated by using the value of 125 Klett units for 10^9 cells/ml as determined directly with a phase-contrast microscope and a Petroff-Hauser counting chamber.

RESULTS

Manganese is growth-inhibitory for *E. coli*. When 10^{-2} M MnCl₂ is added to an overnight culture of *E. coli* at the time of a 100-fold dilution into fresh dilute broth, very little increase in cell mass (turbidity) occurs over a 10-hr period (Fig. 1). This inhibition of growth by manganese is very dependent on the magnesium concentration present. The addition of 10^{-3} M MgCl₂ (to supplement the 3.5×10^{-5} M Mg²⁺ in the broth) at the same time as 10^{-2} M MnCl₂ allows growth after a short delay (about 30 min at 37 C). The final yield of bacteria after 5 hr or more of growth is reproducibly 50% greater with high Mg²⁺ and Mn²⁺ than in the unsupplemented broth (Fig. 1). Furthermore, this increase in final yield requires both Mg²⁺ and Mn²⁺, and the addition of 10^{-3} M MgCl₂ alone to the broth is without effect on the course of growth. Calcium does not replace magnesium in protecting against manganese

inhibition of growth—at least over a 10 hr period at 37 C. In an experiment similar to that in Fig. 1, we determined that 10^{-2} M Mn²⁺ is growth inhibitory but does not kill *E. coli*. Viable colony-forming units per milliliter of culture neither increases nor decreases during a 90-min exposure to 10^{-2} M Mn²⁺.

In the experiment in Fig. 1, 10^{-2} M Mn²⁺, with or without 10^{-3} M Mg²⁺, was added to the cells at the time of dilution of a stationary-phase culture into fresh broth. In the experiment shown in Fig. 2, 10^{-2} M MnCl₂ was added either at the time of dilution into fresh medium or 2 hr later after the cells have entered the log phase of growth. Figure 2 shows that when 10^{-2} M MnCl₂ is added to growing cells there is an approximately twofold increase in turbidity (from 25 to 50 Klett units) before growth stops. An additional aspect of protection against Mn²⁺ inhibition of growth is seen in Fig. 2. The protection shows specificity in that 10^{-3} M Mg²⁺ almost completely prevents Mn²⁺ inhibition when added along with 10^{-2} M MnCl₂ but 10^{-3} M Ca²⁺ does not protect stationary-phase cells as seen also in Fig. 1. However, if the incubation is continued

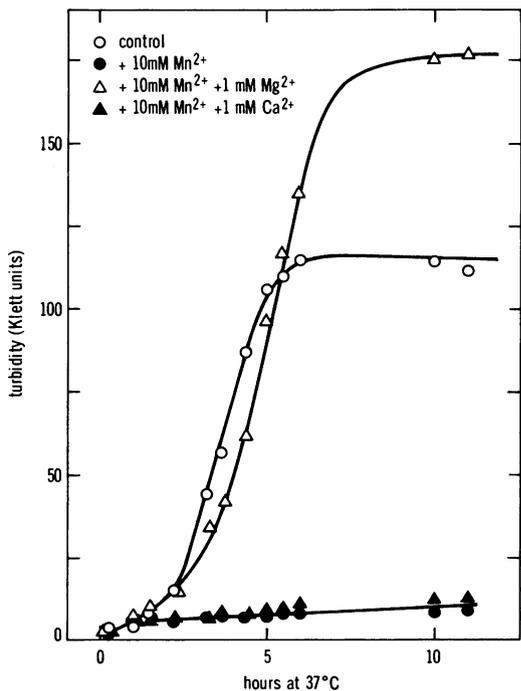


FIG. 1. Manganese inhibition of growth. MgCl₂ or CaCl₂ (1 mM) was added at the time of a 100-fold dilution of the overnight culture into fresh broth; 10^{-2} M MnCl₂ was added immediately thereafter at zero time.

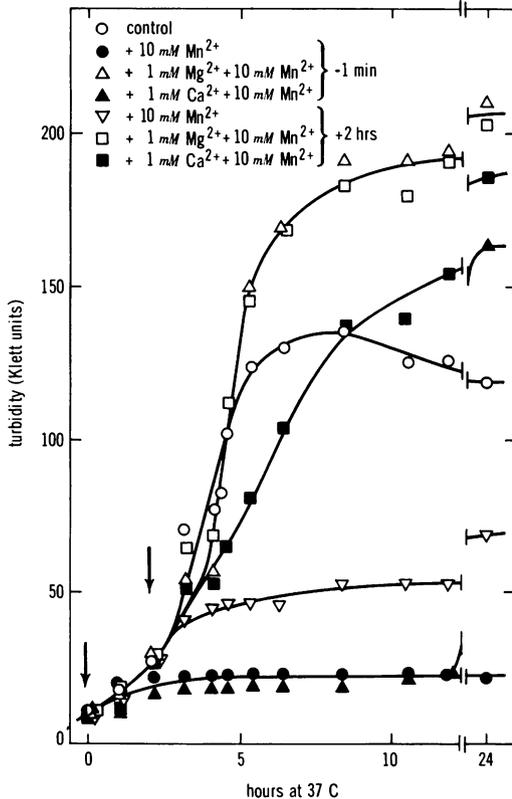


FIG. 2. Manganese inhibition of growth: protection by magnesium and calcium. $MgCl_2$, $CaCl_2$, and $MnCl_2$ were added as indicated either 1 min before or 2 hr after the dilution of the overnight culture into fresh broth.

overnight at 37 C, the cells with Ca^{2+} plus Mn^{2+} begin to grow sometime after 12 hr of incubation. When 10^{-3} M Ca^{2+} is added along with 10^{-2} M $MnCl_2$ to log-phase cells, growth is not completely inhibited although the protection by Ca^{2+} is not as complete as that by Mg^{2+} .

We have not experimentally pursued the difference in response to high manganese between log-phase and stationary-phase cells seen in Fig. 2. Our current model for the mechanism of manganese toxicity is developed below (Fig. 9). It seems plausible that, with stationary-phase cells, high manganese prevents the "recovery" to a high metabolic state and therefore inhibits growth; with log-phase cells, the effects of manganese take some time (Fig. 4 to 8) and therefore growth inhibition is not immediate.

Isolation of manganese-resistant mutants. $MnCl_2$ (10^{-2} M) is also growth inhibitory when incorporated into solid media in petri

dishes. With wild-type *E. coli*, colonies arise with frequencies about 10^{-6} to 10^{-4} only after 36 to 48 hr of incubation at 37 C. The number of colonies appearing is, within limits, a function of the length of incubation time, and more and larger colonies appear with increasing incubation at 37 C. The concentration of $MnCl_2$ also effects the time and number of colonies appearing; with concentrations of $MnCl_2$ above 1.5×10^{-2} to 2×10^{-2} M, colonies do not appear regardless of the length of incubation. Furthermore, the "freshness" of the petri dishes significantly effects the sensitivity of *E. coli* to Mn^{2+} , and we generally obtain reproducible results with tryptone plates stored at 4 C for less than 1 week after pouring. With agar containing 10^{-2} M $MnCl_2$, we have isolated more than 10 independently arising manganese-resistant strains by selecting large colonies appearing early on selective plates and streaking them out repeatedly on Mn^{2+} -containing agar. Some of these mutants were selected after nitrosoguanidine mutagenesis; others without deliberate mutagenesis, but Mn^{2+} itself is mutagenic (8, 19). Because of the repeated selective pressure to which these isolates were subjected, we could not at this stage assume that resistance was due to single rather than multiple mutations in these strains. Figure 3 shows the resistance to Mn^{2+} of one such mutant (*mngK*) in both liquid and solid media when compared to the manganese-sensitive strain from which it was isolated. (All of the resistant mutants described in this paper were isolated in strain K-10—an Hfr Cavalli derivative. Other less extensively studied mutants were isolated from other *E. coli* K-12 strains, both Hfr and F⁻. Each of the more than 10 wild-type K-12 substrains which we have tested was manganese sensitive.) After 16 hr of incubation at 37 C, 40% or more of the control number of colonies appear with the mutant strain on plates containing 5 or 10 mM Mn^{2+} (Fig. 3A). With the manganese-sensitive strain, 10- to 100-fold fewer colonies appear. Similarly, in liquid media containing Mn^{2+} , one can distinguish between the manganese-resistant and manganese-sensitive bacteria over a narrow range of concentrations (Fig. 3B). The distinction in tryptone liquid media can only be made when the magnesium level is elevated, e.g., to 2×10^{-3} M Mg^{2+} in the experiment in Fig. 3B. In unsupplemented 3.5×10^{-5} M Mg^{2+} tryptone, both mutant and wild-type are equally sensitive to Mn^{2+} inhibition. An explanation for this finding may come from the high magnesium content of the agar used to solidify the media in petri dishes.

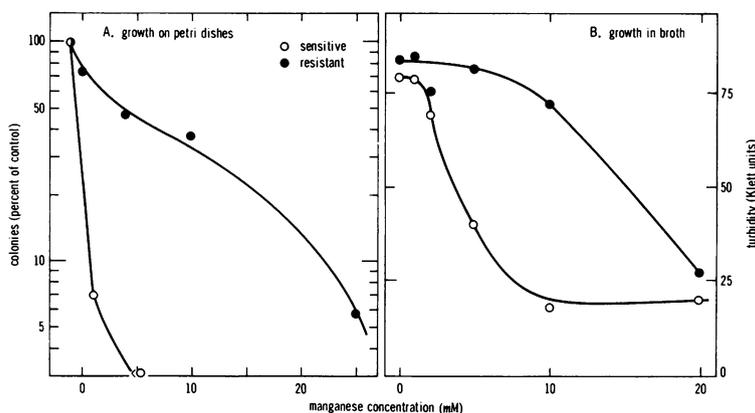


FIG. 3. Manganese inhibition of growth in liquid broth medium and on petri dishes: differences between the sensitive strain and the resistant mutant. (A) Dilutions of stationary-phase cultures of wild-type K-10 (sensitive) and its manganese-resistant mutant *mngK* were spread on petri dishes containing different Mn²⁺ concentrations in tryptone broth-agar. Colony numbers were counted after 16 hr growth at 37 C. "Pin point" colonies occurred only over a very narrow concentration range and time range; either essentially full-sized colonies were found or no growth was observable with a low-power lens. (B) Cultures (16 hr old) of strain K-10 and mutant *mngK* were diluted 100-fold into fresh broth supplemented with 2×10^{-3} M Mg²⁺ and Mn²⁺ as indicated. Growth (turbidity) was measured after 5 hr of aeration on a rotator at 37 C.

Reproducible differences in manganese-resistance among the *mng* mutants were found (data not shown). Mutant *mng-43* is more resistant than *mngK* which is reproducibly more resistant to manganese than *mng-3* or *mng-4*, and *mng-3* and *mng-4* are more resistant than *mng-2*.

Since the distinction in liquid media between the resistant and sensitive strains requires high magnesium concentrations, we made comparisons over a range of Mg²⁺ concentrations and tested whether Ca²⁺ could replace Mg²⁺ in defining conditions for selecting and testing Mn²⁺-resistant strains. Table 2 contains data from a few of hundreds of such tests. Mg²⁺ between 0.5 and 2 mM allows for the best distinction between sensitive and resistant strains. Ca²⁺, at concentrations up to 10 mM, cannot replace Mg²⁺ in this role. Similarly none of the other materials which we have tested can protect the cells from Mn²⁺ inhibition of growth. Among the compounds tested were KCl (0.01 M and 0.1 M), sucrose (0.1 M), and spermidine, putrescine, and spermine at concentrations ranging upwards until the polyamines themselves became growth inhibitory. Magnesium protection against manganese inhibition of growth is specific in another sense. High magnesium does protect the cells against growth inhibition by 5×10^{-5} M Co²⁺ or Zn²⁺ or 5×10^{-4} M Ni²⁺, but in these cases 25 mM Mg²⁺ is much more effective than 1 mM and no difference could be found between the wild-type and the manganese-re-

TABLE 2. Inhibition by manganese and protection by magnesium

Inhibitor (10 mM)	Other additions (mM)	Growth: turbidity after 5 hr at 37 C (Klett units)	
		Sensitive (wild type)	Resistant (mutant)
None (control)	None	105	100
MnCl ₂	None	38	42
MnCl ₂	0.5 MgCl ₂	42	77
MnCl ₂	1 MgCl ₂	65	120
MnCl ₂	1 CaCl ₂	43	34
MnCl ₂	10 CaCl ₂	29	38

sistant mutants. After we learned of Nelson and Kennedy's (17) cobalt-resistant mutants and exchanged strains with them, we determined that the manganese-resistant mutants are still cobalt-sensitive and that the cobalt-resistant mutant *cor-4* (17) is still manganese sensitive. Furthermore, a cobalt-resistant isolate from manganese-resistant strain *mngK* lost its manganese resistance in the course of becoming cobalt resistant (unpublished data). Additional information about the cobalt-resistant mutants and mutational changes in the magnesium transport system will be available shortly (Nelson and Kennedy, manuscript in preparation).

Genetic locus of the manganese-resistance marker. Although the degree of resistance of the mutants is not sufficient to allow direct quantitative selection of manganese-re-

sistant recombinants in crosses by conjugation or transduction, we have selected for other markers in crosses and nonselectively scored for Mn^{2+} sensitivity and resistance on petri dishes as shown in Fig. 3. (The distinction between the mutants and the sensitive wild-type strains is not even sufficient to allow scoring by replica plating, although we have extensively sought conditions which would allow this.) In an early conjugation experiment, mutants *mng-2*, *mng-4*, and *mngK* (all isolated in the prototrophic Hfr strain) were mated with F^- AB1157. All three Mn^{2+} -resistant strains gave rise to normal numbers of recombinants for a variety of markers, indicating the normal order of gene transfer for the Cavalli Hfr strain and therefore showing that the manganese-resistant strains are mutationally derived from the manganese-sensitive Hfr strain. Less than 1% of the *thr⁺ leu⁺ str^r* recombinants or the *arg⁺ str^r* recombinants from these three crosses were *mng^r*, but, when *his⁺ str^r* recombinants were tested, 32, 58, and 44% were *mng^r*, respectively. Next the *mng* locus was placed to the right of the *his* region on the Taylor (25) map by mating Hfr *mng-2* with F^- X36 *purC⁻* (at 48 min) *his⁻* (at 39 min) *try⁻* (at 25 min). In this experiment, 4% of the *purC⁺ str^r* recombinants, 48% of the *his⁺ str^r*, and 14% of the *try⁺ str^r* recombinants were *mng^r*. Furthermore, three-factor analysis suggested a locus between *his* and *try* on the map. In a third conjugation experiment with Hfr *mngK* and F^- AB1360 *his⁻ aroD⁻*, about 60% of both classes of recombinants *his⁺ str^r* and *aroD⁺ str^r* were *mng^r* (Table 3), and the three-factor analysis supports a position on the map

TABLE 3. Location of *mng* between *his* and *aroD*

Relative map order				
<i>str</i> <i>his</i> <i>mng</i> <i>aroD</i>				
Parental genotypes				
<i>s</i>	+	<i>r</i>	+	Hfr K-10 <i>mngK</i>
<i>r</i>	-	<i>s</i>	-	F^- AB1360
Of 98 <i>his⁺ str^r</i> recombinants:				No. found
<i>r</i>	+	<i>r</i>	+	45
<i>r</i>	+	<i>s</i>	-	31
<i>r</i>	+	<i>r</i>	-	14
<i>r</i>	+	<i>s</i>	+	8
Of 96 <i>aroD⁺ str^r</i> recombinants:				
<i>r</i>	+	<i>r</i>	+	40
<i>r</i>	-	<i>s</i>	+	23
<i>r</i>	-	<i>r</i>	+	23
<i>r</i>	+	<i>s</i>	+	10

about one-third to one-half of the distance from *aroD* toward *his* or around 35 min on the Taylor map. The *mng* locus is not cotransducible with either *aroD* or *his* (more than 100 transductants tested in each case). Unfortunately, the region of the map near 35 min does not contain readily selectable markers for transductional analysis, and, with the difficulty in scoring manganese-resistance, we have abandoned more precise transduction mapping experiments until more readily scored mutants are isolated.

Differences between manganese-sensitive and manganese-resistant strains with regard to magnesium metabolism. Seeking the physiological basis for the manganese resistance of the *mng* mutants, we looked for changes in the kinetic parameters (K_m , V_{max} , and K_i) of the specific manganese accumulation system in *E. coli* (3, 22, 23) and of the magnesium transport system for which manganese is a competitive inhibitor of accumulation (20, 21). No differences were found between sensitive and resistant strains for the manganese accumulation system (*data not shown*), and indeed none were anticipated because of the concentration ranges involved (*see below*). With the magnesium system accumulating radioactive ^{28}Mg (15, 17, 20, 21), differences were found consistent with the interpretation that the *mng* mutants have a somewhat reduced affinity for manganese as a competitive inhibitor of magnesium accumulation (Fig. 4-7).

Figure 4 shows the results from a ^{28}Mg accumulation experiment where the time course of accumulation was compared for the sensitive parental strain and mutant *mngK*. When the data are presented as relative accumulation (to the average of the 50- and 60-min data so as to facilitate comparisons), the time course over 60 min at 20 C is the same for the sensitive and resistant strains. The accumulation of ^{28}Mg by both strains is equally inhibited by the addition of 0.5 mM nonradioactive $MgCl_2$ (Fig. 4), giving a first indication that there is no difference in the K_m values for the two strains. However, 5.0 mM $MnCl_2$ causes a slightly greater inhibition of ^{28}Mg uptake with the sensitive strain than with the *mng* mutant. When this question was further pursued by varying both the concentrations of Mg^{2+} (from the 3.5×10^{-5} M Mg^{2+} in the dilute broth) and Mn^{2+} and the data for the initial rate of accumulation presented in a Lineweaver-Burk (14) plot in Fig. 5, we can see that the magnesium accumulation system follows standard kinetics with manganese as a competitive inhibitor of

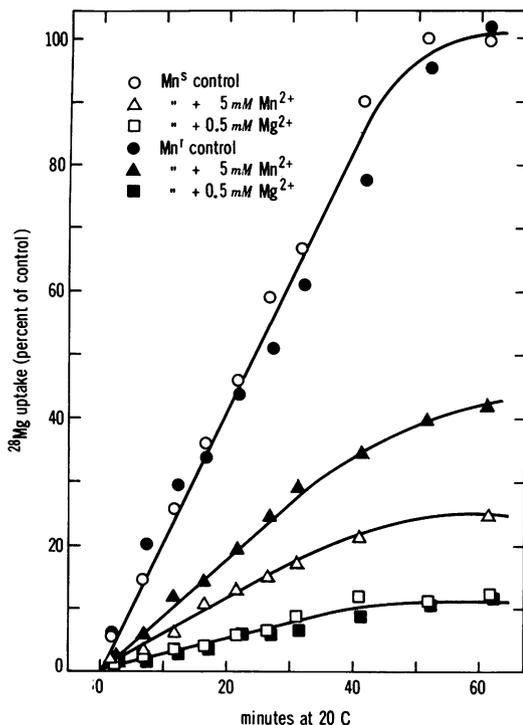


FIG. 4. ²⁸Mg accumulation by manganese-sensitive and -resistant *E. coli*. Strain K-10 and mutant *mngK* were grown in tryptone at 37 C to 2.3×10^8 and 3.5×10^8 cells/ml, respectively. Cells were distributed into small flasks; after the addition of non-radioactive Mn²⁺ or Mg²⁺, as indicated, ²⁸Mg (10^{-5} M; 0.15 μ Ci/ml) was added. Samples were removed, filtered on membrane filters, and washed twice with 5 ml of broth before gluing onto planchets and counting the filters in the gas-flow counter. The data are normalized to the average of the 50- and 60-min values (45% of the ²⁸Mg retained on the filters for strain K-10 and 24% for strain *mngK*).

magnesium accumulation (see reference 21). The K_m values for magnesium accumulation are not significantly different in this or in other experiments directly to this point (3.3×10^{-5} M in Fig. 5 insert). However, there is a small but consistent difference in the V_{max} values with the resistant strain showing less uptake ($V_{max} = 0.14$ μ moles per min per 10^{12} cells in Fig. 5) than the sensitive strain (0.20 μ moles per min per 10^{12} cells). This difference in V_{max} has been seen in several similar experiments but does not result in any difference in growth rate in tryptone since magnesium accumulation is not growth limiting. One can also see a difference in the apparent K_i values for inhibition by manganese with less than 4 mM Mn²⁺ causing more than a twofold inhibition

in the sensitive strain at low Mg²⁺ concentrations and more than 7.5 mM Mn²⁺ required for a similar inhibition with the resistant strain. The data in Fig. 5 will not allow an orthodox determination of K_i values, since with both strains the increase from 4 to 7.5 mM Mn²⁺ has only a small effect. This is perhaps not surprising since we are dealing with intact cells which have recourse to compensatory control mechanisms (see below), but our conclusions from three independent detailed experiments, one of which is shown in Fig. 5, are that the Lineweaver-Burk plots are always linear and consistent with a common V_{max} —indicative of competitive inhibition—but that increasing concentrations of manganese do not always lead to further inhibition of magnesium accumulation. To reach comparable inhibition of ²⁸Mg accumulation with cells of the resistant mutant generally requires two to four times higher concentrations of manganese than with the sensitive strain (which appears to show a K_i value for this inhibition of about 2 mM Mn²⁺; see reference 21).

Manganese at inhibitory concentrations causes a rapid loss of intracellular radioactive magnesium (Fig. 6) and a net loss of cellular magnesium as measured by atomic absorption spectroscopy (Fig. 7). The effect of high manganese concentrations causing a loss of pre-accumulated ²⁸Mg has been previously described in detail (21); in addition, the experiment in Fig. 6 shows that, at given Mn²⁺ concentrations (1 or 5 mM), ²⁸Mg is lost more rapidly and extensively from manganese-sensitive *E. coli* than from cells of the resistant mutant. This difference, again small, has been consistently found in several experiments. If the net loss of cellular magnesium seen in Fig. 6 and 7 is the basic cause of manganese inhibition of growth, then we might expect that adding high magnesium or calcium (to a lesser extent) would protect the cells against magnesium loss just as it protects against growth inhibition (Fig. 2). The protection against Mn²⁺-induced ²⁸Mg loss was previously described (21) and is seen in Fig. 8 for an experiment with an *mng* mutant. The protection against ²⁸Mg loss by 10 mM Ca²⁺ such as seen in Fig. 8 has been variable from experiment to experiment, but at equal concentrations Ca²⁺ never protects against ²⁸Mg loss as completely as Mg²⁺.

DISCUSSION

Manganese has long been known to be mutagenic and growth inhibitory for *E. coli* (1, 8, 19); relations between magnesium and man-

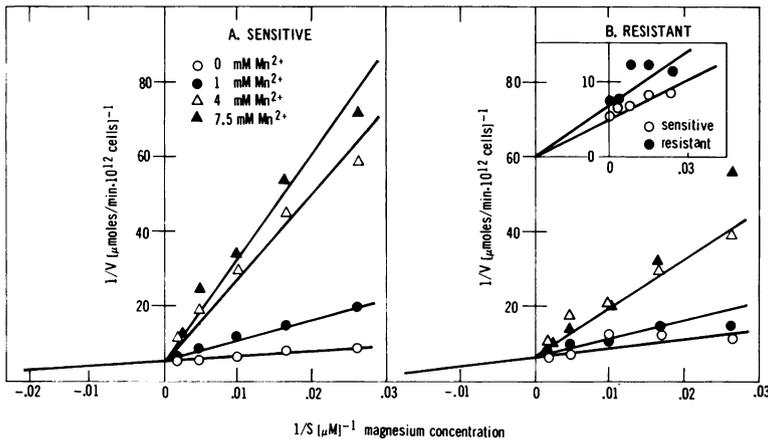


FIG. 5 Kinetics of manganese inhibition. Cells were grown at 37 C to 7.4×10^8 per ml (A, sensitive; K-10) or 7.2×10^8 per ml (B, resistant; mngK) and transferred to room temperature (21 C). In order, nonradioactive $MgCl_2$ (0 to 0.5×10^{-3} M), $MnCl_2$, and ^{28}Mg (from 2.8×10^{-6} to 8.0×10^{-6} M) were added. Samples were filtered at 5-min intervals from 2 to 22 min, and the initial rate of ^{28}Mg accumulation was converted to μmoles per min per 10^{12} cells from the known specific activity and cell concentration. (Inset) Data from the control (no Mn^{2+}) curves are replotted with a change of scale to point out the common K_m values but difference in V_{max} values.

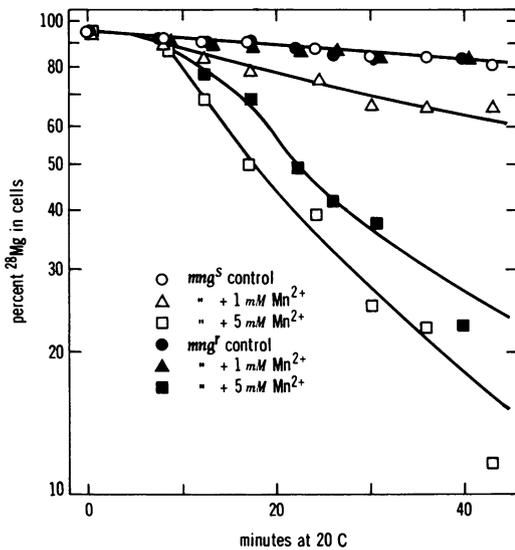


FIG. 6. Manganese-stimulated loss of magnesium: difference between sensitive and resistant strains. K-10 and mngK cells were grown for 3 hr at 37 C in the presence of $0.1 \mu Ci$ of ^{28}Mg per ml, harvested by centrifugation, and suspended in fresh broth. After filtering 1-ml samples without washing (both the filters and dried 0.5-ml samples of the filtrates were counted to determine the actual distribution of radioactivity between cells and culture fluid), $MnCl_2$ was added as indicated (time = 4 min), and additional 1-ml samples were filtered.

gane with regard to bacterial growth conditions have been extensively studied (1, 27). With other transition elements (but not manganese), mutants leading to increased sensitivity (4, 26) and genetic elements conferring resistance (24) have been reported. To our knowledge, this is the first report of mutants specifically resistant to manganese inhibition of growth. One possible reason that these mutants have not been sought before might be the relatively high concentration of manganese (10^{-2} M) needed for inhibition. We isolated the mutants because of our interest in the accumulation mechanisms for manganese and magnesium in *E. coli*.

The manganese transport system in *E. coli* has been described in some detail (2, 22, 23), and here we need only explain why it does not appear to be involved in manganese inhibition of growth and in the manganese resistance of the mutants. First of all, we could not find differences between the manganese-sensitive and the manganese-resistant strains with regard to this system (unpublished data). But we had not expected to for two reasons. (i) The low K_m (2×10^{-7} M) for the manganese transport system means that this system would be saturated at concentrations 1,000 times lower than those needed for growth inhibition. Additional increases in extracellular manganese would not affect the rate of functioning of this transport system. (ii) Magnesium is without effect on the

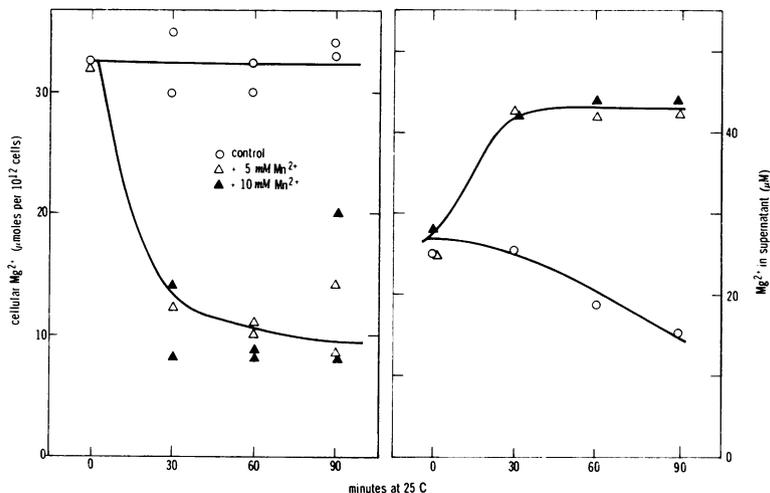


FIG. 7. Manganese-induced net loss of magnesium (atomic absorption spectroscopy). $MnCl_2$ (5 or 10 mM) was added to two of three cell samples (initially at 3.6×10^8 cells per ml). Either 5 min before or 30, 60, or 90 min after the addition of Mn^{2+} , 50-ml samples were centrifuged and the magnesium content of the resuspended cells and the supernatant fluids was measured by atomic absorption spectroscopy. Magnesium content per cell did not differ significantly between the 30-, 60-, and 90-min samples. With the control cells, supernatant fluid magnesium declined with time as the cells grew in number.

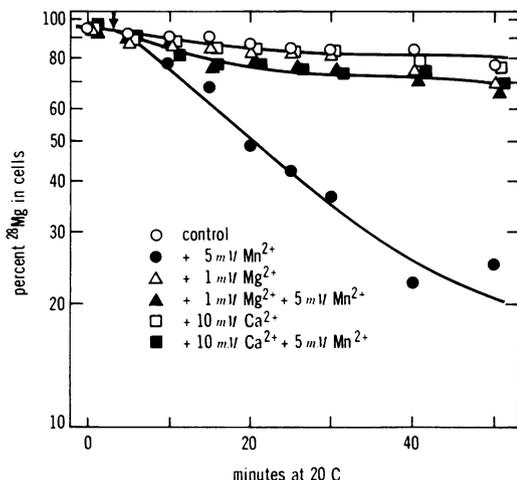


FIG. 8. Manganese-induced loss of ²⁸Mg: protection by magnesium and calcium. In the same experiment as in Fig. 7, 1 mM $MgCl_2$ or 10 mM $CaCl_2$ was added to portions of the radioactive *mngK* cells just prior to the addition of 5 mM $MnCl_2$.

manganese-specific system (22), whereas the manganese inhibition of growth depends critically upon the magnesium-manganese ratio.

The magnesium transport system (15, 17, 20, 21), however, has kinetic parameters consistent with the properties of manganese inhibition and the behavior of the mutants. Manganese is

a competitive inhibitor of magnesium accumulation with a K_i value of about 2 mM Mn^{2+} in dilute tryptone. Assuming that manganese is, in addition, a substrate for the magnesium transport system with a K_m value equal to the K_i value of 2 mM and a V_{max} value of the same order as that for magnesium, we can calculate that the manganese-specific system would be the principal manganese accumulation system only at concentrations below 4×10^{-5} M Mn^{2+} . When the external manganese concentration is raised to 10^{-2} M, 100 times more manganese might be entering the cell via the magnesium transport system than via the manganese-specific system. We have not directly measured ⁵⁴Mn accumulation by the magnesium system since in dilute cultures, such as in the experiments in Fig. 4 and 5, accumulation of radioactivity above background measurements can only be seen when the concentration in (or bound to) the cells is several times the medium concentration; this does not occur with manganese in the millimolar range. However, the carrier-mediated transport of manganese to equilibrium across the membrane would be expected to have drastic effects on cellular function. These effects have been discussed before (see references 17 and 21 for details and references) and will be outlined here in the context of the model in Fig. 9. Manganese might be expected to affect cell membrane

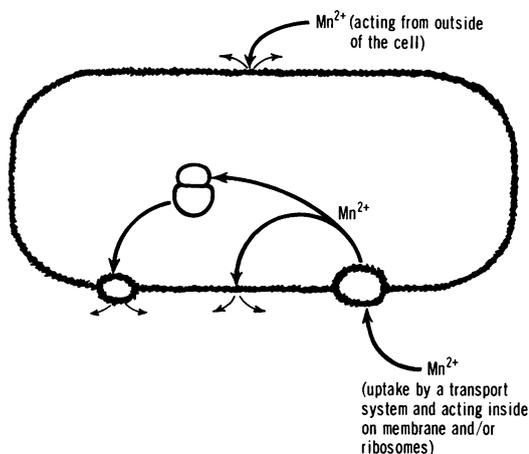


FIG. 9. Inhibitory action of manganese on *E. coli*.

integrity by displacing magnesium involved in maintaining the membrane structure. This might occur by manganese acting either directly on the outside of the membrane or by intracellular manganese displacing magnesium on the inner surface of the cell membrane [experimental evidence (21) suggests membrane permeability changes do not occur at the concentrations of 10^{-2} M or less Mn^{2+}]. Once within the cell, manganese competitively displaces magnesium as the bivalent cation on functioning enzymes which might be growth inhibitory. Quantitatively more significant, however, manganese displaces the bulk of the intracellular magnesium which is bound to ribosomes (13). This results in an increase in the free intracellular magnesium concentration, and the magnesium leaves the cells via the magnesium transport carrier. This model is consistent with and explains the results in Fig. 6-8. The cells lose primarily magnesium when high manganese is added; potassium is retained (21) showing that generalized membrane leakage does not occur. The manganese resistance of the *mng* mutants might simply be due to a lesser affinity for the magnesium accumulation system leading to less Mn^{2+} accumulation under given conditions, and therefore less inhibition of magnesium accumulation (Fig. 4 and 5) and less loss of cellular magnesium (Fig. 6 and 7). Although the differences between the resistant and sensitive cells are small (when measured over the relatively short time periods of the radioactive experiments; Fig. 5-8), they would "amplify" into larger differences in growth over a period of many hours (Fig. 1-3). However, because the differences between the sensitive and resistant strains are small, we do not feel at this time

that we can completely rule out alternative explanations involving mutant-controlled changes in internal magnesium-binding materials (ribosomes?) affecting the ease of manganese displacement of magnesium.

Other classes of magnesium mutants have been described. Cobalt-resistant mutants (17; Nelson and Kennedy, *manuscript in preparation*) appear to be altered in their magnesium transport system. Cobalt as an inhibitor of magnesium accumulation has one striking advantage over manganese for genetic studies. Cobalt is lethal rather than simply growth inhibitory, since once the cobalt is accumulated by the cells, it is firmly fixed and cannot be displaced by magnesium (17). Mutants requiring high magnesium concentrations for growth were previously reported (16), but these appear to have other causes for their high magnesium requirements not associated with accumulation. The magnesium accumulation system for these Mg^{2+} -requiring strains appears unaltered, and the mutants were found to make membranes with unusual lipid composition under conditions of magnesium starvation (Lusk and Kennedy, *J. Bacteriol.*, *in press*). Additional cobalt- and nickel-resistant derivatives of *E. coli*, *Aerobacter aerogenes*, and *Bacillus subtilis* were recently isolated by Webb (28). It seems reasonable that use of this variety of mutant strains will help clarify the nature of the cellular cation transport processes as well as the intracellular location and roles of the cations.

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