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Response to a Metal Ion-Citrate Complex in Bacterial Sensing

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Salmonella typhimurium responds chemotactically to gradients of divalent cations in the presence of citrate ions. The actual chemoeffector is the citrate-metal ion complex, which acts as an attractant. Citrate (which is also a chemoeffector for *Salmonella*) and the citrate-metal ion complex are recognized by different receptors. The response of *Salmonella*, which can transport citrate through its membrane, is quite different than that of *Escherichia coli*, which cannot.

Bacteria respond to gradients of chemicals in their environment by modifying their swimming behavior (1, 7, 12, 19, 20, 22). They swim smoothly when they encounter an increase in concentration of attractant molecules (or a decrease in repellent molecules), but they tumble and randomize direction when they encounter increases in repellents (or decreases in attractants). The net migration, called chemotaxis, enables bacteria to move up gradients of certain sugars, amino acids, and metabolites and down unfavorable gradients of compounds like phenol and indole. Generally, bacteria move toward molecules that promote survival (such as carbon sources) and away from molecules detrimental to survival (such as uncouplers of oxidative phosphorylation).

Divalent cations, however, do not fit neatly into this general pattern. Although beneficial metals such as Mg^{2+} and Ca^{2+} can act as attractants at high concentrations, toxic cations such as cobalt and nickel are also attractants for *Salmonella typhimurium* (32). The same divalent cations can act as repellents for *Escherichia coli* (30). Because of this anomaly and the importance of cations in cellular metabolism and signalling systems, a study of metal ions in chemotaxis was made and has led to partial characterization of the divalent cation receptor for chemotaxis.

MATERIALS AND METHODS

Materials. L-Malic acid, D-(+)-malic acid, *cis*-aconitic acid, *trans*-aconitic acid, and DL-isocitric acid were obtained from Sigma Chemical Co. Citric acid and sodium citrate were from Mallinckrodt. Nutrient broth was from Difco Laboratories. Bacterial strains ST1, ST171, ST356, and ST382 have been described previously (5, 6, 9). Strains AN1, M72, and M272, *S. typhimurium* strains mutant in the tricarboxylic acid transport system, were provided by Ko Imai, Institute for Fermentation, Osaka, Japan. Mutants in the *unc* genes (adenosine triphosphatase [ATPase] protein) of *Salmonella*, TT1039 and TT1042, were provided by John Roth, University of Utah, and Jen-Shiang Hong, Brandeis University.

Chemotaxis assay. Cultures (3 ml) of bacteria were grown to the midlog phase at 30°C with shaking. After the cells were collected by centrifugation at 3,000 × *g* for 10 min, the bacteria were resuspended at a density of about 10^8 cells per ml in 50 mM morpholinepropanesulfonic acid (MOPS)-10 μM EDTA and were then immediately diluted to 10^6 cells per ml in the desired assay medium. The cells were then incubated at 30°C with shaking for 15 to 45 min before chemotactic responses were tested.

The temporal assay was performed by the general procedure of Tsang et al. (29). To 9 drops of bacteria in a test tube at 30°C, 1 drop of 10× attractant was added, and the contents of the tube were mixed rapidly. From this mixture a sample was removed and added to a microscope slide. The behavior of the population was recorded by either of two methods. The first involved measuring the time after chemoeffector addition before an individual bacterium returned to a tumbling mode and averaging the values obtained from individual trials. The second method involved estimating the time required for 80% of the population to return to the unstimulated swimming pattern. The methods correlated well with each other.

Capillary assays were performed by the procedure of Adler and Templeton (3).

Enzyme assays. Membranes were prepared from bacteria grown to midlog phase at 30°C and collected by centrifugation. The cells were washed in 50 mM MOPS-10 mM $MgCl_2$ (pH 7.0), suspended in the same buffer containing 10 μg of DNase per ml, and disrupted by two passages through a French pressure cell at 20,000 lb/in^2. After the cell debris was removed by centrifugation at 10,000 × *g* for 10 min, the membranes were collected by centrifugation at 100,000 × *g* for 90 min. The membranes were washed in 50 mM MOPS-10 mM $MgCl_2$, pH 7.0, and then resuspended in 50 mM Tris-hydrochloride-10 mM $MgCl_2$, pH 8.0.

ATP hydrolytic activity was measured on these membranes by a modification of the method of Evans (10). Buffer and cofactor (30 μl of 0.33 M Tris [pH 8.0] -0.33 M $MgCl_2$) and various amounts of enzyme were

diluted to 480 μl with water. ATP (20 μl of 0.1 M ATP, pH 7.0) was added to initiate the reaction, which was allowed to proceed for 10 min at 37°C. The reaction was terminated by the addition of 500 μl of 14% trichloroacetic acid. If a precipitate formed in any tube, 0.1 ml of 10% sodium dodecyl sulfate was added to dissolve it. The inorganic phosphate released was immediately measured by the Fiske-SubbaRow colorimetric method (11).

Succinate dehydrogenase activity was assayed spectrophotometrically following the reduction of dichlorophenolindophenol at 600 nm after the addition of enzyme, succinate, and phenazine methosulfate (17, 18).

Protein concentration was determined by the method of Lowry et al. after trichloroacetic acid precipitation (21).

RESULTS

Role of citrate in divalent cation chemotaxis. Citrate is required for *S. typhimurium* to respond to divalent cations. If the bacteria are suspended in media without citrate, such as 50 mM MOPS, no change in swimming behavior is observed when 0 to 10 mM temporal gradients (23) of either $MgCl_2$ or $CaCl_2$ are applied. The bacteria respond normally to amino acids and sugars in this buffer. If the buffer contained 10 mM citrate, a smooth swimming response of about 1 min was observed (Table 1). This smooth swimming response in the presence of citrate was observed with a variety of metal ions (Table 2). The original finding that citrate was a chemoattractant was made a number of years ago (R. M. Macnab and D. E. Koshland, Jr., unpublished data) and has now been expanded in a recent study (M. Kihara and R. M. Macnab, personal communication) and in this paper.

L-Malate also facilitated the divalent cation response, but a number of other compounds tested were inactive (Table 3). Although L-malate was less effective than citrate, it was a strong attractant when added in the absence of divalent cations (24; M. Kihara and R. M. Macnab, manuscript in preparation).

This chemotactic response was shown in a conventional capillary assay. Bacteria moved up a spatial gradient of divalent cations when citrate was present in uniform concentration. They accumulated inside a capillary containing either $MgCl_2$ or $CaCl_2$ if citrate was present (Fig. 1). The drop in bacterial accumulation at 0.1 M $CaCl_2$ could have been due to paralysis produced at high $CaCl_2$ concentration or to the saturation effect observed for serine by Adler (2).

In the absence of citrate many of these divalent cations produce paralysis, even at concentrations as low as 10 μM (3). At concentrations below the level causing paralysis, the divalent cations tested did not show any effect, indicating that the free metal ions are neither attractants nor repellents. The concentrations of free metal

TABLE 2. *Effects of divalent cations on Salmonella in citrate*[a]

Stimulus (0–1 mM)	Smooth swimming response time (min)
$MgCl_2$	0.9
$CaCl_2$	1.2
$CoCl_2$	0.7
$NiCl_2$	0.6
$CuCl_2$	0.6
$FeSO_4$	0.6
$ZnCl_2$	0.7

[a] The experimental procedure was as discussed in the text except that 10 mM citrate was added to MOPS buffer. Tumbly mutant ST171 was used for the assay.

TABLE 3. *Specificity of the cofactor requirement in divalent cation chemotaxis*[a]

Compound	Smooth swimming response to 0–10 mM compound (min)	Smooth swimming response to 0–10 mM $MgCl_2$ for bacteria suspended in 10 mM compound (min)
Citrate	0.8	1.1
L-Malate	0.9	0.5
D-(+)-Malate	0.6	<0.2
cis-Aconitate	<0.2	<0.2
trans-Aconitate	<0.2	<0.2
DL-Isocitrate	<0.2	<0.2

[a] Experimental conditions as described in the text were applied to strain ST171. Compounds were neutralized to pH 7 before being added to buffer. The response to a 0 to 1 mM ribose stimulus was used as a control for each culture. In all cases, the ribose response was near 0.6 min.

TABLE 1. *Effect of citrate on divalent cation responses in wild type (ST1)*[a]

Stimulus	Resuspension buffer	Response
0–10 mM $MgCl_2$	50 mM MOPS, pH 7.0	None (wild-type swimming behavior)
0–10 mM $MgCl_2$	50 mM MOPS–10 mM citrate, pH 7.0	1-min smooth swimming response
0–10 mM $CaCl_2$	50 mM MOPS, pH 7.0	None (wild-type swimming behavior)
0–10 mM $CaCl_2$	50 mM MOPS–10 mM citrate, pH 7.0	1.2-min smooth swimming response

[a] For experimental procedure, see text.

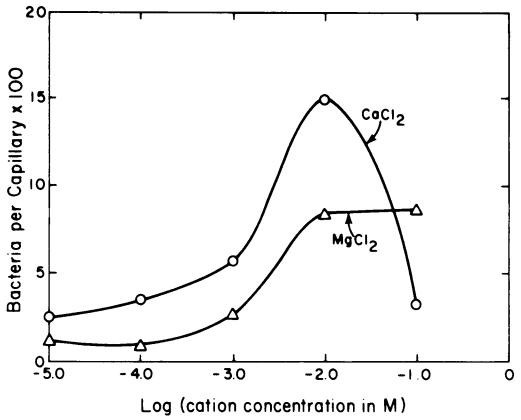

FIG. 1. *Bacterial accumulation inside cation-containing capillaries. Bacteria (strain ST1) were grown to midlog phase in nutrient broth and then washed by centrifugation and resuspended in 50 mM MOPS–10 mM citrate, pH 7.0. The bacteria were diluted to 5 × 10^6 bacteria per ml and added to sterile chambers. Microcaps (1 μl) containing the same buffer plus varying concentrations of either $MgCl_2$ or $CaCl_2$ were then added to the chambers. After 30 min at 30°C, the microcaps were removed and wiped clean, and the contents were added to sterile saline. The saline was diluted and plated out on nutrient broth plates. The number of bacteria per capillary was determined by counting colonies on the plates and correcting for dilution. Each point is the average of three determinations. Symbols: ○, $CaCl_2$ in the capillaries; △, $MgCl_2$ in the capillaries.*

ion and citrate-metal ion complex at equilibrium could be calculated from the stability constants, which range from $10^{3.3}$ for Mg^{2+} to $10^{5.9}$ for Cu^{2+} (16). The calculated final free metal ion concentrations in 10 mM citrate for Co(II), Ni(II), and Cu(II) were well below the concentrations that cause paralysis.

Since changes in levels of metal ions per se caused no change in tumbling frequency, the appearance of smooth swimming in a temporal gradient in the presence of citrate must have been due to either the increase in metal ion-citrate complex or the reduction in free citrate concentration. Free citrate by itself is an attractant, however, which means that a reduction in free citrate should produce tumbling, not smooth swimming. Therefore, the smooth swimming response to gradients of divalent cations was due to increases in the concentration of the metal ion-citrate complex, and this stimulus overrode the effects of reduction in free citrate concentration.

The attractant responses were not due to a lower K_m for the binding of the citrate-metal ion complex compared with that of citrate alone. As Fig. 2 shows, citrate acted at lower concentrations than the citrate-metal ion complex. Since the dissociation constants (K_d) determined through behavioral changes correspond to the K_d values of effector molecule to purified receptor proteins for the ribose (4, 5) and galactose (33) receptors, it is likely that the K_d values for citrate and metal ion-citrate binding to receptors are approximately equal to the half-maximal value for the behavioral responses. On this basis, the metal-citrate receptor K_d is higher than the citrate receptor K_d.

High concentrations of citrate did not inhibit the chemotactic response to the citrate-metal ion complex. Bacteria responded with smooth swimming to a 0 to 1 mM temporal gradient of the citrate-metal ion complex even in the presence of 50 mM citrate. Thus, citrate does not compete with the citrate-metal ion complex for binding to the chemotaxis receptor. This indication that the citrate and metal ion-citrate receptors are different was reinforced by growth experiments. *S. typhimurium* strains ST1 (wild type) and ST171 (a *cheT* mutant with a tumbly phenotype), when grown in Vogel-Bonner minimal salts medium plus citrate (VBC) plus 0.4% glucose, did not respond to temporal gradients of divalent cations in the presence of citrate but did respond to citrate alone (Table 4). The response of these bacteria to a simultaneous administration of 10 mM citrate and 10 mM $MgCl_2$ was the same as the response to citrate alone. If these bacterial strains were grown in nutrient broth or VBC, they responded with smooth swimming to divalent cation gradients and also to citrate alone (Table 4).

Response of ATPase mutants to divalent

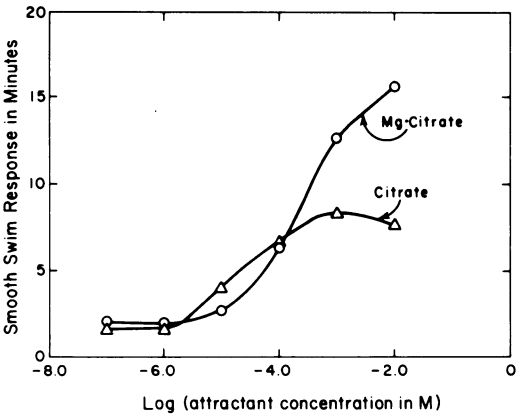

FIG. 2. *Sensitivity of the chemotactic response to citrate and magnesium citrate. Strain ST171, a tumbly mutant, was grown in nutrient broth and resuspended in 50 mM MOPS, pH 7.0 (see Table 1). The responses to either sodium citrate, pH 7.0 (△) or an equimolar mixture of $MgCl_2$ and sodium citrate, pH 7.0 (○) were determined as described in Table 2, footnote a.*

TABLE 4. *Effect of growth conditions on citrate and divalent cation taxis*[a]

Growth medium	Response to 0–10 mM $MgCl_2$ (min)[b]	Response to 0–10 mM Citrate (min)[c]	Response to 0–10 mM Mg·citrate (min)[c]
Nutrient broth	0.9	0.8	1.3
VBC	0.9	0.8	1.3
VBC + 0.4% glucose	<0.2	0.6	0.6

[a] The experimental conditions were as described in the text and were applied to strain ST171 (tumbly mutant).

[b] Bacteria were resuspended in 50 mM MOPS–10 mM citrate–10 μM EDTA, pH 7.0.

[c] Bacteria were resuspended in 50 mM MOPS–10 μM EDTA, pH 7.0.

cations. An earlier report implicated the Mg^{2+},Ca^{2+}-ATPase as the chemotaxis receptor for divalent cations (32). The current findings on the role of citrate led to a re-examination of that conclusion. This was done by using additional ATPase mutants. The *unc* mutant *Salmonella* strain TT1039, obtained from Jên-Shiang Hong (personal communication), was tested and demonstrated normal chemotactic responses to citrate and divalent cations. The mutation in this strain is due to insertion of the Tn10 (tetracycline resistance-conferring) transposon. The tetracycline resistance genes were 44% cotransducible with *asn* (using P22HT transducing phage) and 3% cotransducible with *rbsP*, which establishes a gene order of *unc*, *asn*, *rbsP*, *ilvC*. Uncoupled strain TT1039 possesses less than 3% of wild-type membrane-associated ATP hydrolytic activity. Immune precipitates of radiolabeled extracts of TT1039, when antiserum directed against *E. coli* coupling factor is used, contain only one polypeptide (molecular weight, 55,000), whereas immune precipitates of wild-type strains contain five polypeptides (molecular weights, 58,000, 52,000, 31,000, 21,500, and 13,000). To facilitate the chemotaxis measurements, the mutant *unc* gene was transduced into ST171, a tumbly *cheT* mutant described previously (6). The chemotactic responses to temporal gradients of divalent cations for this *unc* strain (ST399) were equivalent to the responses of ST171, the *cheT* mutant with a fully effective ATPase.

Another ATPase mutant, strain TT1042, was also tested and found to have normal divalent cation taxis. This strain possessed about 12% of the wild-type membrane-associated ATP hydrolytic activity. Thus, mutants possessing very low ATPase activity and mutants possessing partial activity responded normally to temporal gradients of divalent cations.

A different type of uncoupled mutant, an *nfe* mutant (Ingolia and Koshland, manuscript in preparation), was isolated and tested, and this mutant also possessed normal chemotactic responses to gradients of divalent cations. This *nfe* mutant is genotypically distinct from *unc* mutants (Ingolia and Koshland, manuscript in preparation), but it has the phenotype of an uncoupled ATPase species (8). When the mutant *nfe* gene was transferred to a *cheT* background, the divalent cation responses were once again found to be indistinguishable from the responses of the isogenic parent strain containing the functional ATPase.

Finally, under growth conditions that eliminate divalent cation chemotaxis in *Salmonella*, the levels of membrane-associated ATPase activity remained high. As noted above, growth of bacteria in VBC plus 0.4% glucose eliminated divalent cation chemotaxis, whereas bacteria grown on VBC responded with smooth swimming to gradients of divalent cations. Under these same growth conditions, the specific activity of membrane-associated ATP hydrolysis was the same in both cultures (Table 5).

Several other *Salmonella* mutants were examined for their responses to divalent cations and citrate. Since chemotaxis receptors are often transport proteins as well, tricarboxylic acid transport (*tct*) mutants (13–15) were studied. (These mutants were kindly provided by Ko Imai.) A *tct* mutant (strain M72), a *tct*III mutant (strain M272), and a double mutant in *tct*I and *tct*III (strain AN1) responded normally to temporal gradients of divalent cations and citrate.

Besides mutant studies, two other lines of evidence were used to implicate the ATPase as the chemotaxis receptor for divalent cations. First, dicyclohexylcarbodiimide, a known inhibitor of the ATPase, eliminated divalent cation chemotaxis in *S. typhimurium*, whereas ribose

TABLE 5. *Effect of growth conditions on ATP hydrolytic activity and divalent cation chemotaxis*[a]

Growth medium	Sp act of ATP hydrolysis[b]	Sp act of succinate oxidation[c]	Smooth swimming response to 0–10 mM $MgCl_2$ (min)
VBC	0.83	1.90	0.9
VBC + 0.4% glucose	0.76	0.51	<0.2

[a] Bacterial strain ST356, a derivative of LT2-Zinder, was grown and tested for enzymatic activity as described in the text.

[b] ATP hydrolytic activity is reported as micromoles of inorganic phosphate released per 20 min per milligram of protein.

[c] Succinate oxidation is reported as micromoles of dichlorophenolindophenol reducing equivalents produced per minute per milligram of protein.

chemotaxis was unaffected. However, when this experiment was repeated, it was found that dicyclohexylcarbodiimide eliminated the chemotactic response to citrate as well as the response to the citrate-metal ion complex. Dicyclohexylcarbodiimide may block divalent cation chemotaxis by inhibiting the receptor for the citrate-metal ion complex. The second piece of evidence was that the divalent cations that are attractants for *S. typhimurium* correspond to the cations that stimulate the ATP hydrolytic activity of the ATPase complex. Since the function of the metal ions in chemotaxis is now known to involve binding to citrate, the fact that these cations also stimulate the ATPase is fortuitous.

Divalent cation responses in *E. coli*. *E. coli* responds differently than *S. typhimurium* to temporal gradients of divalent cations (30). W3110, an *E. coli* strain whose chemotaxis has been studied extensively (1, 26), did not respond to temporal gradients of Mg(II) or Ca(II) but responded with tumbling to gradients of Ni(II) or Co(II) (Table 6) (1). The presence or absence of citrate in the medium did not affect the lack of response to Mg(II) or Ca(II). In the presence of citrate, higher concentrations of Ni(II) or Co(II) were required to elicit the tumbly response, probably because of the ability of citrate to complex these ions.

Another strain of *E. coli*, AN180, responded with smooth swimming to temporal gradients of Mg(II) and Ca(II) (32). The response was not dependent on the presence of citrate. Inclusion of citrate actually reduced the sensitivity of the bacteria to the divalent cations because of the citrate complexing.

DISCUSSION

Metal ion-citrate complex attraction in *S. typhimurium*. A general chemotaxis to metal ion-citrate complex was found for *S. typhimurium*. Metal ions alone are not chemoeffectors. Citrate alone is an attractant, but the attractant stimuli of citrate and the citrate-metal ion complex are separate and appear to act through different receptors. Evidence for this conclusion is that (i) certain growth conditions eliminate the citrate-metal ion response but not the citrate response, and (ii) citrate is not a competitive inhibitor of the chemotactic response to the citrate-metal ion complex.

Although citrate does not seem to bind to the citrate-metal ion complex receptor, the receptor protein recognizes the citrate portion of the complex. L-Malate was the only acid tested that could substitute for citrate in the chemotactic response, even though many other acids bound metal ions tightly. All divalent cations tested, however, functioned efficiently as cofactors in the citrate-metal ion response. The role of the metal ion could be to hold citrate in the proper conformation and/or to shield part of the negative charge in the citrate molecule.

Differences between *S. typhimurium* and *E. coli*. It is intriguing that the chemotactic responses of *S. typhimurium* and *E. coli* to citrate, metal ions, and the citrate-metal ion complex are different despite the great similarity of chemotaxis in *E. coli* and *S. typhimurium* (9). *E. coli* does not respond to citrate or the citrate-metal ion complex, whereas *Salmonella* does. This can be explained by the inability of *E. coli* to ingest citrate, in contrast to *Salmonella* which can do so. *E. coli* does respond to metal ions as chemoeffectors, whereas *S. typhimurium* does not. Also, in an *E. coli* strain that recognizes magnesium as an attractant, mutation of the ATPase protein causes the loss of the response to magnesium. ATPase mutations in *S. typhimurium*, however, do not affect chemotactic responses. Perhaps the attractant response to magnesium in the *E. coli* strain is due to an influx of magnesium (facilitated, presumably, by a relatively magnesium-leaky membrane), which stimulates the ATPase protein and therefore causes a transient rise in membrane potential. It is known that increases in membrane potential can cause an attractant response (25). An ATPase mutant would be unable to increase the membrane potential after an increase in magnesium concentration and therefore would not respond chemotactically to magnesium. It is logical that *E. coli* would not respond chemotactically to citrate or the citrate-metal complex since

TABLE 6. *Comparison of metal ion chemotaxis in S. typhimurium and E. coli*

Bacterium	Response to Mg, Ca	Response to Ni, Co	Response to citrate-metal ion complex	Response to citrate	Chemotactic response to Mg^{2+} by *unc* derivative	Growth on citrate
S. typhimurium	0	0	Attractant	Attractant	Yes	Yes
E. coli W3110[a]	0	Repellent	0	0		No
E. coli AN180[b]	Attractant	0	0	0	No	No

[a] Wild type was used for chemotaxis studies (1).
[b] *unc* mutant was used for energy coupling studies (29).

chemotactic responses would be expected to develop evolutionarily only toward utilizable compounds.

The situation with respect to iron appears to be different. *E. coli* possesses an iron transport system which utilizes citrate as a cofactor (31), even though *E. coli* cannot transport the citrate itself. Conversely, iron transport in *S. typhimurium* mutants lacking enterobactin (*enb* mutants) is inhibited by citrate (27), indicating that *S. typhimurium* cannot utilize citrate as an iron scavenger. The experiments described in this paper show that these iron transport systems are not associated with metal ion chemotaxis because *S. typhimurium* responds chemotactically to the iron-citrate complex, whereas *E. coli* does not.

Physiological benefits of a citrate-metal ion complex response. The chemotactic response to the citrate-metal ion complex could have developed as a means of accumulating required metals. Bacteria utilize magnesium as a cofactor in many enzymatic reactions, and they require metals, such as calcium, copper, zinc, cobalt, and iron, as cofactors and electron carriers. Since *S. typhimurium* utilizes citrate as a carbon and energy source, and since citrate binds divalent cations tightly, citrate could have been utilized as a convenient metal ion scavenger. Chemotaxis toward the metal ion-citrate complex would then maximize the ability of the cells to accumulate required metals. This would be especially important if the citrate concentration were high, since under these conditions the free metal ion concentrations would be very low and the transport systems specific for the free metal ions would be ineffective.

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