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Chemotactic Properties of *Escherichia coli* Mutants Having Abnormal Ca^{2+} Content

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The *calA*, *calC*, and *calD* mutants of *Escherichia coli* are known to be sensitive to Ca^{2+} (R. N. Brey and B. P. Rosen, J. Bacteriol. 139:824–834, 1979). In the absence of any added stimuli for chemotaxis, both the *calC* and the *calD* mutants swam with a tumbly bias. Both the *calC* and the *calD* mutants were defective in chemotaxis as measured by computer analysis, use of swarm plates, and capillary assays. The *calA* mutant was only slightly defective in motility and only slightly impaired in chemotaxis. Chemotactically wild-type cells had an intracellular free-Ca^{2+} level of about 105 nM. The intracellular free-Ca^{2+} levels of the mutants, as determined by use of the fluorescent Ca^{2+} indicator dye fura-2 or fluo-3, were about 90, about 1,130, and about 410 nM for *calA*, *calC*, and *calD*, respectively. Lowering the intracellular free-Ca^{2+} levels in wild-type cells and in the tumbly *cal* mutants by use of Ca^{2+} chelators promoted running (smooth swimming). Overexpression of CheZ (which causes dephosphorylation of CheY-phosphate) in the wild type and in the tumbly *cal* mutants decreased the level of tumbliness (which is caused by CheY-phosphate). The *calA* mutant was 4- to 10-fold more resistant than the wild type to the inhibitory effect of ω-conotoxin on chemotaxis. ω-Conotoxin had no effect on Ca^{2+} extrusion by wild-type *E. coli*; that result suggests that ω-conotoxin affects Ca^{2+} transport at the point of entry instead of exit.

In eukaryotic cells, calcium ions play a role as a second messenger for many events, such as signal transduction, behavior, and differentiation (8, 9, 26). The role of Ca^{2+} in prokaryotes, however, is not as well defined; for reviews, see references 25 and 28. Calcium ions have been shown to participate in chemotaxis by *Bacillus subtilis* (20, 21, 29) and in both chemotaxis and phototaxis by *Halobacterium halobium* (now called *Halobacterium salinarium*) (6, 27, 36). There is also evidence indicating calcium ion involvement in gliding motility by myxobacteria (49) and cyanobacteria (11a, 24, 27).

We have been reinvestigating the role of calcium ions in *Escherichia coli* chemotaxis. Several lines of evidence already indicate that calcium ions do participate in *E. coli* chemotaxis (27, 41–43, 48; see also Discussion in reference 7). Free Ca^{2+}, produced from caged Ca^{2+} compounds such as nitr-5 upon irradiation, results in tumbling; the receptor proteins (methyl-accepting chemotaxis proteins) are not required for this, but CheW, CheA, and CheY are required (41). By use of the fluorescent Ca^{2+} indicator dye fura-2, we have been able to show that repellents cause a temporary rise in cytoplasmic free-Ca^{2+} levels, while attractants cause a temporary fall, and for that the receptor proteins (methyl-accepting chemotaxis proteins) are required (42).

There are two calcium efflux systems in *E. coli*: a Ca^{2+}/PO_4^{2-} symporter and a Ca^{2+}/H^+ antiporter (5, 33). The mechanism of Ca^{2+} entry is unknown. Several calcium-sensitive mutants have been isolated, and three different calcium-sensitive loci were identified: *calA*, *calC*, and *calD* (7). (*calB* was also identified [7], but it seems to have been lost.) All three of these loci are associated with Ca^{2+}/PO_4^{2-} symporter activity. Mutations in either the *calC* or the *calD* locus result in defective Ca^{2+}/PO_4^{2-} symporter activity, while the *calA* mutation results in an increase in Ca^{2+}/PO_4^{2-} symporter activity. The *calA* phenotype, Ca^{2+} sensitivity, is expressed in cells only in a *corA* (Co^{2+} resistance) background; however, the *calA* locus by itself will still cause an increase in Ca^{2+}/PO_4^{2-} symporter activity. Recently, another Ca^{2+} transport gene, *chaA*, was identified, cloned, and sequenced (14); the *chaA* gene is involved in Ca^{2+}/H^+ antiporter activity, but mutants of this gene have not yet become available.

The chemotactic properties of these *calA*, *calC*, and *calD* mutants were investigated in this study. To correlate the inhibitory effect of ω-conotoxin, a calcium channel blocker, on *E. coli* chemotaxis (43) with Ca^{2+} transport, we tested the effect of ω-conotoxin on these *cal* mutants.

MATERIALS AND METHODS

Abbreviations. OD_{590}, optical density at 590 nm; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; BAPTA, bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Strains. All strains of *E. coli* used are derivatives of K-12 and are listed in Table 1. RB202, parent of the *calC* mutant, is apparently no longer available. Since RB202 was derived from KBT001 (the parent of *calD*) (7), the results for the *calC* mutant were compared with those for KBT001.

Growth conditions. Cells were grown in tryptone broth consisting of 1% tryptone (Difco Laboratories) and 0.5% NaCl or in Vogel-Bonner medium (47) containing the required amino acids at 1 mM each and 50 mM glycerol (minimal glycerol medium) or 50 mM DL-lactate (minimal lactate medium). Doubling times were determined by measuring the OD_{590}.

Chemotaxis assays. Cells were grown in tryptone broth by being shaken at 35°C until they reached an OD_{590} of 0.4 to 0.6. Then they were harvested by centrifugation at 6,000 × *g* for 3 min. The supernatant fluid was discarded, the pellet was resuspended, and chemotaxis medium (10 mM K^+ phosphate [pH 7.0], 0.1 mM K^+ EDTA, and 1 mM L-methionine) was added. This wash was followed by two more such washes in chemotaxis medium, and finally the cells were resuspended in chemotaxis medium to an OD_{590} of 0.01 (about 7×10^6 bacteria per ml). Chemotaxis was assayed by the capillary method (3).

Chemotactic ability was measured also by use of tryptone swarm plates (2). In this assay, bacteria migrate in response to a gradient of amino acids created by their metabolism. Tryptone swarm plates containing 1% tryptone, 0.5% NaCl,

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TABLE 1. Bacterial strains used

Strain	Relevant characteristics	Reference or source
AW574	Chemotactically wild type; *thr leu his lac xyl rpsL*	16
B275	Chemotactically wild type; *thr leu met rpsL lac phoA ton* λ^r *tsx mal* (λ)	4
KBT001	Chemotactically wild type; *purE trp leu proC ara lac rpsL metE lysA*	7
2561	AW574 *calA corA*	7
RB063	*calC purE trp leu proC ara lac rpsL metB argH cycA*	7
RB073	KBT001 *calD*	7
RP1616	Δ*cheZ rpsL metF leuB hisG thr thi ara mtl xyl tonA tsx*	19
RP3098	Δ*fhlCD thi thr leu his met rpsL*	31
CAG18433	*zbf-3057*::Tn*10*	38
CAG18594	*proC zaj-3099*::Tn*10kan*	38
AW833	B275 *calC zbf-3057*::Tn*10*	This study
AW834	B275 *calD zaj-3099*::Tn*10kan*	This study
pBB1	*cheZ* under *trp* promoter	A. Wolfe

and 0.25% Bactoagar (Difco Laboratories) were inoculated in the center with a stab of approximately 10^6 cells and incubated at 35°C for 8 to 14 h.

Analysis of free-swimming behavior. Bacterial swimming behavior was observed at 30°C by phase-contrast microscopy at a magnification of ×400. The cells were suspended in chemotaxis medium or in filtered used growth medium to an OD_{590} of 0.1. The microscopic behavior was videotaped and analyzed by computer (35). Alternatively, the swimming pattern was analyzed by a photographic method (13) (see the legend to Fig. 1).

Tris-EDTA permeabilization. Cells grown in minimal glycerol or minimal lactate medium were made permeable by Tris-EDTA treatment (17, 40, 46).

Calcium sensitivity assay. Calcium sensitivity was measured by the growth inhibition assay of Brey and Rosen (7). The *cal* mutant strains were tested for Ca^{2+} sensitivity before and after each of the chemotactic assays to make sure that the *cal* mutation had been retained.

Electroporation of Ca^{2+} chelators and Ca^{2+} indicator dyes. According to a procedure described previously (41), the Ca^{2+} chelators diazo-2 (1), diazo-3 (1), BAPTA (1, 44), and EGTA (11, 44) were electroporated into cells. Direct measurements of intracellular free Ca^{2+} were determined by electroporating the fluorescent calcium indicator dye fura-2 (11) or fluo-3 (23). For all these electroporations, the following procedure was used.

Cells were incubated in tryptone broth by being shaken at 35°C until they reached an OD_{590} of 0.4 to 0.6. Then they were harvested by centrifugation at 6,000 × *g* for 3 min. The supernatant fluid was discarded, the pellet was resuspended, and 5 ml of electroporation buffer (1 mM HEPES [pH 7.2] and 10% glycerol) was added. This was followed by two more such washes in electroporation buffer, and finally the cells were suspended in 1 ml of electroporation buffer at an OD_{590} of 2 to 6 (about 1.5×10^9 to 4.5×10^9 bacteria per ml). The washed cells were stored on ice until electroporation. Sometimes cells from overnight tryptone broth cultures were used instead; in those experiments, the cells were diluted into fresh tryptone broth to an OD_{590} of 0.1 and incubated by being shaken at 35°C for two or three doublings before washing and electroporation.

Diazo-2 (in water at 300 μM), diazo-3 (in water at 300 μM), BAPTA (in water at 1 mM), EGTA (in water at 1 mM), fura-2 (in water at 25 to 100 μM), or fluo-3 (in water at 25 to 100 μM) was introduced into *E. coli* (a 200-μl suspension of the washed cells) by electroporation by use of a single pulse at a capacity of 25 μF with a field intensity of 5 kV/cm at 200 Ω for 4 to 6 ms in a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). Immediately after electroporation, the cells were diluted with 1 ml of filtered used (OD_{590}, 0.4 to 0.6) tryptone broth and incubated at room temperature for 15 min without shaking.

The above procedure was also performed with minimal glycerol or minimal lactate medium replacing tryptone broth. The cells were collected by centrifugation at 6,000 × *g* for 3 min. The supernatant fluid was discarded, the pellet was resuspended, and 5 ml of HMK buffer (50 mM HEPES [pH 7.5], 1 mM $MgCl_2$, and 100 mM KCl) was added. This was followed by an additional wash in HMK buffer, and finally the cells were resuspended in HMK buffer. Then they were electroporated as described above.

Fluorescence measurements. Fluorescence measurements were made with a spectrofluorometer (SLM Instruments, Inc., c/o Milton Roy Co., Rochester, N.Y.) interfaced with a computer. During the measurements, the cell suspension was continuously stirred. For fura-2 measurements, an excitation scan was performed with the emission monochrometer set at 510 nm. For fluo-3 measurements, an emission scan was performed with the excitation monochrometer set at 490 nm. In both cases, the excitation and emission slit widths were 8 and 4 nm, respectively. The excitation spectra were corrected for variations in the excitation light intensity.

Measurement of $[Ca^{2+}]_i$. The intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated by use of the following equation (45): $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$ where F is the fluorescence intensity of the dye in the cells, F_{max} and F_{min} are the intensities at saturating and zero calcium concentrations, respectively, and K_d is the dissociation constant for Ca^{2+} and fura-2 or fluo-3. At 30°C, fura-2 and fluo-3 had K_ds for Ca^{2+} of 192 and 574 nM, respectively. After the measurement of fluorescence intensity, cells were disrupted by sonication. Then F_{max} was measured by the addition of 1 mM $CaCl_2$, and F_{min} was measured by the addition of 10 mM EGTA.

As an alternative method for fura-2 measurements, Ca^{2+} levels were determined from the ratio of fluorescences at 340 versus 380 nm as described by Grynkiewicz et al. (11). R_{max} (Ca^{2+}-saturated fluorescence ratio) and R_{min} (free-dye fluorescence ratio) were determined by the addition of 1 mM $CaCl_2$ or 10 mM EGTA, respectively, to cells disrupted by sonication. Autofluorescence was always measured and subtracted from the fluorescence data.

Membrane vesicle preparations. Everted membrane vesicles were prepared by the method of Rosen (32). Cells grown in Vogel-Bonner lactate medium were harvested by centrifugation at 10,000 × *g* for 5 min at 4°C. The supernatant fluid was discarded, and the pellet was resuspended and washed once with buffer I (10 mM Tris-HCl [pH 7.6], 0.14 M choline chloride, 0.5 mM dithiothreitol, and 0.25 M sucrose). The washed cells were disrupted by a single passage through a French pressure cell at 4,000 lb/in^2. The crude lysate was centrifuged at 10,000 × *g* at 4°C for 10 min to sediment cellular debris. To sediment the membrane fraction, the resulting supernatant fluid was centrifuged for 1.5 h at 47,000 rpm in a 70 Ti rotor at 4°C. The membrane pellet was resuspended in 1.0 ml of buffer I. Everted membrane vesicles were either used on the day of preparation or quickly frozen and stored at −70°C; membrane vesicles stored in this manner retained functional activity.

Calcium transport assays. Calcium transport was assayed by $^{45}Ca^{2+}$ uptake of everted membrane vesicles by the method of Rosen (32). ω-Conotoxin was added directly to these everted membranes or else to spheroplasts before passages through a French pressure cell. The latter method allowed both sides of the membrane to be exposed to ω-conotoxin.

Transduction of *cal* mutations. The *calC* and *calD* mutations were transferred to chemotactically wild-type strain B275 by P1 transduction by use of the $P1_{vir}$ phage according to the method of Miller (22). To facilitate the transfer of *calC* and *calD*, markers for Tet^r and Kan^r, respectively (from CAG18433 and CAG18594, whose chromosomes carry Tet^r and Kan^r, respectively [38]), located within 1 min of the *cal* mutations, were first transduced into the mutant strains. The *cal* mutations and the resistance markers were cotransduced into strain B275 by P1 transduction. The resulting transductants, AW833 and AW834, were selected for antibiotic resistance and screened for calcium sensitivity.

Chemicals. BAPTA, diazo-2, diazo-3, fura-2, and fluo-3 were purchased from Molecular Probes Co. EGTA was purchased from Sigma Chemical Co. ω-Conotoxin GIVA was purchased from Research Biochemical Inc. and is here called ω-conotoxin.

RESULTS

In *E. coli*, three genes, *calA*, *calC*, and *calD*, are involved in the efflux of Ca^{2+} by means of a Ca^{2+}/PO_4^{2-} symporter (7). Here, we report the effect of each of these *cal* mutations on motility and chemotaxis.

Swimming behavior of calcium transport mutants. Under unstimulated conditions, chemotactically wild-type cells swim with a pattern of alternating runs and tumbles. Both the *calC* and the *calD* mutant strains exclusively tumbled, unlike the wild-type parent (Fig. 1). The *calA* mutant (not shown) was only slightly more tumbly than its wild-type parent.

To provide an objective analysis of swimming behavior, such microscopic observations were subjected to computer analysis. The behavior of free-swimming bacteria was videotaped, digitized, and analyzed by computer (35). This technique measures both the average angular speed (the rate of change in direction) and the average linear speed (the rate of movement in a straight line) of a population of motile bacteria. The angular and linear speeds are directly and inversely proportional, respectively, to the tumbling frequency: an increase in the angular speed reflects an increase in the time spent tumbling, while a decrease corresponds to a reduction in tumbling (an increase in running).

Figure 2 shows the computer analysis of the swimming behavior of the mutant strains and the wild-type *E. coli*. In com-

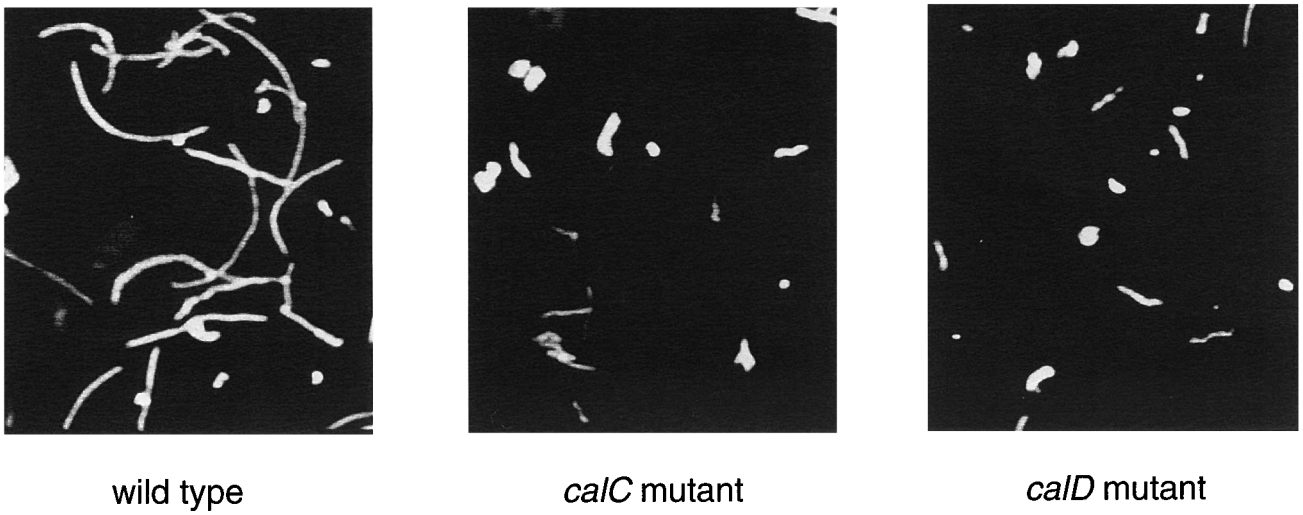

FIG. 1. Comparison of the free-swimming behavior of chemotactically wild-type strain KBT001, *calC* mutant RB063, and *calD* mutant RB073. Swimming cells grown in tryptone broth at 35°C were observed in tryptone broth at 30°C under a phase-contrast microscope and recorded on videotape. A reverse-image recording was generated from the videotape, and swimming tracks were photographed from the reverse-image videotape with an exposure time of 1 s.

parison with its chemotactically wild-type parent (AW574), the *calA* mutant had a slightly increased angular speed and a somewhat decreased linear speed, consistent with its slightly increased degree of tumbliness. Both the *calC* and the *calD* mutant strains had decidedly increased angular speeds and decreased linear speeds compared with their chemotactically wild-type parent (KBT001); this is expected from their tumbly behavior. These results for *calA*, *calC*, and *calD* were found to the same degree for cells grown in tryptone broth or minimal glycerol medium (data not shown).

Chemotactic behavior of calcium transport mutants. Figure 2 shows that the addition of an attractant (0.1 mM L-serine) to the parental wild-type strains or the *calA* mutant strain resulted in decreased angular speed and increased linear speed. The addition of repellent (3 mM Na^+ acetate) to these strains resulted in the opposite effect, increased angular speed and decreased linear speed. In contrast, the addition of attractant or repellent to the *calC* or *calD* mutants changed their angular and linear speeds little or not at all (Fig. 2).

Wild-type *E. coli* cells form two or more chemotactic rings on a tryptone swarm plate; the outer ring represents L-serine chemotaxis, while the inner ring represents L-aspartate chemotaxis (2). The *calA* mutant was hardly defective in swarming, while both the *calC* and the *calD* mutants were totally defective in swarming (Fig. 3). For comparison, we used a Δ*cheZ* strain (RP1616), which is a tumbly mutant, and a Δ*flhCD* strain (RP3098), which is a nonmotile mutant; the Δ*cheZ* plate looked like the plates for *calC* and *calD*, while the Δ*flhCD* plate showed no spreading at all (data not shown).

Analysis of chemotactic behavior by use of tryptone swarm plates is influenced by growth rate. This factor is eliminated in the capillary assay by the use of chemotaxis medium (Fig. 4). While the *calA* mutant strain was slightly defective, both the *calC* and the *calD* mutant strains were largely defective in chemotaxis toward L-serine or L-aspartate, compared with the parental wild types.

The above results show that both the *calC* and the *calD* mutants are defective in chemotaxis (Fig. 2 to 4). To eliminate possible strain-dependent effects, the *calC* and *calD* loci were transferred by P1 transduction (see Materials and Methods, "Transduction of *cal* mutations") to B275, a different chemotactically wild-type strain (4). The chemotactic defects were found to cotransduce with the *cal* genes in the B275 derivatives, AW833 and AW834 (data not shown). This indicates that the *cal* genes are responsible for these chemotactic defects.

Measuring intracellular free-Ca^{2+} levels by use of Ca^{2+} indicator dyes. Gangola and Rosen (10) measured intracellular free-Ca^{2+} levels in *E. coli* by use of the fluorescent Ca^{2+} indicator dye fura-2 (11) produced intracellularly. Uncoupled Tris-EDTA-permeabilized cells were incubated with the acetoxymethyl ester of fura-2 (10), which is membrane permeable and thus freely crosses the cytoplasmic membrane. Once the ester of fura-2 was inside, the cells then slowly hydrolyzed the ester to form fura-2. Once all five ester linkages are cleaved, the fura-2 is in the membrane-impermeable form and is able to bind free Ca^{2+} (10). To circumvent these requirements, we have loaded cells with fura-2 itself, though it is membrane impermeable, by electroporation (41). Cells were loaded in the same way with another fluorescent Ca^{2+} indicator dye, fluo-3 (23).

The following data indicate that electroporation had loaded fura-2 into the cell cytoplasm, not primarily into the periplasm. Cells were loaded with fura-2 by electroporation and were then treated with Tris-EDTA to break the outer membrane barrier (17, 40, 46); these cells did not release fura-2 (data not shown). However, cell disruption by sonication did release the dye (data not shown).

The intracellular free-Ca^{2+} levels for the wild-type and *cal* mutant strains of *E. coli* were measured by use of the Ca^{2+} indicator dyes fura-2 and fluo-3. The results are shown in Table 2. Our measurement for intracellular free Ca^{2+} for wild-type *E. coli*, around 105 nM, is similar to the value of 90 ± 10 nM reported by Gangola and Rosen (10). The *calC* and *calD* mutants, for which Ca^{2+} values have not been previously reported, had elevated levels of intracellular free Ca^{2+}. This indicates that their tumbly swimming bias was caused by these elevated levels of intracellular free Ca^{2+}. It is possible that fura-2 itself was buffering cytosolic Ca^{2+}, so we also used as an indicator dye fluo-3, which has a lower affinity for Ca^{2+} than does fura-2 (23), but we obtained similar measurements for intracellular free Ca^{2+} (Table 2). These similar results with two dyes indicate that the intracellular free-Ca^{2+} measurements reflect the true values and are not the result of an individual indicator.

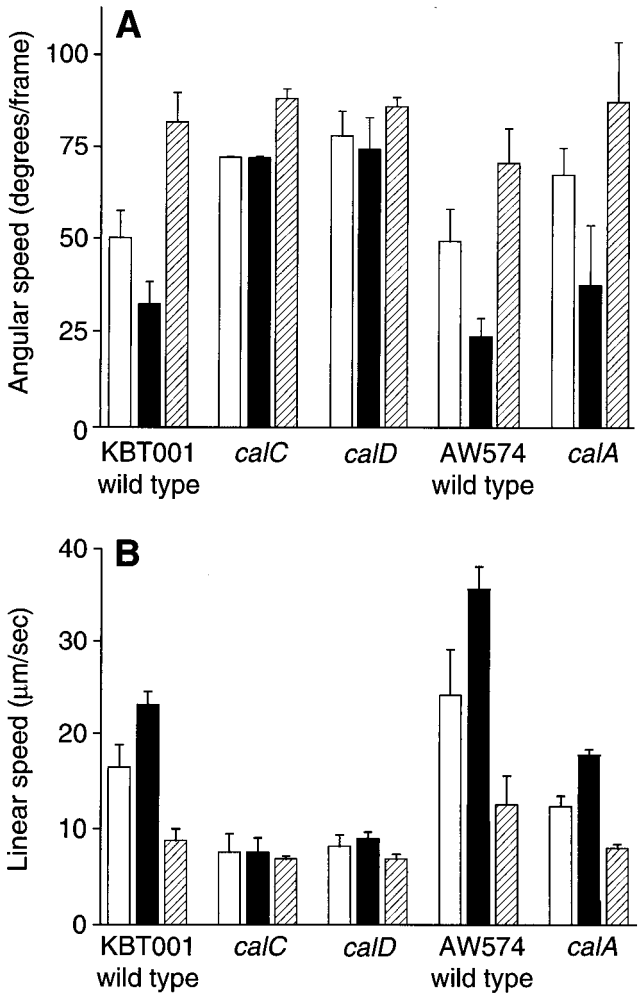

FIG. 2. Comparison of the average angular and linear speeds of free-swimming wild-type and calcium-sensitive mutant strains in chemotaxis medium. The free-swimming behavior was videotaped and analyzed by computer as described in Materials and Methods (35). Averages for three to six measurements of different fields are plotted; the ranges of the results are indicated by the error bars. (A) Average angular speed; (B) average linear speed. The *calC* mutant is RB063, the *calD* mutant is RB073, and the *calA* mutant is 2561. Open bars, unstimulated (neither attractant nor repellent added); closed bars, 0.1 mM L-serine added; hatched bars, 3 mM Na^+ acetate added. These observations were made within 10 s of addition of attractant or repellent.

Overnight cultures of cells were inoculated fresh and loaded with fura-2 by electroporation. The cells were then grown further for several generations, with no effect of fura-2 on their generation time or cell viability. This result shows that fura-2 was not harmful to the cells. During this incubation, cytosolic Ca^{2+} was measured and found to remain constant (data not shown), whether the cells were grown in tryptone broth or minimal glycerol or minimal lactate medium. This illustrates that fura-2 was not buffering cytosolic Ca^{2+}.

Either without added external Ca^{2+} or with various elevated levels of external Ca^{2+}, these bacteria maintained around 100 nM basal levels of cytosolic Ca^{2+} (Fig. 5, bottom curve). Treatment with a Ca^{2+} ionophore, ionomycin (18), allowed free access of externally added Ca^{2+} to the fura-2 in the cytoplasm (Fig. 5, top curve); this resulted in loss of control of cytoplasmic Ca^{2+} levels.

Changing the intracellular free-Ca^{2+} levels by use of Ca^{2+} chelators. The Ca^{2+} chelator BAPTA (1, 44) and the more-or-less Ca^{2+}-specific chelator EGTA (11, 44) were electroporated into the cytoplasm of *E. coli* cells (see Materials and Methods). Lowering of the intracellular Ca^{2+} concentration by the introduction of Ca^{2+} chelators into the cell cytoplasm promoted running of the *calC* and *calD* mutants and the chemotactically wild-type parent.

Diazo-2 is a caged Ca^{2+} chelator which takes up Ca^{2+} upon illumination (1). The increased affinity of diazo-2 for Ca^{2+} is the result of a photochemical rearrangement of a diazoacetyl group to a carboxymethyl group. Diazo-3 is a control compound which does not change its affinity for Ca^{2+} upon photolysis (1). Electroporation of the *calD* mutant RB073 with 0.3 mM diazo-2 results in tumbling cells before photolysis by 370-nm-wavelength light but running cells after photolysis. Electroporation of the *calD* mutant with 0.3 mM diazo-3 results in tumbling cells whether or not there is photolysis. Thus, lowering the concentration of intracellular Ca^{2+} promotes running in the *calD* mutant. Similar results were obtained with the *calC* mutant.

Overexpression of CheZ in the *calC* and *calD* mutants. The CheZ protein increases the dephosphorylation rate of the tumble-causing phosphorylated CheY protein (12). We tested the effect of increased levels of CheZ on the swimming behavior of both the *calC* and the *calD* mutants by turning on a *cheZ* plasmid (pBB1) and analyzing the results by computer analysis. Increasing the level of CheZ decreased the angular speed in the wild type and also in both the *calC* and the *calD* mutants (data not shown), and the bacteria were observed to swim more smoothly. This might be expected if the higher levels of Ca^{2+} in *calC* and *calD* mutants result in a high level of phosphorylated CheY, which would promote tumbling.

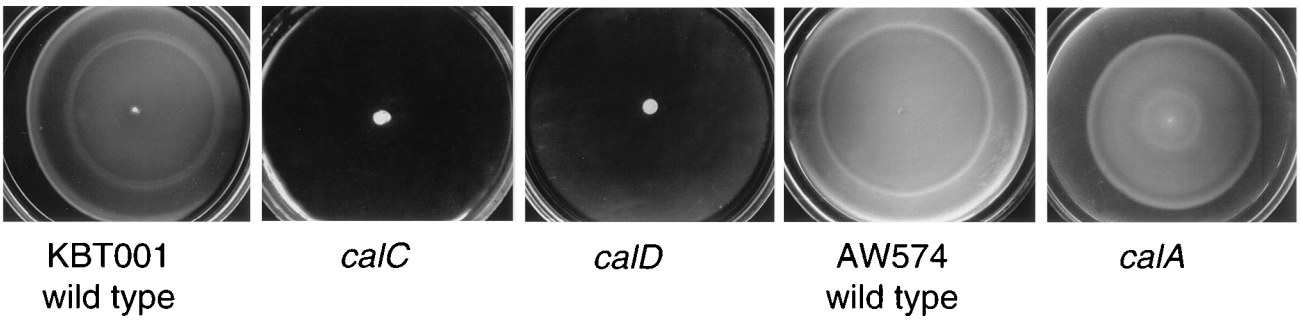

FIG. 3. Chemotactic properties of wild-type and calcium-sensitive mutant strains of *E. coli* as determined by the tryptone swarm assay. Bacteria which had been grown overnight in tryptone broth at 35°C were used as inocula for the tryptone swarm plates. The swarm plates were incubated at 30°C for 9 h. Results are shown for parental chemotactically wild-type strains KBT001 and AW574 and *calC* mutant RB063, *calD* mutant RB073, and *calA* mutant 2561.

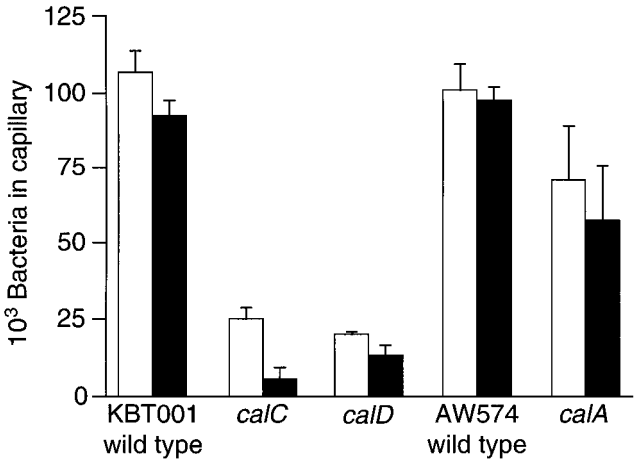

FIG. 4. Chemotactic properties of wild-type and calcium-sensitive mutant strains of *E. coli* as measured by capillary assay. Bacteria were grown in tryptone broth and treated as described in Materials and Methods. Cells were incubated for 30 min at 30°C in chemotaxis medium and were then presented with capillaries containing either no attractant or 1 mM L-serine or 1 mM L-aspartate in chemotaxis medium. Each point is the average of three determinations; the ranges of results are indicated by the bars. This experiment has been repeated a number of times with similar results. Open bars, aspartate chemotaxis; closed bars, serine chemotaxis. When no attractant was added to the capillaries, the numbers of bacteria entering were in every case too small to show up on this *y*-axis scale. We did not test concentrations of attractant other than 1 mM, so we cannot exclude a shift of the dose-response curve in the mutants.

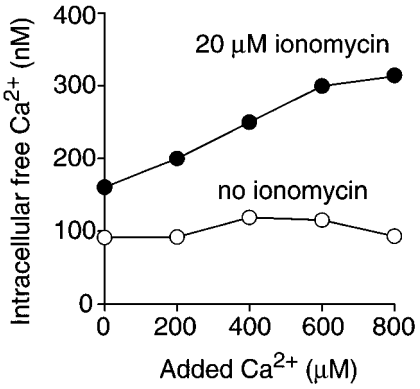

FIG. 5. Effect of extracellular Ca^{2+} on cytosolic Ca^{2+} levels in wild-type *E. coli* AW574. Cells were loaded with fura-2 by electroporation, and fluorescence measurements were made as described in Materials and Methods. Ionomycin was added outside the cells after they had been electroporated with fura-2.

For the *calC* and *calD* mutants and the wild type, the amount of CheZ found in cells without the CheZ plasmid was quantified by immunoblotting with anti-CheZ antibodies. The *cal* mutants had levels of CheZ similar to that of the wild type (data not shown). This indicates that the tumbly behavior of the mutants was not due to a lack of CheZ.

Effect of ω-conotoxin on calcium transport mutants. The effect of ω-conotoxin on motility and chemotaxis of the *calA* mutant was assayed by the capillary method and compared with the effect on parental strain AW574 (Fig. 6). The *calA* mutant was more resistant to ω-conotoxin than the wild-type cells. The mutant required nearly fourfold-more ω-conotoxin to inhibit chemotaxis by 50% than did the wild type (Fig. 6A). *calA* cells treated with Tris-EDTA to allow access of ω-conotoxin to the cytoplasmic membrane required for 50% inhibition of chemotaxis about 10-fold-more ω-conotoxin than did the wild type (Fig. 6B). This indicates that the ω-conotoxin resistance of the *calA* mutant was not due to changes in its permeability to ω-conotoxin. Resistance occurs presumably because of an alteration of an ω-conotoxin receptor on the cytoplasmic membrane or an alteration at some point downstream of such a receptor.

Although the two other mutants, *calC* and *calD*, were largely defective in chemotaxis (Fig. 2 to 4), the effect of ω-conotoxin on these two strains was nevertheless tested. ω-Conotoxin slightly inhibited the residual chemotaxis by the *calD* mutant and slightly stimulated the residual chemotaxis by the *calC* mutant (data not shown).

ω-Conotoxin and $^{45}Ca^{2+}$ uptake by everted wild-type membrane vesicles. The effect of ω-conotoxin on wild-type *E. coli* calcium exit was measured by use of an everted wild-type membrane-transport system (32). Everted membrane vesicles accumulated $^{45}Ca^{2+}$ in the presence of an energy source such as NADH; no accumulation was observed in the absence of NADH. The addition of ω-conotoxin to everted membrane vesicles had no effect on rates of Ca^{2+} exit carried out by either the Ca^{2+}/PO_4^{2-} symporter or the Ca^{2+}/H^+ antiporter (data not shown). Calcium transport by membrane vesicles which were preloaded with ω-conotoxin by the addition of ω-conotoxin during spheroplast formation was also not inhibited (data not shown). The effect of ω-conotoxin on calcium entry into intact cells and right-side-out membrane vesicles of *E. coli* was not tested, because these cells and vesicles accumulate Ca^{2+} only at a low temperature or in the presence of an uncoupler (37); under these conditions, Ca^{2+} accumulates passively and presumably not by active transport. These data imply that ω-conotoxin affects calcium transport of *E. coli* cells at the point of entry instead of exit.

TABLE 2. Measurement of the intracellular free-Ca^{2+} levels of *cal* mutants

Strain	Intracellular $[Ca^{2+}]$ (nM) determined by[a]:	
	Fura-2	Fluo-3
Chemotactically wild-type AW574	108 ± 11	104 ± 3
Chemotactically wild-type KBT001	104 ± 4	105 ± 5
calA mutant 2561	94 ± 16	87 ± 10
calC mutant RB063	1,327 ± 662	928 ± 211
calD mutant RB073	381 ± 139	434 ± 162

[a] Intracellular free-Ca^{2+} concentrations were determined as described in Materials and Methods. Cells were loaded with Ca^{2+} indicator dye fura-2 or fluo-3 by electroporation. The averages for 3 to 12 measurements and the ranges of the results are presented.

DISCUSSION

There is a strong correlation between the cytosolic free-Ca^{2+} level and the tumbliness of *E. coli* cells. Both the *calC* and the *calD* mutant strains, which have high levels of cytoplasmic free Ca^{2+}, swim with a tumbly bias (Fig. 1 and 2), while the chemotactically wild-type strain and the *calA* mutant, which have relatively low levels of free Ca^{2+}, run and tumble (Fig. 1 and 2). Running was promoted by lowering the intracellular levels of cytosolic free Ca^{2+} through use of Ca^{2+} chelators or of a caged Ca^{2+} chelator which takes up Ca^{2+} upon illumination. The two mutants with high levels of Ca^{2+}, *calC* and *calD* mutants, were defective in chemotaxis, as measured by angular and linear speeds (Fig. 2), use of tryptone swarm plates (Fig. 3), and capillary assays (Fig. 4). Brey and Rosen (7) reported that none of these mutants showed any

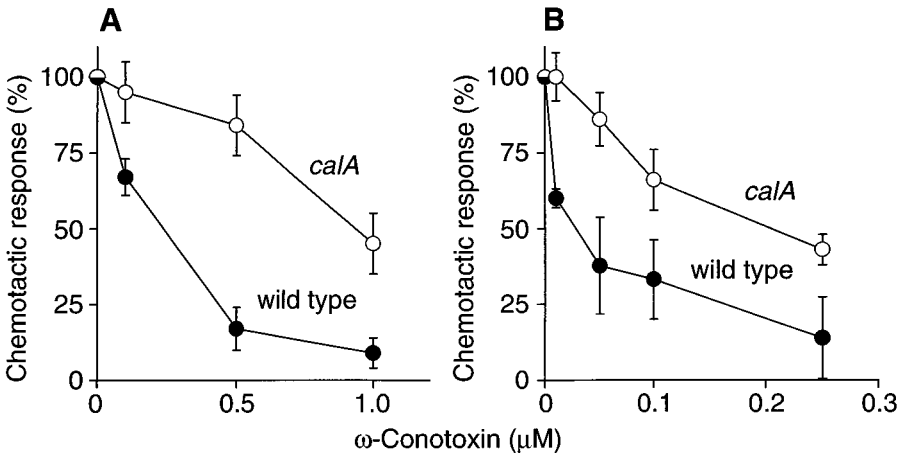

FIG. 6. ω-Conotoxin inhibition of chemotaxis in the *calA* mutant 2561 compared with that of the parental wild-type strain AW574. The cells were grown in minimal glycerol medium and treated as described in Materials and Methods. Bacteria were incubated for 30 min at 30°C in chemotaxis medium with different concentrations of ω-conotoxin, and then they were presented with capillaries containing the attractant L-serine (1 mM) in chemotaxis medium. In each case, the concentration of ω-conotoxin in the capillary was the same as in the pond of bacteria. Each point is the average of three determinations; the ranges of results are indicated by the bars. This experiment has been repeated a number of times with similar results. (A) Untreated *E. coli*. The responses without ω-conotoxin (100%) were 56,200 ± 8,300 and 71,800 ± 10,000 bacteria in the capillary for the *calA* mutant and the parental wild type, respectively. (B) Tris-EDTA-treated *E. coli* (40). The responses without ω-conotoxin (100%) were 43,900 ± 7,700 and 51,500 ± 5,000 bacteria in the capillary for the *calA* mutant and the parental wild type, respectively.

obvious chemotactic defects (although see Discussion in reference 7).

By electroporating into *E. coli* the fluorescent Ca^{2+} indicator dye fura-2 or fluo-3, we directly measured the cytosolic free-Ca^{2+} levels in wild-type and mutant strains (Table 2). This is the first direct measurement of cytosolic free-Ca^{2+} levels in *E. coli* mutants defective in calcium transport (*calA*, *calC*, and *calD*). Previously, the cytosolic free-Ca^{2+} level in wild-type uncoupled *E. coli* cells was measured by Gangola and Rosen by the use of fura-2 produced intracellularly from its acetoxymethyl ester (10) and by Knight et al. by the use of aequorin, a Ca^{2+}-sensitive photoprotein (15). Both of these studies found that wild-type *E. coli* tightly regulates and maintains its cytosolic free-Ca^{2+} levels. Gangola and Rosen reported that the cytosolic free-Ca^{2+} content was 90 nM (10), while Knight and coworkers did not report an actual value for cytosolic Ca^{2+} (15). In our study, by the direct use of fura-2 and fluo-3, the cytosolic content of free Ca^{2+} for wild-type *E. coli* was found to be about 105 nM (Table 2), which is close to the value of 90 ± 10 nM reported by Gangola and Rosen (10). Calcium transport mutants *calC* and *calD*, whose Ca^{2+} contents had not been measured previously, had elevated levels of cytosolic free Ca^{2+}, while the *calA* mutant strain had a level of cytosolic free Ca^{2+} that was slightly lower than the wild-type level (Table 2).

The electroporation method of loading cells makes it possible to use fura-2 with intact *E. coli* cells and not just with uncoupled cells. The technique allows rapid loading of the cells, and cells from any stage in the growth cycle can be loaded by this method. With eukaryotic cells, electropermeabilization is a useful tool for biochemical and pharmacological studies (30). The electroporation technique should be generally applicable for loading bacteria with other molecules. From this study and our previous report with caged calcium compounds (41), we know that we can load small molecules, such as fura-2, caged calcium compounds, and calcium chelators, into the prokaryotic cytoplasm. Freeze fracture electron microscopy has shown that both the outer membrane and the cytoplasmic (inner) membrane of *E. coli* are disrupted by electroporation (34). By measuring the extent of ATP leakage, cell permeabilization of *E. coli* by electroporation has been documented (39). Our inability to release fura-2 from electropermeabilized cells by Tris-EDTA treatment provides further evidence that this technique allows the loading of materials into the bacterial cytoplasm, not the periplasm.

The *calA* mutant was relatively resistant to ω-conotoxin. This mutation is known to cause an increase in Ca^{2+}/PO_4^{2-} symporter activity (7, 33). Here, we report that ω-conotoxin had no effect on either of the calcium extrusion systems (the Ca^{2+}/PO_4^{2-} symporter or the Ca^{2+}/H^+ antiporter). These data imply that ω-conotoxin affects calcium transport at the point of entry instead of exit. However, the *calA* mutant had nearly wild-type levels of cytosolic Ca^{2+}; this suggests that the *calA* mutation may affect also other calcium transport systems. Preliminary binding studies show that ω-conotoxin binds to *E. coli* membranes with saturation kinetics, which implies a specific binding site(s) (42a). There are two explanations for the ω-conotoxin resistance of the *calA* mutant strain. First, the *calA* mutation may directly alter the target site of ω-conotoxin and cause a change in its affinity for the toxin. Alternatively, the *calA* mutation could have no effect on the target site of ω-conotoxin or its affinity for the toxin, but, rather, the effect of the *calA* mutation could occur downstream from an ω-conotoxin receptor. We are presently attempting to determine which of these two explanations is correct and to identify the site of action of ω-conotoxin.

It is likely that not all of the possible *cal* mutants have yet been isolated. We are attempting to isolate more calcium-sensitive mutants and to isolate new ω-conotoxin-resistant mutants. These mutants should help elucidate the mechanism of Ca^{2+} homeostasis and the role of Ca^{2+} in chemotaxis.

We have proposed a working model involving changes in Ca^{2+} levels occurring during bacterial chemotaxis (41), and this model has been advanced by directly measuring changes in cytosolic Ca^{2+} levels upon addition of attractants or repellents (41, 42, 48). In the absence of stimuli, cells maintain a steady-state level of cytosolic Ca^{2+} and swim in an unbiased random-walk pattern of runs and tumbles. Upon addition of a repellent, cells tumble; repellents cause a transient rise in cytosolic Ca^{2+} level, which then returns to the steady-state level. In contrast, the addition of an attractant promotes running; at-

tractants cause the level of cytosolic Ca^{2+} to drop transiently and then return to the steady-state level.

The results presented in this report and in our previous studies (41–43) support in addition the following points. (i) Certain mutant strains defective in Ca^{2+} transport, *calC* and *calD*, have elevated levels of cytosolic Ca^{2+} and are found to be tumbly and defective in chemotaxis (this report). (ii) Release of intracellular Ca^{2+} from caged calcium compounds promotes tumbling (41). (iii) ω-Conotoxin, a Ca^{2+} channel blocker, inhibits chemotaxis by promoting running and blocking tumbling (43).

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