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Original Article

Calcium Homeostasis in *Escherichia coli*: Characterization of Mutants and Genome Expression of MG1655

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While the role of calcium ions as secondary messengers has been well described in eukaryotic cells, little is known about calcium homeostasis in bacteria at the physiological and molecular levels. Genetic and genomic approaches were used to address calcium regulation and to identify genes (*cal*) involved in calcium homeostasis. Transposon mutagenesis of *Escherichia coli* generated several calcium-sensitive mutants that fell into three categories: (i) Ca^{2+}-sensitive chemotaxis mutants, (ii) Ca^{2+}-sensitive cell division mutants, and (iii) Ca^{2+}-sensitive mutants that showed no defects in cell division or chemotaxis. The physiological properties of these Ca^{2+}-sensitive mutants were determined. Besides calcium-sensitivity to 75 m*M* calcium, all of the mutants exhibited increased sensitivities to several divalent cations including Ni^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+}. To identify the *cal* gene sequence in the Ca^{2+}-sensitive mutants, the region of the genes fused to the reporter gene (*phoA*) on the transposon Tn*phoA* was amplified by PCR and sequenced. The sites of the gene fusion for three *cal* mutants were at the *fdoG*, *gpt* and *pqi5* genes. The pleiotropic nature for the *cal* mutations suggested that many genes may be globally regulated by calcium. We then investigated global gene expression patterns of wild-type *E. coli* under calcium-depleted (addition of 10 m*M* EGTA) and calcium-elevated (addition of 75 m*M* Ca^{2+}) conditions as compared to cultures grown under unstressed conditions. A comprehensive transcriptome analysis using macroarrys exhibited a global regulation of diverse genes within the *E. coli* genome during calcium homeostasis.

Keywords: Calcium, Homeostasis, *Escherichia coli*, Transposon, Cations, Genome expression profile

Introduction

Escherichia coli tightly regulate cytosolic free calcium at approximately 100 n*M*, a level similar to that of eukaryotic cells[1]. Calcium ions serve as secondary chemical messengers in eukaryotic cells and tissues including muscle, neural, and cardiovascular tissue. However, the specific role of calcium ions in prokaryotic cells is not as well defined.

The role of calcium ion participation in bacterial behaviour and signal transduction has been established by several lines of evidence[2-7]. Previous studies demonstrate the following observations: (1) Changing cytoplasmic free Ca^{2+} levels in cells of *E. coli* alters their behavior[2]. Tumbling ensues following the release of free Ca^{2+} that is produced from caged Ca^{2+} compounds such as nitr-5 upon irradiation. (2) Changes in bacterial behaviour are correlated with changes in cytoplasmic free Ca^{2+} level[4]. Fluorescent Ca^{2+} indicator dye fura-2 was used to show that repellents caused a temporary rise in cytoplasmic free Ca^{2+} levels, while attractants caused a temporary fall, and the receptor proteins were required for these effects. (3) Some mutants defective in calcium transport have elevated levels of cytoplasmic free Ca^{2+}, tumble continuously, and are defective in chemotaxis[3]. (4) Calcium ion channel blockers inhibit *E. coli* chemotaxis[5-6].

Ca^{2+}-stimulated kinase activities have been found in *E. coli*[8-10] and other bacteria[11-12]. Ca^{2+}-binding proteins and calmodulin-like proteins have been reported to be present in a wide variety of bacteria[13].Immunological evidence indicates the presence of three calmodulin-like proteins in *E. coli*, which were suggested to be involved in calcium ion regulation[14].

The mechanism of calcium entry is unknown. Four genes (*calA, calC, calD,* and *chaA*) have been identified in *E. coli* that are involved in calcium homeostasis. All three of these loci, A, C, and D, are associated with Ca^{2+}/PO_4^{2-} symporter activity[15]. The *chaA* gene is involved in Ca^{2+}/H^+ antiporter activity[16]. Recently, ATP has been proposed to regulate calcium efflux in *E. coli* through an ATPase[17].

To explore the regulatory role of calcium, we decided to generate calcium homeostasis mutant strains and physiologically characterize the mutant strains. In addition, our efforts included a comprehensive assessment of the genome-wide regulation of calcium homeostasis in *E. coli* MG1655 wild-type strain to identify candidate calcium homeostasis genes.

Materials and Methods

Strains, plasmids and phages

All strains of *E. coli* used in this study are derivatives of K12 and are listed in Table 1.

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Table 1. *List of bacterial strains*

Strain	Phenotype	Reference or Source
CC118	araD139 △(ara,leu)7697 △lacX74 phoA △20 galE□galKthirpsErpoBargEamrecAl	Colin and Beckwith, 1985[18]
Cal102	CC118::TnphoA	This study
Cal117	CC118::TnphoA	This study
Cal134	CC118::TnphoA	This study
Cal512	CC118::TnphoA	This study
Cal504	CC118::TnphoA	This study
Cal719	CC118::TnphoA	This study
Cal526	CC118::TnphoA	This study
Pho43	CC118::TnphoA	This study
PhoA4	CC118::TnphoA	This study
PhoC23	CC118::TnphoA	This study
MG1655	Wildtype K12 strain, sequenced first.	Blattner*et al.*, 1997[19]
ëTn*phoA*	Tn5 IS50L::phoA (KmR)	Guttierrez *et al.*, 1987[20]

Growth conditions

E. coli K12 strain CC118 (Ä*phoA*, spectinomycin resistant) was the parental strain that was used for mutagenesis and subsequent generation of calcium-sensitive mutants. Cells were grown and maintained in LB medium (1.0% bactopeptone, 0.5% NaCl, 0.5% yeast extract) containing the appropriate antibiotics. The calcium-sensitive mutants were designated: Cal117, Cal512, Pho43, Cal102, Cal134, PhoA4, PhoC23, Cal504, Cal719, and Cal526. For cation sensitivity assays, cells were grown in E medium (0.5% bactopeptone with 120 m*M* Tris, 70 m*M* NaCl, 20 m*M* KCl, 20 m*M* NH_4Cl, 3 m*M* Na_2SO_4, 1 m*M* $MgCl_2$, 2 ì*M* $ZnCl_2$ and 0.4% glycerol) at a pH of 7.8[21]. For motility and chemotaxis assays, cells were grown in tryptone broth consisting of 1% Bacto-tryptone and 0.5% NaCl and incubated at 35°C. In some cases, cells were grown in Vogel-Bonner medium[22] containing the required amino acids at 1 m*M* and 50 m*M* glycerol (minimal glycerol medium) or 50 m*M* DL-lactate (minimal lactate medium).

Transposon Tn*phoA and mutagenesis of CC118*

The transposon Tn*phoA* was introduced into strain CC118, the parental wild type *E. coli* strain, by phage ë infection (ë Tn*phoA*) according to the method of Manoil and Beckwith[18] at a multiplicity of infection (MOI) of 0.05. Cells of strain CC118 were incubated at 37°C in LB medium containing 10 m*M* $MgSO_4$ and 0.4% maltose. Overnight grown cells were harvested by centrifugation and resuspended in 10 m*M* $MgSO_4$. The washed cells were infected with ë Tn*phoA* lysate as described in the next section.

Selection of calcium-sensitive mutants

For one series, each 100 ìl sample of cells was infected with 10, 100, 200, or 500 ìl of lysate. The mixture was incubated at 30°C for 20 min. Following the addition of 1 ml of LB medium, the cells were allowed to outgrow for 30 min at 30°C. The cells were harvested by centrifugation at 6,000 x g for 10 min and resuspended in E medium containing glycerol (glycerol-E medium). This procedure was repeated twice and the cells were finally resuspended in 1 ml of glycerol-E medium containing 50 m*M* $CaCl_2$ and 60 ìg/ml kanamycin. Following an incubation at 35°C for 1 h, penicillin G (15,000 U) was added and the cells were allowed to incubate for 3 h. Penicillin G was removed by centrifugation at 6,000 x g for 10 min and resuspending the cell pellet in glycerol-E medium. This washing step was repeated twice. The cells were finally resuspended in 1 ml of glycerol-E medium and 100-ìl samples were plated on glycerol-E medium plates containing 5 m*M* $CaCl_2$, 60 ìg/ml kanamycin and 40 ìg/ml 5-bromo-4-chloro-3-indolyl phosphate (XP) to screen for TnphoA insertions exhibiting PhoA activity. The plates were incubated at 37°C for 2 days before colonies were picked.

For the second series, a 100-ìl sample of cells was infected with 100-ìl of phage lysate for 20 min at 37°C and directly plated on either (1) glycerol-E medium containing 50 m*M* $CaCl_2$, 30 ìg/ml kanamycin and 40 ìg/ml XP or (2) glycerol-E medium containing 5 m*M* $CaCl_2$, 180 ìg/ml kanamycin and 40 ìg/ml XP. These plates were also incubated at 37°C for 2 days before colonies were picked.

Atypically small colonies on the plates were judged to be potential calcium-sensitive mutants. Those colonies that grew on glycerol-E medium but not on glycerol-E medium containing 50 m*M* $CaCl_2$ (thus indicating sensitivity to calcium) were purified and retained.

Calcium-sensitivity assay

Calcium-sensitivity was later defined as the inability to grow in the presence of 75 m*M* $CaCl_2$. Calcium-sensitivity was measured by two different growth inhibition assays. Initially, calcium-sensitivity was measured by growth inhibition assay of Brey and Rosen[23]. Cells were screened for their ability to grow on E medium plates supplemented with 0 m*M*, 25 m*M*, 50 m*M*, 75 m*M*, or 100 m*M* $CaCl_2$. The plates were incubated at 37°C and the growth

results were monitored at 24 h and 48 h. After 48 h, calcium-sensitive cells are unable to grow on E medium plates supplemented with 75 m*M* $CaCl_2$. Growth inhibition was also determined from turbidity measurements in broth cultures. During this assay, cells were inoculated in E medium broth supplemented with glycerol and different concentrations (0 m*M*, 25 m*M*, 50 m*M*, 75 m*M*, or 100 m*M*) of $CaCl_2$, and were incubated at 37°C for 8 h. The optical density at a wavelength of 600 nm was measured at 0 and 8 h.

Cation sensitivity assay

The sensitivity to various other cations was monitored by growth inhibition assay of the calcium-sensitive cells as described by Brey and Rosen[23]. The minimum inhibitory concentration (MIC) values were determined for each cation tested. MIC is the lowest concentration of cation at which all bacterial growth is stopped.

Chemotaxis assay

Chemotactic ability was measured by swim-ring migration assay[24]. Tryptone swarm plates containing 1% bacto-tryptone, 0.5% NaCl, and 0.25% bactoagar (Difco Laboratories, Franklin Lakes, New Jersey) were inoculated with a stab of approximately 10^6 cells at the centre of the plates and incubated at 30°C for 8 to 14 h. The ring diameters were measured at the end of the experiment.

Analysis of free-swimming behaviour

Bacterial swimming behaviour was observed at 30°C by phase-contrast microscopy at a magnification of 400x. The cells in these behavioural assays were suspended in chemotaxis medium (10 m*M* potassium phosphate pH 7.0, 0.1 m*M* potassium EDTA, and 1 m*M* L-methionine) or in filtered spent/used growth medium to an optical density at 590 nm of 0.1. The microscopic behaviour was videotaped and analyzed by computer[25].

Alkaline phosphatase assay

Alkaline phosphatase activity was measured using both solid (for all mutants) and liquid media (only for calcium-sensitive transposon mutant Pho43). Cells were grown at 37°C in LB medium containing appropriate antibiotics. Overnight cultures were then streaked for isolation on E medium plates containing the chromogenic alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). The parental strain CC118 lacks functional alkaline phosphatase. Only colonies that produced alkaline phosphatase fusion on the outside of the cell or in the periplasmic space would turn blue. Following overnight growth at 37°C, successful calcium-sensitive mutants with functional alkaline phosphatase were monitored for blue color production.

DAPI experiments

Prior to staining the cells were first fixed with toluene and treated with chloramphenicol to condense their genomes. The fixed cells were incubated with the fluorescent DNA binding dye DAPI (4',6'-diamidino-2-phenylindole 2 HCl), and observed by the use of phase contrast microscopy and fluorescence microscopy at a total magnification of 1,000x.

Arbitrarily primed PCR

To identify the *cal* gene sequence, the region of the genes fused to the reporter gene (*phoA*) were amplified by PCR and sequenced. To amplify partial regions of *cal* genes fused to the transposon, we used the arbitrarily-primed PCR method[26-27]. This method involved two rounds of PCR amplification using arbitrary primers to prime from the chromosome and primers specific to Tn*phoA*. During the first round of PCR, the primer (Tn-R) with sequence homology to the right end of the transposon Tn*phoA* and arbitrary primer ARB1 were used. The PCR parameters for first round were as follows: (1) 95°C 5 min, (2) 6 cycles of 95°C for 30 s, 30°C for 30 s, 72°C for 1.5 min, (3) 30 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 2 min. During the second round of PCR, a 5 ìl aliquot of first round PCR product was used as template and the primers ARB2 and Tn-I were used for amplification.The PCR parameters for second round were as follows: 30 cycles of 95°C for 30 s, 45°C for 30 s, 72°C for 2 min. The final PCR products were purified from an agarose gel with the aid of â-agarase.The primers (ARB1, ARB2, Tn-I and Tn-R) used in this experiment are listed in Table 2. These PCR amplified products were sequenced using primers (Tn*phoA*-int and Tn-R) located close to the fusion site on Tn*phoA*. DNA sequence data were analyzed by sequence analysis software DNAStar for Mac.The resultant edited sequences, ORFs present in the edited sequences, as well as translated sequences were used to query the *E. coli* genomicdatabase[28].

Bacterial growth conditions for DNA macroarray experiments

To provide a comprehensive insight into calcium homeostasis, the global expression profiles of wild type *E. coli* MG 1655 were

Table 2. *Primers used in arbitrarily-primed PCR and sequencing reaction*

Primer	Primer sequence (5'→3')	Reference
ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	This study
ARB2	GGCCACGCGTCGACTAGTAC	This study
Tn-I	CCTTCGGCATAATTACGTGC	This study
Tn-R	GCAGTCTGATCACCCGTTAAA	This study
TnphoA-int	TTTCCAGAACAGGGCAAAAC	This study
TnphoA-F	TGCAAGTTGAAGGTGCGTCAATCG	This study
TnphoA-R	ATGAGATGCCCTGCAAGCAATTCG	This study

determined under three different conditions: (1) growth medium containing elevated levels of calcium (the addition 75 m*M* CaCl2), (2) growth medium containing depleted levels of calcium (the addition of 10 m*M* EGTA), and (3) growth medium control (untreated). Overnight cultures of *E. coli* MG 1655 were used to inoculate fresh E media and the cultures were incubated at 37°C with rotary oration. For the array experiments cultures were grown to an OD_{600} of 0.4 to 0.5 and then either 75 m*M* calcium or 10 m*M* EDTA was added. After 30 min of rotary aeration at 37°C, total RNA was quickly extracted by using QiagenRNeasy kit (Qiagen, Inc., Valencia, CA). Proper precautions were taken to avoid contamination with RNases and a non-denaturing electrophoresis gel was run with the extracted RNA to check the integrity of RNA.

Synthesis of ^{33}P-labeled cDNA probe

Sigma-Genosys Biotechnologies, Inc. had developed commercially available cDNA primers that are specifically designed to preferentially label cDNA from mRNA. These C-terminal primer sets (4,290 ORF-specific C-terminal primers) were used to generate the hybridization probe according to the manufacturers recommendations. As recommended by the manufacturer, ^{33}P-á-dCTP was used to label the cDNA. The ^{33}P-labeled cDNA was purified and unincorporated-labeled nucleotides were removed by the use of Sephadex G-25 gel-filtration spin columns. An estimation of percentage incorporation of ^{33}P-dCTP into the cDNA was determined by using a hand-held Geiger counter or by scintillation counts of samples before and after column purification. The labeled cDNA probe was then ready to be used in a hybridization reaction with the Panorama *E. coli* Gene Arrays (Sigma-Genosys Biotechnologies, Inc.).

Hybridization with panorama E. coli gene array

Following its preparation and purification, the ^{33}P-labeled cDNA was hybridized to the Panorama gene array by the use of roller bottles in a hybridization oven. The hybridization and washing steps were performed according to the protocol specified in Panorama *E. coli* cDNA Labeling and Hybridization Kit. The DNA array blots were rinsed in 2X SSPE (1X SSPE: 0.18 *M* NaCl, 10 m*M* NaH2PO4, 1 m*M* EDTA, pH 7.7) and pre-hybridized in pre-warmed hybridization solution (5X SSPE, 2% SDS, 1X Denhardt's reagent, 100 ìg/ml sheared salmon sperm DNA) at 65ºC for 1 hr at 6 rpm. The entire ^{33}P-labeled cDNA was first denatured at 95ºC for 10 min and then added to 3 ml of hybridization buffer and the blot were hybridized in this solution for 15 h at 65ºC. After overnight incubation, the blots were washed with buffer (0.5X SSPE, 0.2% SDS) three times at room temperature. The blots were then washed three more times with pre-warmed (65ºC) buffer in the hybridization oven at 65ºC for 20 min at 6 rpm. Washed blots were finally air-dried briefly for 5 min and wrapped in clear plastic food wrap and exposed to a phosphorimager screen (Bio-Rad Laboratories) for 24 h.

Data acquisition by phosphorimaging and analysis of the arrays

The gene expression signals were measured by the use of a Bio-Rad PhosphorImager with a Kodak Low Energy Storage Phosphor Screen. Typically, the arrays were exposed overnight to yield quantifiable results. For quantification, imaging screens were scanned at a 50ìm pixel size and analyzed by Quantity One software (Bio-Rad Laboratories, Hercules, CA) based on the spot coordinates. Each gene has two corresponding spots and the pixel density (intensity) of each spot representing each gene in the array was measured and corrected for the background. The average signal of the pair of duplicate spots were subsequently determined and normalized for comparative studies. To standardize the data from experiments the average intensity for each spot was expressed as a percentage of the total of intensities for all of the spots on the DNA array.

Results and Discussion

Isolation of calcium-sensitive mutants of E. coli by Tn*phoA mutagenesis*

The 7.7 kb transposon Tn*phoA* contains Kan^r gene as a selective marker and the reporter gene for alkaline phosphatase *phoA* (Figure 1). The transposon, Tn*phoA*, used as a mutagenic agent for generation and isolation of new calcium-sensitive mutants. The reporter gene for alkaline phosphatase *phoA* has no promoter or Shine Dalgarno sequences, and is only expressed when the fusion is in-frame. When inserted in frame and in proper orientation, Tn*phoA* will randomly mutagenize bacterial chromosome by disrupting the gene the transposon will insert itself in. In addition, Tn*phoA* will fuse alkaline phosphatase to the amino terminal of the protein product of the disrupted gene. Alkaline phosphatase is functional when it is fused to a signal that promotes the export of the protein to the envelope of the cell (such as periplasmic, outer membrane or cytoplasmic membrane proteins). Our search for calcium sensitive *E. coli* mutants was targeted to membrane protein genes by essentially isolating *phoA* gene fusions. Infection of a *phoA* deletion strain CC118 with ëTn*phoA*, followed by penicillin enrichment and subsequent screening of blueness of colonies on XP resulted in mutants with Tn*phoA* insertions exhibiting PhoA activity.

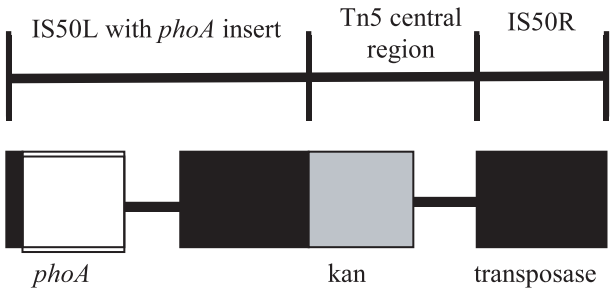


Figure 1. *The transposon TnphoA (Manoil and Beckwith[18]). The 7.7 Kbp transposon is derived from Tn5 with the selective marker Kan and the reporter gene phoA. Successful (in frame) gene-transposon fusion allows for identification of secreted and transmembrane proteins.*

Following transposon mutagenesis, penicillin enrichment was used to aid in the isolation of calcium-sensitive mutants. Several new calcium-sensitive mutants were identified by this transposon mutagenesis procedure. The calcium-sensitive mutants generated are described in Table 1 and photomicrographs of some mutants are included in Figure 2. The mutants fell into three

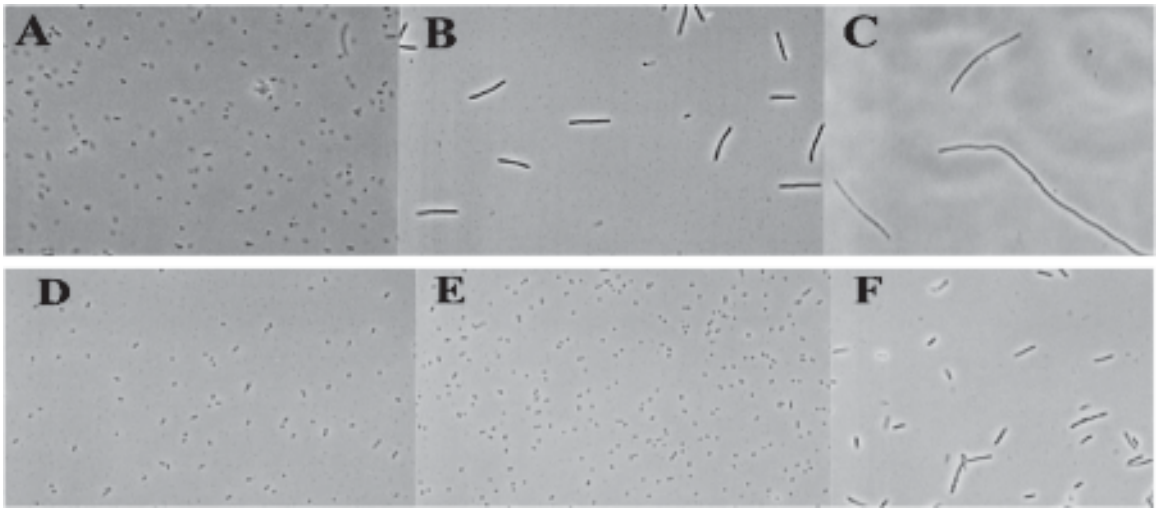


Figure 2. *Photomicrograph of wild type Escherichia coli and calcium-sensitive mutants. (A) CC118 with wild type morphology, (B) Cal102 cells with a snake-like morphology consist of up to 10 times the length of the wild type CC118, (C) Cal134 cells were more elongated at about 10 to 20 times the length of CC118, (D) and (E) Cal 117 and Pho43, respectively, had wild type morphology, and (F) Pho43 are long like Cal 102.*

general classes: (i) Ca^{2+}-sensitive chemotaxis mutants, (ii) Ca^{2+}-sensitive cell division mutants, and (iii) Ca^{2+}-sensitive mutants that showed no defects in cell division or chemotaxis (Table 3). While some of the mutants were sensitive to 50 m*M* $CaCl_2$, sensitivity measured by growth inhibition assay revealed the inability of most mutants to grow in the presence of 75 m*M* $CaCl_2$ on plates or in broth cultures.

Physiological and Biochemical Properties of Calcium-Sensitive Mutants

The cell division mutants (*e.g.*, Cal134) were easily identified microscopically as long "snake-like" cells (Figure 2). One of the Ca^{2+}-sensitive cell division mutants (Cal134) grew as a long filamentous cell that was over 20 cell-body-length in size.

Multiple chromosomes within these snake-like cells were observed following DAPI staining of this mutant (Figure 3). These results imply that this mutant was defective in cell division, but not DNA replication.

The generation of these Ca^{2+}-sensitive cell division mutants was not unexpected. Holland and co-workers[29-30] isolated mutants that were resistant to different calcium antagonists and are defective in cell division. They proposed a model suggesting that calcium plays a role in the control of bacterial growth and its cell cycle[31-32]. The sensitivity of these calcium-sensitive mutants to other cations was tested and their minimal inhibitory concentration (MIC) values for these cations are shown in Figure 4. Besides calcium-sensitivity, all of the mutants exhibited increased sensitivities to several divalent cations (Ni^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+}). Mutant Cal117 was sensitive to all of the cations tested. Mutants Cal134 and Pho43 exhibited sensitivity to Co^{2+} and Zn^{2+}, while Cal134 was also sensitive to Cu^{2+}. Cal102 was sensitive to all of the cations except Ni^{2+}, and

Table 3. *Properties of calcium-sensitive mutants*

Strain	Morphology	Motility	Swim rate mm per 8 hours	Alkaline phosphatase (plate assay)
CC118 (WT)	Short rods	Smooth-tumbly	49	–
Cal117	Medium snakes	Smooth	0	++
Cal512	Medium snakes	Smooth	2	+
Cal102	Medium snakes	Tumbly	14	++
Pho43	Sausage snakes	Non-motile	0	++
Cal134	Long snakes	Smooth	4	++
PhoA4	Short rods	Smooth-tumbly	37	+++
PhoC23	Short rods	Tumbly	23	–
Cal 719	Medium snakes	Smooth-tumbly	31	ND
Cal526	ND	ND	11	ND
Cal504	ND	ND	12	ND

Medium = 5-8 cell lengths; Long = 10-20 cell lengths; – = No color; + = Light blue; ++ = Medium blue; +++ = Dark blue; ND = Not determined.

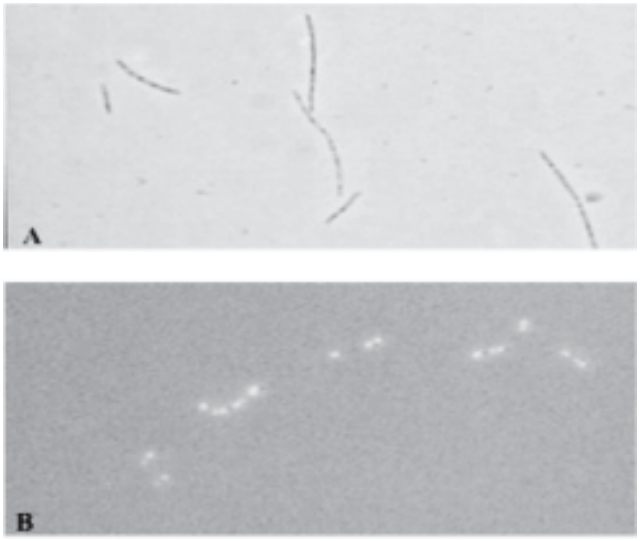


Figure 3. *The Cal134 mutant is defective in cell division, but not DNA replication. DAPI stained phase contrast (A) and fluorescent (B) pictures of Cal134 long snake cell division mutants.*

Cal512 exhibited a similar pattern of sensitivity as Cal117 though it was not sensitive to Mn^{2+}. It is noteworthy to mention that all calcium-sensitive mutants were resistant to Mg^{2+} as shown in Figure 4A.

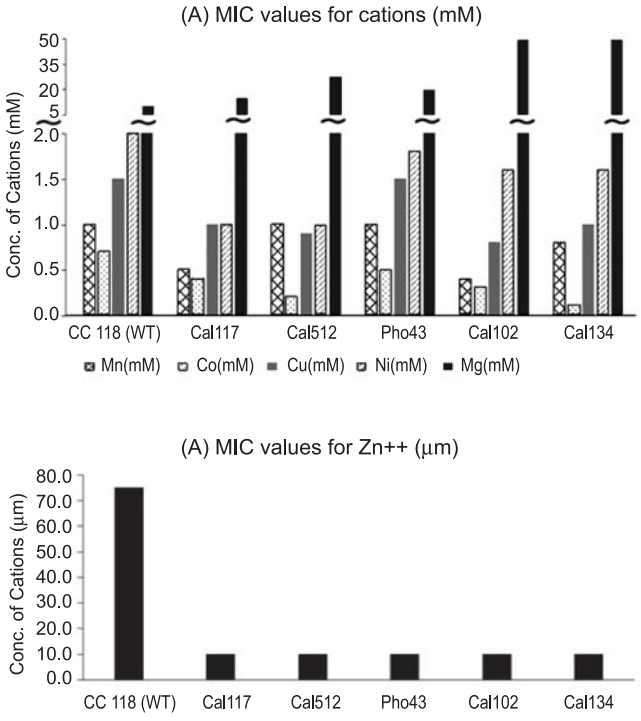


Figure 4. *Ca-sensitive mutants were sensitive to other divalent cations. MIC values for cations tested were determined as described in the Methods. Mutants Cal117 and Cal512 exhibit a similar pattern of cation sensitivity (except for Mg), while Cal102 and Cal134 exhibit similar profiles. The wild type CC118 and mutant Pho43 have identical MICs for Mn^{2+} and Cu^{2+}.*

In other ion transport systems, mutants defective in ion transport show increased sensitivity to diverse additional ions. Na^{+} transport mutants are resistant to Li^{+} [33], while Mg^{2+} transport (*corA*) mutants are resistant to Co^{2+} and sensitive to Ca^{2+}[34]. We expected that the *cal* mutants isolated above would show sensitivities to other cations or that they would show an increased resistance to some cations.

Table 3 summarizes the physiological and biochemical characterization data for a comprehensive representation of observations made on the transposon mutants.

Molecular characterization of calcium-sensitive mutants

The sites of the gene fusions for three mutants were identified by arbitrarily-primed PCR (arbPCR). Since the PCR products of arbPCR contained part of the upstream regions of the transposon Tn*phoA* fused to the disrupted gene, the start of *cal* gene was easily recognized. The resulting partial gene sequence was used to search the compiled DNA sequence databank of *E. coli* genome by the use of the BLAST program at NCBI. The sites of the gene fusion for three *cal* mutants were at the *fdoG*, *gpt* and *pqi* genes (Table 4). The *fdoG*gene codes for the á-subunit of formate dehydrogenase[35], while the *gpt* gene codes for guanine-xanthine phosphoribosyl transferase phosphotransferase[36] and *pqi* gene is induced by paraquat and regulated by SoxRS[37]. The arbitrarily-primed PCR fragment of Cal134 exhibited 96% sequence homology with *fdoG* gene and also a significant match as evidenced by the e value of 5e-12. Similarly, the cal genes of PhoC23 and PhoA4 showed significant matches to genes *gpt* and *pqiB* (e value of 2e-44 and 1e-25, respectively) with 98% sequence identity for both.

The site of the Tn*phoA* insertion was detected by hybridization of a DIG-labeled Tn*phoA* derived probe to membrane blotted nucleic acids. Hybridization experiments using this Tn*phoA* derived probe confirmed that each mutation was distinct and the result of the single insertion of Tn*phoA* (data not shown). The site of insertion was also confirmed by PCR (data not shown).

The sites of the gene fusion for three *cal* mutants were the following: gene (Cal134), *gpt* gene (PhoC23), *pqi* gene (PhoA4). Our initial observations with the Tn*phoA*-generated *cal* mutants suggest that their calcium-sensitivity was the result of a number of diverse genes being disrupted by the insertion of the transposon. These results suggest a pleiotropic nature for the *cal* mutations and indicate that many genes may be regulated by calcium. Because of the results from these experiments, we wanted to investigate global gene expression under growth conditions with and without calcium with a broader goal of identifying all of the calcium-regulated genes in *E. coli*.

Panorama E. coli gene array

Panorama *E. coli* DNA macroarrays (Sigma_Genosys) representing all 4,290 protein-coding genes were used to identify those genes that were globally regulated by calcium and to observe the effects

Table 4. *cal gene mutations identified by ARB-PCR*

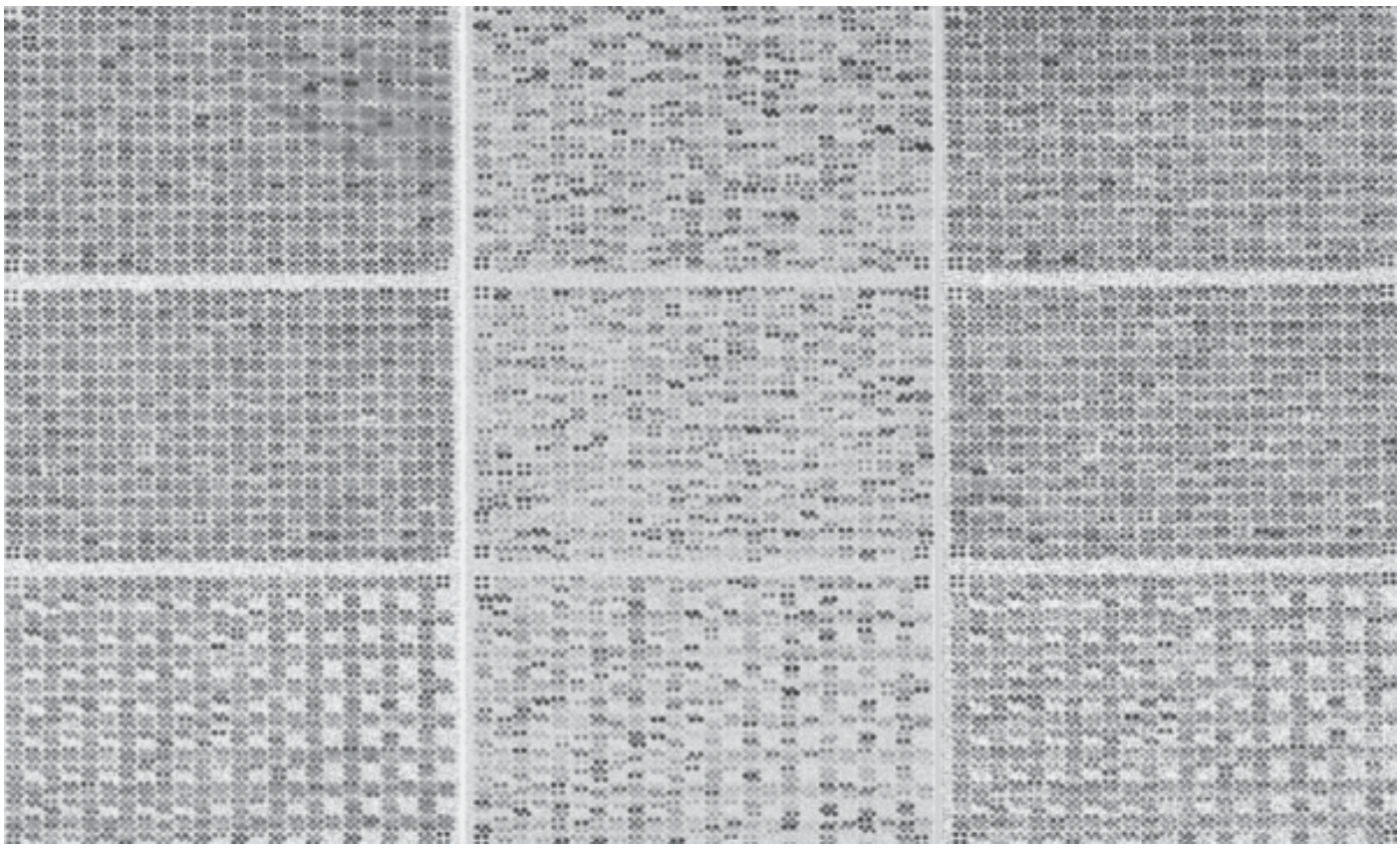
Mutant	*cal*gene	Gene function	e-value	% identity
Cal134	*fdoG*	Formate dehydrogenase (Allows the use of formate as major electron donor during aerobic respiration)	5e-12	96
PhoC23	*gpt*	Guanine-hypoxanthine phosphoribosyltransferase (Involved in salvage of nucleosides and nucleotides)	2e-44	98
PhoA4	*pqiB*	Paraquat-inducible protein (Inducible by superoxide generating radical paraquat and regulated by SoxRS)	1e-25	98

of elevated (75 m*M* Ca) and depleted calcium (10 m*M* EGTA) levels on the global transcription profile (Figure 5).

The 23S and 16S ribosomal RNA bands were clearly visible at about 2:1 ratio (23S:16S) of staining intensity. The processed RNA samples were devoid of any genomic DNA contamination as evidenced by agarose gel electrophoresis.

The corresponding spots from samples on arrays were compared to identify the fold-induction (up-regulation) or fold-reduction (down-regulation) in expression between the samples (Figure 5). By this method of array analysis a 2-fold difference in expression was considered as important. Changes in expression (fold change) of genes during elevated and depleted calcium conditions are shown in Table 5.

The findings from our study of calcium homeostasis represent a dynamic state with hitherto unknown regulatory activity throughout *E. coli* genome. It is very likely that diverse genes are under calcium regulation as evidenced by changes in gene



Control Calcium added EGTA added

Figure 5. *Panorama E. coli gene array exhibiting global gene expression profiles of E. coli MG1655 following growth on E medium (control), E medium supplemented with 75 mM calcium, and E medium supplemented with 10 mM EGTA.*

expression in elevated or depleted calcium. The next logical extension of our study will include an investigation into quantitative analysis of gene expression of subsets of genes in wild-type and mutant strains with addition of calcium and chelation of calcium.

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