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pH and monovalent cations regulate cytosolic free Ca^{2+} in *E. coli*

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ABSTRACT

The results here show for the first time that pH and monovalent cations can regulate cytosolic free Ca^{2+} in *E. coli* through Ca^{2+} influx and efflux, monitored using aequorin. At pH 7.5 the resting cytosolic free Ca^{2+} was 0.2–0.5 μM. In the presence of external Ca^{2+} (1 mM) at alkaline pH this rose to 4 μM, being reduced to 0.9 μM at acid pH. Removal of external Ca^{2+} caused an immediate decrease in cytosolic free Ca^{2+} at 50–100nM s^{-1}. Efflux rates were the same at pH 5.5, 7.5 and 9.5. Thus, ChaA, a putative Ca^{2+}/H^{+}exchanger, appeared not to be a major Ca^{2+}-efflux pathway. In the absence of added Na^{+}, but with 1 mM external Ca^{2+}, cytosolic free Ca^{2+} rose to approximately 10 μM. The addition of Na^{+}(half maximum 60 mM) largely blocked this increase and immediately stimulated Ca^{2+} efflux. However, this effect was not specific, since K^{+} also stimulated efflux. In contrast, an increase in osmotic pressure by addition of sucrose did not significantly stimulate Ca^{2+} efflux. The results were consistent with H^{+} and monovalent cations competing with Ca^{2+} for a non-selective ion influx channel. Ca^{2+} entry and efflux in *chaA* and *yrbG* knockouts were not significantly different from wild type, confirming that neither ChaA nor YrbG appear to play a major role in regulating cytosolic Ca^{2+} in *Escherichia coli*. The number of Ca^{2+} ions calculated to move per cell per second ranged from <1 to 100, depending on conditions. Yet a single eukaryote Ca^{2+} channel, conductance 100 pS, should conduct >6 million ions per second. This raises fundamental questions about the nature and regulation of Ca^{2+} transport in bacteria, and other small living systems such as mitochondria, requiring a new mathematical approach to describe such ion movements. The results have important significance in the adaptation of *E. coli* to different ionic environments such as the gut, fresh water and in sea water near sewage effluents.

1. Introduction

Bacteria such as *Escherichia coli* exist and grow in a variety of ionic environments, including the gut, fresh water and in sea water, especially close to sewage effluents. These ionic environments involve a range of pH, monovalent cation and divalent cation concentrations, particularly calcium. The role of calcium ions as a universal signal, through μM changes in cytosolic free Ca^{2+}, is well established in excitable and non-excitable eukaryote cells [1–6]. Using the photoprotein aequorin as a reporter we have shown that intracellular free Ca^{2+} in *E. coli* is indeed tightly regulated in the μM range [7–9], although the role of Ca^{2+} in bacteria remains poorly defined [9–14]. There is nevertheless some indirect evidence for the importance of Ca^{2+} [12] suggested by; (1) the presence of putative primary and secondary Ca^{2+} transporters; (2) identification of calcium binding proteins in a number of bacteria; (3) regulation of a low level of cytosolic free Ca^{2+} in the presence of external Ca^{2+}; (4) effects of manipulation of external Ca^{2+} on bacterial physiology. Furthermore, we have recently described the first Ca^{2+} transients in bacteria, analogous to Ca^{2+} transients in eukaryotic cells, activated by the fermentation products butane 2,3 diol and methylglyoxal [8,9]. Changes in intracellular Ca^{2+} are classified as transient when an agonist provokes a rise in free Ca^{2+} to a peak within seconds or minutes, which then declines towards the resting cell level. To understand why *E. coli*, and other bacteria, maintain a large gradient of free Ca^{2+} across their cytoplasmic membrane, and thus can generate Ca^{2+} transients, it is essential to understand how Ca^{2+} influx and efflux are regulated.

The aim of the work described here was to investigate whether pH, and the monovalent cations Na^{+} and K^{+}, could regulate cytosolic free Ca^{2+}, and if so whether this was through control of Ca^{2+} influx or efflux. Two possible Ca^{2+} transporters have been identified in *E. coli*, a Ca^{2+}/H^{+} exchanger, ChaA [15–17], and a putative Na^{+}/Ca^{2+} exchanger, YrbG, identified from the *E. coli* genome [18]. Depending on the Na^{+} or Ca^{2+} (and K^{+}) gradient and membrane potential, Na^{+}/Ca^{2+} exchangers can operate in eukaryotes in either the forward (Ca^{2+} exit) or reverse (Ca^{2+} entry) direction to move Ca^{2+} out or into the cytosol in exchange for Na^{+}. In bacteria there is also a putative Ca^{2+}-phosphate co-transporter (PitB), which may be involved in calcium transport [19,20].

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Finally, Reusch et al. [21–23] have described a complex in *E. coli*, polyhydroxybutyrate–polyphosphate (PHB–PP), that *in vitro* can act as a Ca^{2+} channel inhibited by La^{3+}. Although evidence has been obtained using membrane vesicles that proteins such as ChaA can transport Ca^{2+}, there is at present no direct evidence that ChaA, YrbG, PitB or PHB–PP can regulate cytosolic free Ca^{2+} in live cells. This can only be achieved by testing the effect of changes in external Ca^{2+}, pH and Na^{+} on cytosolic free Ca^{2+} in normal cells, followed by measurements in knockout mutants, where cytosolic free Ca^{2+} is measured directly in live cells.

E. coli, like many bacteria, does not have a large internal Ca^{2+} store analogous to the endoplasmic reticulum or mitochondria of eukaryotes, though some Ca^{2+} may be bound to DNA [24]. Thus, it is possible to quantify Ca^{2+} efflux directly in live cells by monitoring the decrease in cytosolic free Ca^{2+} when Ca^{2+} is removed from the external medium. Here we describe the use of the Ca^{2+}-activated photoprotein aequorin to measure absolute Ca^{2+} influx and efflux in *E. coli*, and how they are regulated. The results show that pH and monovalent cations can regulate Ca^{2+} influx apparently through ionic competition for a Ca^{2+} influx channel. However, neither ChaA nor YrbG appeared to be major pathways for Ca^{2+} influx or efflux.

2. Methods

2.1. Chemicals

Coelenterazine, the prosthetic group in aequorin that is oxidised to produce the excited state light emitter coelenteramide [25,26], was a gift from Dr Bruce Bryan, ProLume Inc. Bacterial media components were from Oxoid, Hampshire UK, RNeasy mini kit from Qiagen and Impron-II Reverse Transcription system from Promega. All other AnalaR chemicals were from Sigma Chemical Co. (Dorset, UK).

2.2. Gene knockouts

To generate MG1655 Δ*chaA* and MG1655 Δ*yrbG* strains, the null mutations were transferred from the Keio collection [27] to the MG1655 strain by P1 transduction. In this collection, each *E. coli* non essential gene was replaced by a kanamycin resistance cassette using a technique devised by [28]. After transducing the mutations into MG1655, the resulting *kan*R transductants were checked by PCR to confirm the correct structure of the knockouts. In addition, the absence of *chaA* and *yrbG* genes was examined in the appropriate strain by RT-PCR, confirming the absence of expression from the respective mutant (Fig. 1B).

2.3. Ecoli strains and growth conditions

The *E. coli* strains JM109 and MG1655 (transformed with pMMB66EH containing the aequorin gene) were grown in LB (Luria Bertani) medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl pH 7.2) with carbenicillin (100 μg ml^{-1}) at 37 °C as previously described [8,9,29]. The absorbance at 600 nm was used to estimate the rate of growth. Viability of the cells was confirmed under all conditions by growth measured through absorbance at 600 nm and colony forming units.

2.4. Induction of apoaaequorin and aequorin reconstitution

Bacteria were transformed with the vector containing aequorin DNA, and the aequorin activated using coelenterazine (2 μM) as previously described [13,14,29]. Under all conditions reported there was no effect on cell viability, as checked by viable counts (colony forming units). Following activation of the aequorin the cells were kept on ice throughout the 4–6 h necessary to complete any particular experiment.

2.5. Reverse Transcriptase-PCR (RT-PCR) analysis

Bacterial cells (JM109, MG1655, Δ*chaA* and Δ*yrbG*) were grown overnight from a single colony. Total RNA was extracted using the Qiagen RNeasy mini kit. RT-PCR was then conducted in two steps. In the first step cDNA was synthesized at 42 °C for 60 min from 1–2 μg RNA using the random hexamer and Impron-II Reverse Transcriptase System. In the second step PCR was carried out using gene specific primers in a 50 μl reaction volume containing 2 μl of the synthesised cDNA, 0.5 μM of each primer and 5 units of Biotaq DNA polymerase.

chaA up: 5 -ATGTCAAATGCTCAAGAGGCGGTAAAAAC-3
chaA down: 5 -TCAGGCAAATATCGTCATCAAATAGGCG-3
yrbG up: 5 -GCTTTTAGCTACGGCACTGTTAATTGTTGG-3
yrbG down: 5 -CAACGAGTATTGGCGATAACCAGTACAACAT-3

PCR was conducted under the following conditions: 95 °C, 5 min; followed by 25 cycles of 95 °C, 30s, 60 °C, 30s, 68 °C, 2 min; an extension of 68 °C, 2 min. The PCR products were then analysed using a 1% agarose gel (Fig. 1A and B). Thus RT-PCR from normal MG1655 cells, and *chaA* and *yrbG* knockouts confirmed the presence of the genes in normal cells and their loss in the knockouts (Fig. 1B). No bands were observed when RNA was added without the RT step (Fig. 1A). This control, together with the absence of detectable gene expression in the knockouts, ruled out the possibility of the results being affected by genomic contamination.

2.6. Luminescence measurements and cytosolic free Ca^{2+} estimation

(a) Light emission: This was measured as previously described [8,9,29]. All the active aequorin was in the cytoplasmic compartment. Thus, changes in aequorin light emission were due wholly to changes in cytosolic free Ca^{2+} in *E. coli* dependent on Ca^{2+} influx and efflux across the inner membrane. Some apoaequorin can be trapped inside inclusion bodies, but there was no evidence for this being released under the conditions of the experiments described here. The total aequorin light yield, estimated by summation of the light emitted throughout a complete experiment (see section (d) below), showed that all of this aequorin was exposed to cytosolic free Ca^{2+} during the experiment, ruling out any apparent artefact due to aequorin trapped within inclusion bodies, and not exposed to changes in cytosolic Ca^{2+}.
(b) Ca^{2+} influx measurements: Bacterial cells expressing aequorin as described above were resuspended in the required buffer (buffer A: 25 mM HEPES, 125 mM added NaCl, 1 mM $MgCl_2$ or buffer AA: 25 mM HEPES, 1 mM $MgCl_2$) and light production monitored, under different conditions. The rate of initial Ca^{2+} influx was measured from the slope of cytosolic free Ca^{2+} against time. This was justified since there is no evidence for significant stores or Ca^{2+} buffering inside *E. coli*, though some Ca^{2+} may be bound to DNA [24].
(c) Ca^{2+} efflux measurements: To measure Ca^{2+} efflux, external Ca^{2+} was removed rapidly by addition of the Ca^{2+} chelator EGTA (5 mM) that does not bind Mg^{2+} significantly. The luminescence counts were recorded and converted to free Ca^{2+}. In *E. coli* there are no internal Ca^{2+} stores such as mitochondria or endoplasmic reticulum, or significant Ca^{2+} buffering by proteins such as calmodulin, that are found in eukaryotic cells. Thus any increase in cytosolic free Ca^{2+} in *E. coli* must arise primarily from influx into the cell from outside via Ca^{2+} channels, while a decrease should reflect Ca^{2+} efflux out of the cell. The estimates for Ca^{2+} influx and efflux rates were made assuming no significant internal Ca^{2+} buffering, and are therefore minimum values. Even if there were significant internal Ca^{2+} buffering the comparisons of effects on the estimated Ca^{2+} influx and efflux rates under different conditions were always valid. This is not possible in eukaryotic cells where mitochondria, the ER and calmodulin prevent estimation of absolute influx and efflux from measurements of cytosolic free Ca^{2+}. It is well established that, unlike the fluorescent Ca^{2+} indicators, the level of expression of aequorin, together with its binding constant for Ca^{2+}, mean that when used as an intracellular indicator aequorin does not significantly buffer cytosolic Ca^{2+}.
(d) Residual active aequorin: This was estimated by permeabilising the cells with the detergent NP40 (Nonidet 40) in the presence of 12.5 mM Ca^{2+}. The active aequorin remaining at the end of the time course was in all cases sufficient (from 10–90% of the initial value, depending on the conditions of the experiment) to enable estimation of the free Ca^{2+} concentration to be made throughout, provided the

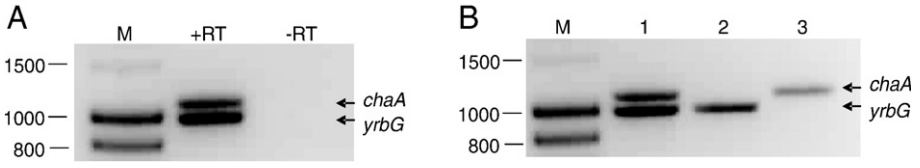

Fig. 1. RT-PCR of putative calcium transporter genes. Total RNA was extracted from *E. coli* wild type strains MG1655 and JM109, and knockouts of *chaA* and *yrbG* in MG1655 respectively, according to the Qiagen RNeasy protocol. RT-PCR of calcium transporter genes was then performed using cDNAs prepared from the total RNA as described in the Methods. A: Total RNA from JM109 bacterial cells was isolated and the RT (reverse transcription) reaction was performed using 1.5 μg RNA containing plus and minus RT (reverse transcriptase) as control. B: Total RNA from MG1655, Δ*chaA* and Δ*yrbG* bacterial cells was isolated and RT-PCR reactions were performed using synthesised cDNAs of MG1655 (lane 1), Δ*chaA* (lane 2) *and* Δ*yrbG* (lane 3). 5 μl of each PCR reaction was electrophoresed on a 1% agarose gel containing EtBr for detection of *chaA* (1101bp) and *yrbG* (978 bp).

progressive consumption of the aequorin was taken into account by calculating the rate constant at each time point, as originally described [25,30]. These rate constants (k s^{-1}) at each time point were estimated as (counts s^{-1})/(total counts remaining at each time point), and converted to free Ca^{2+} concentrations using a standard curve [29–31]. The standard curve was obtained using apoaequorin extracted from the bacteria, activated *in vitro* to aequorin using coelenterazine. This recombinant aequorin was then incubated in 0.1 μM to 1 mM free Ca^{2+}, EGTA buffers being used at low Ca^{2+}, in order to measure the rate constant of the chemiluminescent decay at each Ca^{2+} concentration. A plot of log *k* versus pCa was linear over the range used to measure free Ca^{2+} in the cells. The equation from this plot was $y=0.612x+3.745$; where $x=-\log k$ and $y=$pCa, i.e.–log Ca. There was no reproducible effect of conditions of pH or monovalent cations on the total aequorin measured.

2.7. Estimation of number of Ca^{2+} ions moving into, and out of, a single *E. coli* cell

(a) Ca^{2+} influx: A typical *E. coli* cell is a cylinder (0.5μ diameter, 1.3 μ long), total volume 0.26 fl, and inner cytosol 0.21fl (periplasmic space 16% of total). A typical membrane capacitance is 1 μF cm^{-2}, giving a total capacitance for each *E. coli* cell of 2.4×10^{-14} F (total surface area = 2.4 μ^2). The key equations in Table 1 are:

1. Charge for Ca^{2+} equivalent to the EMF = Q = CE coulombs = CE/2F moles = CEL/2F ions
   E = EMF = ElectroMotive Force = Em − (RT/2F) ln (Ca_o/Ca_i)
   Em = membrane potential = −150 mV in a typical *E. coli*
   Ca_o = external Ca^{2+} concentration
   Ca_i — cytosolic Ca^{2+} concentration
   R = Universal gas constant = 8.314 J $mole^{-1}$ $°K^{-1}$
   T = temperature in °K = 293.2 at 20 °C
   F = the Faraday constant = 96470 coulombs $mole^{-1}$
   L = The Avodagro constant = 6.022×10^{23}
2. Current for Ca^{2+} moving through one channel = EG amps = EG/2F moles s^{-1} = EGL/2F ions s^{-1}
   G = conductance (Siemens)
3. Number of Ca^{2+} ions per cell = $Ca^{2+}{}_i\times$ volume × L.
   Reusch et al. [21] reported the conductance of a single PHB–PP channel *in vitro* to be 100 pS for Ca^{2+}. Here a value of 10 pS was used taking account of the 18:1 competition with Na^+:$Ca^{2+}{}_i$ concentrations are those measured prior to determining Ca^{2+} efflux.

(b) Ca^{2+} efflux: Numbers for efflux were based on the rate of decrease in cytosolic free Ca^{2+} measured when EGTA, Na^+, K^+ or sucrose were added respectively. Estimated values were based on one Ca^{2+} transporter per cell obeying Michaelis–Menten kinetics. A turnover number of 10 s^{-1} was used, and a Km of 1 μM. Data from Inesi [32], Steiger and Luterbacher [33], Verkhovskaya et al. [34], Guerini et al. [35], Sarism and Carafoli [36].

3. Results

3.1. RT-PCR of putative calcium transporters

It was necessary to show first that the genes *chaA* and *yrbG* were expressed in *E. coli* under test conditions. As is conventional, RT-PCR analysis was performed using gene specific primers to confirm the expression of *chaA* and *yrbG* genes under test conditions, both before and after the induction and reactivation of the aequorin, i.e. immediately prior to the Ca^{2+} measurements (data not shown). DNA, contaminating RNA preparations, can serve as a template in PCR to produce a false positive signal from RT-PCR, although these can be easily identified by looking at the outcome of a "minus RT" control. The minus RT control indicates whether DNA is contaminating the RT-PCR reaction. Therefore cDNA synthesis was performed plus or minus reverse transcriptase, using total RNA extracted from JM109 cells. These cDNAs were then used to perform PCR step. Bands, separated by electrophoresis in agarose, corresponding to the correct lengths of *chaA* and *yrbG*, 1101 and 978 bp respectively, confirmed the expression of these genes. Absence of bands in the minus RT (−RT) sample indicated that results are not affected by genomic DNA contamination at this stage (Fig. 1A).

As a further negative control it was also necessary to show that PCR products were not obtained from the corresponding knockout Δ*chaA* and Δ*yrbG*. RT-PCR analysis of MG1655, Δ*chaA*, and Δ*yrbG* cells confirmed the expected absence of the respective bands (Fig. 1B). In summary, the presence of full length mRNA confirmed that these proteins must be expressed in the *chaA*$^+$ and *yrbG*$^+$ strains, although it was not possible to estimate the precise level of protein expression produced, since no antibodies are available. Thus, both proteins were being expressed immediately prior to the measurements of cytosolic Ca^{2+}. This result was repeated on several occasions using different RNA extracts.

Table 1
Estimation of number of Ca^{2+} ions moving into and out of a single *E. coli* cell

Ca_i μM	Ca_o mM	Ca^{2+} ions/ cell	Reversal potential mV	Full EMF (E) mV	Ca^{2+} influx (ions/ channel/s)	Measured Ca^{2+} efflux nM/s	Measured Ca^{2+} efflux ions/cell/s	Estimated Ca^{2+} efflux ions/cell/s
0.8	1	104	87.8	−238	7.42E+06	2.9	0.4	4.5
1.1	1	134	84.7	−235	7.32E+06	6.6	0.8	5.1
1.4	1	182	80.9	−231	7.21E+06	14.3	1.8	5.9
2.0	1	253	76.8	−227	7.08E+06	20.9	2.6	6.7
2.2	1	278	75.6	−226	7.04E+06	25.9	3.3	6.9
2.3	1	291	75.1	−225	7.03E+06	25.4	3.2	7.0
2.3	1	295	74.9	−225	7.02E+06	26.9	3.4	7.0
3.3	1	419	70.6	−221	6.89E+06	95.3	12.1	7.7
3.8	1	478	68.9	−219	6.83E+06	68.4	8.7	7.9
4.3	1	546	67.3	−217	6.78E+06	84.8	10.7	8.1
5.0	1	627	65.6	−216	6.73E+06	84.8	10.7	8.3
5.6	1	713	64.0	−214	6.68E+06	91.8	11.6	8.5
6.3	1	795	62.7	−213	6.64E+06	140.3	17.7	8.6
8.0	1	1005	59.8	−210	6.55E+06	202.1	25.6	8.9
25.3	1	3199	45.4	−195	6.10E+06	421.7	53.3	9.6

The absolute number of Ca^{2+} ions moving into or out of individual cells was estimated using measured rates of Ca^{2+} influx and efflux (Figs. 2–7), and the equations shown in the Methods section. The key conclusion was that very small numbers of Ca^{2+} ions moving in and out of each cell per second could explain the changes in cytosolic free Ca^{2+} observed. Furthermore, there is a clear lack of correlation between the measured Ca^{2+} efflux (see penultimate column) and that predicted by simple Michaelis–Menten kinetics (see last column — Estimated Ca^{2+} efflux). i = inside; o = outside.

3.2. Regulation of cytosolic free Ca^{2+} by pH and Na^+

It has been reported that ChaA, as an exchanger of Ca^{2+} for H^+ in or out of the cell, is most active in cells at alkaline pH [37]. Therefore, if ChaA was an influx mechanism for regulating cytosolic free Ca^{2+} in *E. coli*, then the cytosolic free Ca^{2+} should be high at alkaline pH but low at acid pH, in the presence of added external Ca^{2+}. Similarly, if Na^+ was able to regulate cytosolic free Ca^{2+} via Ca^{2+} influx, for example by the putative Na^+/Ca^{2+} exchanger YrbG, then cytosolic free Ca^{2+} should be higher when Na^+ was low in the external medium. Thus, the effects on Ca^{2+} influx of pH 5.0–9.0 and addition of Na^+ from 10–300 mM were investigated.

Addition of Ca^{2+} to the external medium resulted in a small initial spike in light emission (Fig. 2). This could be explained by a small number of bacteria being highly permeable to Ca^{2+}, or to the release of a small amount of aequorin into the external medium. The kinetics of the subsequent light emission could only be explained by light emission from within the cell, and not by any significant and extremely unlikely leakage of aequorin into the medium. At 1 mM external Ca^{2+} the cytosolic free Ca^{2+} rose much higher at pH 9.0 than at either pH 5.0 or 7.5 (Fig. 2). Thus, at pH 9 the basal level of approximately 0.5 μM free Ca^{2+} rose to a peak of 3.6 μM within 5 min, compared to peaks of 0.9 μM or 1.3 μM at pH 5.0 and pH 7.5 respectively. The initial rates of calcium influx at pH 5.0, 7.5 and 9.0, measured by the initial rate of rise of cytosolic free Ca^{2+}, were 0.9 nM s^{-1}, 5.8 nM s^{-1} and 17.6 nM s^{-1} respectively. This was likely to be an underestimate since these values were not corrected for any unidirectional Ca^{2+} efflux. This result could be explained either by ChaA acting as a Ca^{2+} influx channel or by H^+ inhibiting this influx channel.

Lowering the external Na^+ in buffer A from 137.5 mM to 12.5 mM in buffer AA also caused a large increase in cytosolic free Ca^{2+} (Fig. 3). Thus, addition of 1 mM calcium in buffer AA caused the cytosolic free Ca^{2+} to rise from 3 μM to a peak value of approximately 10 μM within less than 5 min, whereas with buffer A with a total of 137.5 mM Na^+ the peak cytosolic free Ca^{2+} was only 2 μM. A second addition of Ca^{2+} in the same buffer caused a further small increase in cytosolic free Ca^{2+} that was again most marked when there was no additional added Na^+ (Fig. 3). Although these results were apparently consistent with a role for YrbG in Ca^{2+} influx, the question arose whether all the above

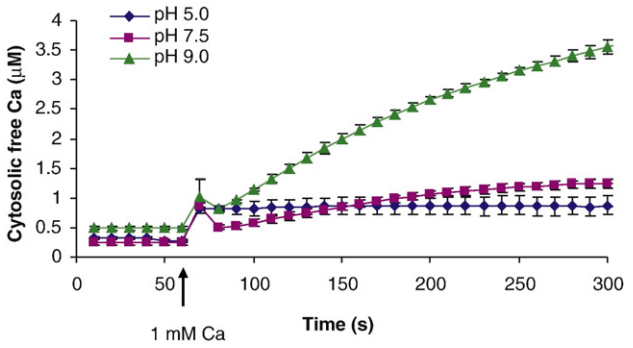

Fig. 2. Effect of pH on cytosolic free Ca^{2+} through Ca^{2+} influx. In order to study the effect of pH on Ca^{2+} influx it was necessary to prepare solutions of buffer A (25 mM HEPES, 125 mM added NaCl, 1 mM $MgCl_2$) at different pH: 5.0, 7.5 or 9.0. JM109 cells expressing active aequorin were therefore added to 0.5 ml of buffer A at pH 5.0 (◆, 7.5 (■) or 9.0 (▲) respectively and the luminescence counts recorded for 1 min. 0.5 ml of buffer A was then added containing 2 mM Ca^{2+} (final 1 mM) at pH 5.0, 7.5 or 9.0 respectively. The luminescence counts were recorded for a further 5 min, when 0.5 ml of NP40 (4%) and 50 mM $CaCl_2$ was added to expose the remaining active aequorin to Ca^{2+} over the following 5 min. The luminescence counts were then converted to cytosolic free Ca^{2+} as described in the Methods section. Results represent the mean+/−SEM of three determinations.

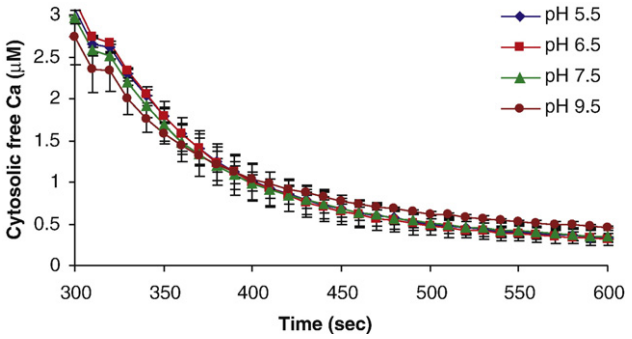

Fig. 4. Effect of pH on Ca^{2+} efflux. In order to study the effect of pH on Ca^{2+} efflux, JM109 cells expressing active aequorin were first added to 0.5 ml of buffer A: 25 mM HEPES, 125 mM added NaCl, 1 mM $MgCl_2$, at pH 7.5 and the luminescence counts recorded for 1 min. Then 0.5 ml of buffer A at pH 7.5 was added containing 2 mM Ca^{2+} (final 1 mM) for 5 min to elevate the cytosolic free Ca^{2+}. A further 0.5 ml of buffer A at different pH values was then added, containing EGTA (final concentration 5 mM) to remove the external Ca^{2+}, and so that the final pH was 5.5(◆, 6.5 (■), 7.5 (▲) or 9.5 (●) respectively. The luminescence counts were recorded for a further 5 min. 0.5 ml of NP40 (4%) and 50 mM $CaCl_2$ was then added to expose the residual active aequorin to Ca^{2+} over the following 5 min. The luminescence counts were then converted to cytosolic free Ca^{2+} as described in the Methods section. Results represent the mean+/−SEM of three determinations.

results could be explained alternatively via competition by either H^+ or Na^+ for a Ca^{2+} influx channel.

3.3. The effect of pH and Na^+ on Ca^{2+} efflux

Thus, the question arose whether the elevations in cytosolic free Ca^{2+} observed when cells were incubated with external Ca^{2+}, at either pH 9 or without added Na^+, were caused by an effect of pH or Na^+ on Ca^{2+} influx or efflux. Therefore, in order to investigate whether these observed elevations in cytosolic free Ca^{2+} at high external pH (i.e. by lowering the external H^+ concentration), or at low external Na^+, were caused by an *activation* of Ca^{2+} influx or an *inhibition* of efflux, the effects of pH or Na^+ on Ca^{2+} efflux were measured. This was achieved by first elevating the cytosolic free Ca^{2+} by addition of external Ca^{2+} (final 1 mM) to the cells, followed by removal of the external Ca^{2+} using EGTA (final 5 mM) to stop any further Ca^{2+} influx (Figs. 4 and 5). Bacteria were therefore first incubated in 1 mM external Ca^{2+} at a constant pH of 7.5 for 5 min, either in normal buffer A (i.e. 25 mM HEPES neutralised with 12.5 mM Na^+, 125 mM Na^+, 1 mM Mg^{2+}) or buffer AA with no additional Na^+(25 mM HEPES neutralised with 12.5 mM Na^+, 1 mM Mg^{2+}). The absolute initial rate of Ca^{2+} efflux increased as the initial cytosolic free Ca^{2+} increased (Fig. 6).

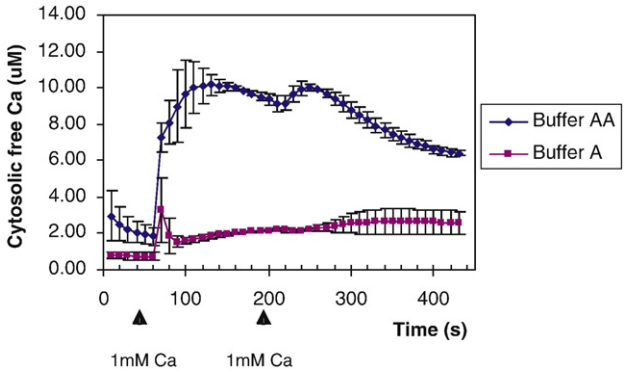

Fig. 3. Role of external sodium in controlling cytosolic free Ca^{2+} through Ca^{2+} influx. In order to investigate the effect of external Na^+ on cytosolic free Ca^{2+}, buffer A was prepared with or without added NaCl. Thus JM109 cells expressing active aequorin were added to 0.5 ml buffer A (■, 25 mM HEPES, 125 mM added NaCl, 1 mM $MgCl_2$, pH 7.5) or buffer AA (◆, 25 mM HEPES, 1 mM $MgCl_2$ pH 7.5) and the luminescence counts recorded for 1 min. 0.5 ml of buffer A (added Na^+) or buffer AA (no added Na^+) containing 2 mM Ca^{2+} (final 1 mM) was then added. The luminescence counts were recorded for a further 5 min when 0.5 ml of NP40 (4%) and 50 mM $CaCl_2$ was added to expose the remaining active aequorin to Ca^{2+} over the following 5 min. The luminescence counts were then converted to cytosolic free Ca^{2+} as described in the Methods section. Results represent the mean+/−SEM of three determinations.

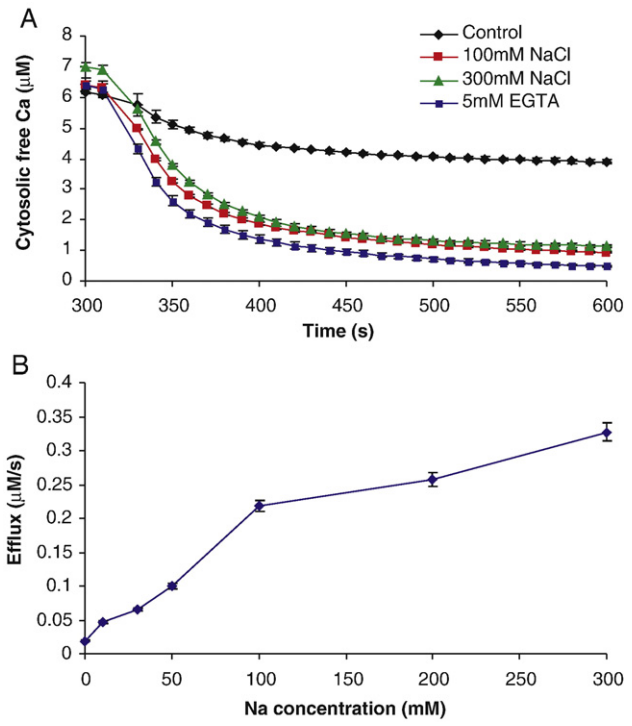

Fig. 5. Effect of sodium on Ca^{2+} efflux A. JM109 cells expressing active aequorin were incubated in 0.5 ml of buffer AA (no added NaCl, 25 mM HEPES, 1 mM $MgCl_2$, pH 7.5) for 1 min in a luminometer and the luminescence counts recorded. 0.5 ml of the same buffer containing 2 mM Ca^{2+} (final 1 mM) was then added and the luminescence counts recorded for a further 5 min. 0.5 ml of buffer AA containing either Na^+(added at a concentration of ■ 100 mM, or ▲ 300 mM), or EGTA (final concentration 5 mM, ■) was then added and the luminescence counts recorded for 5 min to monitor Ca^{2+} efflux. 0.5 ml of NP40 (4%) and 50 mM $CaCl_2$ was then added to expose the residual active aequorin to Ca^{2+} over the following 5 min. The luminescence counts were then converted to free Ca^{2+} as described in the Methods, and compared with the control with no added EGTA or Na^+ (◆). B) Initial calcium efflux rates were plotted against the added extracellular NaCl concentration (10–300 mM). Results represent the mean of three determinations at each point.

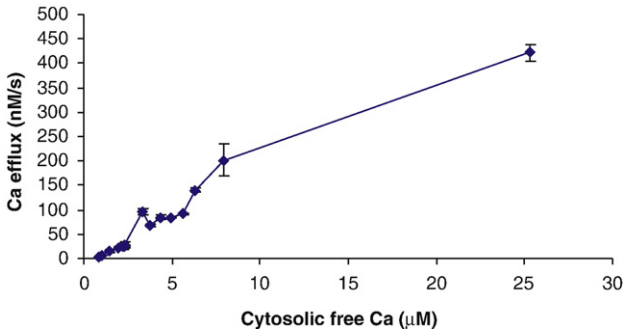

Fig. 6. Ca^{2+} efflux versus cytosolic free Ca^{2+}. JM109 cells were added to 0.5 ml buffer A (25 mM HEPES, 125 mM added NaCl, 1 mM $MgCl_2$, pH 7.5) or buffer AA (25 mM HEPES, 1 mM $MgCl_2$, pH 7.5 and no added Na^+) and the luminescence counts recorded. 0.5 ml of buffer A or buffer AA containing 2 mM Ca^{2+} (final 1 mM) was then added and the luminescence counts recorded for a further 5 min. The rate of decrease in the luminescence counts was then measured following addition of 0.5 ml buffer A or buffer AA containing EGTA (final 5 mM), NaCl (added at 10–300 mM) or KCl (final 100 mM). The luminescence counts were recorded for a further 5 min. 0.5 ml of NP40 (4%) and 50 mM $CaCl_2$ was then added to expose the remaining active aequorin to saturating Ca^{2+} over the following 5 min. The luminescence counts were then converted to cytosolic free Ca^{2+} as described in the Methods section. The initial rate of decrease in cytosolic free Ca^{2+} was then plotted against the absolute cytosolic free Ca^{2+}, demonstrating that the rate of efflux increased as the cytosolic free Ca^{2+} increased. Values represent the means from a total of 12 experiments.

In buffer A with high Na^+, addition of 1 mM Ca^{2+} caused a ten fold rise in cytosolic calcium from 0.3 μM to 3 μM. External Ca^{2+} was removed by adding 5 mM EGTA, and the pH changed from 7.5 to 5.5–9.5 (Fig. 4), or in buffer AA Na^+ (10–300 mM) added in the absence of EGTA (Figs. 5A and B). Addition of EGTA (5 mM) caused a rapid decrease in cytosolic free Ca^{2+}, detectable within 10–20 s, reaching a basal cytosolic free calcium level of 0.5 μM within 5 min. Under these conditions, the initial rate of Ca^{2+} efflux was 23 nM s^{-1}, but was not significantly different at pH 5.5, 7.5 or 9.5. These results showed that the cells contained an efficient Ca^{2+} efflux pathway which, however, appeared distinct from the pH dependent ChaA.

Strikingly, as shown in Fig. 5A, addition of 100 mM Na^+(without addition of EGTA) to cells loaded with Ca^{2+} also caused an apparent rapid efflux of Ca^{2+}, and therefore a decrease in cytosolic free Ca^{2+} in a manner that was dependent on the concentration of added Na^+ (Fig. 5B). This latter result was again consistent with a role of a Na^+/Ca^{2+} transporter such as YrbG in regulating cytosolic free Ca^{2+} via an exchange mechanism, reaching a new balanced rate where Ca^{2+} influx=Ca^{2+} efflux. However, as with the effects of pH, the effect of added Na^+ apparently stimulating Ca^{2+} efflux could still also be explained by a competition between Ca^{2+} and Na^+ for a non-selective ion influx channel with conductance for both monovalent and Ca^{2+} ions. Further experiments were therefore necessary (see below) to determine whether the effects of Na^+ on Ca^{2+} efflux could be specifically ascribed to the activity of the putative YrbG exchanger.

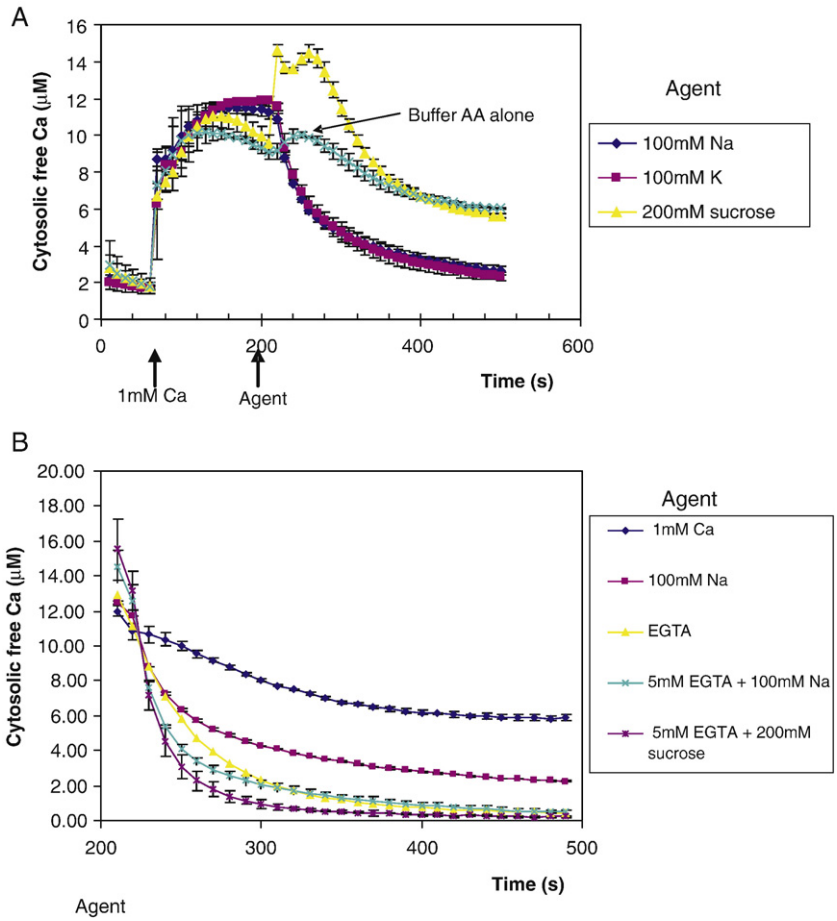

Fig. 7. Effect of sodium, potassium and osmotic pressure on calcium efflux. JM109 cells expressing active aequorin were added to 0.5 ml of buffer AA (no added NaCl) and the luminescence counts recorder for 1 min in a luminometer. 0.5 ml of buffer AA containing 2 mM Ca^{2+} (final 1 mM) was then added and the luminescence counts recorded for a further 2 min. A. 0.5 ml of buffer AA was then added containing Ca^{2+} (1 mM) with NaCl (◆ added at 100 mM), KCl (■ final 100 mM) or sucrose (▲ final 200 mM) respectively; B. 0.5 ml of buffer AA was then added with EGTA (final 5 mM) to remove external Ca^{2+}, with (✕) or without (▲) added NaCl (■ final 100 mM), or with sucrose (✖ final 200 mM) respectively, and the luminescence counts recorded for a further 5 min. 0.5 ml of NP40 (4%) and 50 mM $CaCl_2$ was then added to expose the residual active aequorin to Ca^{2+} over the following 5 min. The luminescence counts were then converted to free Ca^{2+} as described in the Methods section, and compared with the control with no further additions (◆). Results represent the mean of 3 determinations.

3.4. Reduced levels of cytosolic Ca^{2+} were not specific for Na^+

In order to test whether the apparent stimulation of Ca^{2+} efflux was specific for Na^+, cells were first loaded with Ca^{2+} in the absence of added Na^+ or K^+(i.e. in buffer AA), and the effect of adding Na^+ (100 mM) compared with the addition of K^+ (100 mM), in the absence of EGTA as shown in Fig. 7. The maximum efflux rate observed with 100 mM K^+ was 184nM s^{-1}, not significantly different from the efflux rate provoked by 100 mM Na^+ (175 nM s^{-1}). Note that the control, involving adding medium with 1 mM Ca^{2+} alone in the absence of added monovalent cation or EGTA, caused a small increase in cytosolic free Ca^{2+}, followed by a slow decline at an initial rate of 26 nM s^{-1}.

K^+ is not normally thought to act as an exchanger for Ca^{2+} in the Ca^{2+} efflux pathways of eukaryotes. K^+ has only been shown to cotransport with Ca^{2+}, exchanging together for Na^+ [38,39]. Since both K^+ and Na^+ had the same effect on cytosolic Ca^{2+}, we conclude that this is likely due to competition for an influx system rather than an exchange mechanism. Nevertheless, the results left the possibility that the effects of Na^+ or K^+ could be explained by osmotic changes affecting Ca^{2+} influx and efflux in some way. This was therefore investigated as follows.

3.5. Stimulation of Ca^{2+} efflux by Na^+ was not due to osmosis

To demonstrate whether changes in osmotic pressure could affect the movement of calcium, the effect on Ca^{2+} efflux of Na^+ or K^+ was compared with the effect of sucrose (200 mM; Fig. 7A). The results showed that, in the presence of external Ca^{2+}, sucrose actually caused an initial increase in cytosolic Ca^{2+}, which decreased to the same level as the control in the absence of sucrose. A similar result was observed with either 100 mM sucrose or 100 or 200 mM dextran respectively (data not shown). Therefore, sucrose clearly did not initiate the immediate rapid Ca^{2+} efflux seen with either Na^+ or K^+. As a control, to check the effect of sucrose in the absence of external Ca^{2+}, the effect of sucrose on cytosolic free Ca^{2+} was compared with Na^+ in the presence of added EGTA (5 mM; Fig. 7B). In this experiment the rate of Ca^{2+} efflux initiated by addition of Na^+ was similar to that seen on addition of EGTA (Fig. 7B). Thus, comparing the effect of sucrose in the presence or absence of EGTA in Fig. 7A and B, sucrose itself did not significantly stimulate Ca^{2+} efflux. Thus, the activation of Ca^{2+} efflux by the monovalent cations Na^+ or K^+ could not be explained by osmosis. On the other hand, these results showed that changes in external osmotic pressure can affect cytosolic free Ca^{2+} in *E. coli*, with an increase in external osmotic pressure being accompanied by an initial increase in cytosolic free Ca^{2+}, presumably due to loss of water from the cells.

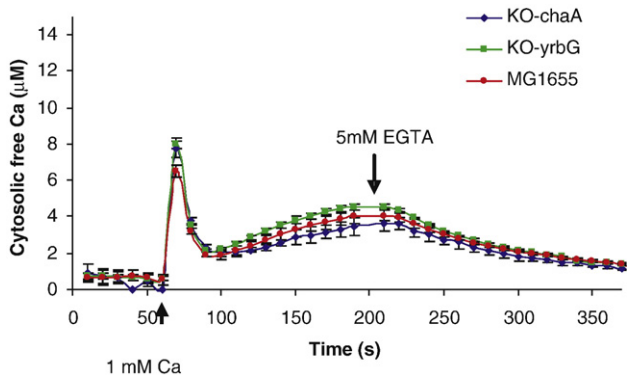

Fig. 8. Ca^{2+} efflux in knockouts of *chaA* and *yrbG*. Cells (◆Δ*chaA*, ■Δ*yrbA* or ●MG1655) expressing active aequorin were added to 0.5 ml of buffer A (25 mM HEPES, 125 mM added NaCl, 1 mM $MgCl_2$, pH 7.5) for 1 min in a luminometer and the luminescence counts recorded. 0.5 ml of buffer A containing 2 mM Ca^{2+} (final 1 mM) was then added and the luminescence counts recorded for 5 min to study the effect of gene knockouts on Ca^{2+} influx. 0.5 ml of EGTA (final 5 mM) was then added to remove the external Ca^{2+} in order to study effects of gene knockouts on Ca^{2+} efflux, the luminescence being recorded for a further 3 min. 0.5 ml of NP40 (4%) and 50 mM $CaCl_2$ was then added to expose the residual active aequorin to Ca^{2+} over the following 5 min. The luminescence counts were then converted to cytosolic free Ca^{2+} as described in the Methods section. Results represent the mean+/−SEM of three determinations.

3.6. Ca^{2+} influx and efflux in knockouts of genes chaA and yrbG

As a final test for the possible involvement of ChaA and YrbG in the calcium influx and efflux pathways under our conditions, it was necessary to use bacteria which were defective in these genes. Therefore, knockouts of *chaA* and *yrbG* in strain MG1655 were constructed (see Methods) from the Keio collection [27] and shown to be true knockouts by RT-PCR as described in the Methods section (Fig. 1B). Incubation of wild type cells and the two mutants in 1 mM Ca^{2+} caused the cytosolic free Ca^{2+} in each case to rise from 0.3 μM to approximately 4 μM within 2 min of incubation, showing that there were no apparent changes in Ca^{2+} influx in the knockout cells (Fig. 8). Similarly, when 5 mM EGTA was added to cause a decrease in free Ca^{2+} in the cytosol, the efflux rates between these strains (MG1655, Δ*chaA* and Δ*yrbG*) were not significantly different, 30nM s^{-1}, 29nM s^{-1} 38nM s^{-1} respectively. This further ruled out the possibility that ChaA and YrbG constitute Ca^{2+} efflux mechanisms in *E. coli* under the conditions of our experiments.

4. Discussion

The results here show, for the first time, that H^+ and monovalent cations (Na^+ and K^+) can regulate cytosolic free Ca^{2+} in *E. coli*. Importantly, we were able to induce rapid changes in cytosolic free Ca^{2+} by manipulation of external ions, supporting the idea of specific influx mechanisms for Ca^{2+}. The fact that changes in external Ca^{2+} alone induced changes in cytosolic Ca^{2+} suggested that some of the Ca^{2+} influx channels must already be open in non-stimulated cells. We propose that the rise in cytosolic free Ca^{2+} provoked by increased pH, or by a large reduction of Na^+ in the external medium, is best explained by an inhibition or competition by H^+ or Na^+/K^+ for a Ca^{2+} influx channel, rather than stimulation of efflux by a specific exchange mechanism because: (a) there was no effect of pH on Ca^{2+} efflux (Fig. 4), yet there was a reduction in Ca^{2+} influx at acid pH (Fig. 2); (b) the apparent stimulation of Ca^{2+} efflux by Na^+ was not specific since K^+ caused an identical effect (Fig. 7A); (c) although changes in external osmotic pressure may have effects on cytosolic free Ca^{2+}, for example as a result of cell shrinkage or swelling, the effect of Na^+ and K^+ could not be explained by osmosis (Fig. 7A and B); (d) there appeared to be no differences in Ca^{2+} influx or efflux between the wild type strain and *chaA* or *yrbG* knockouts (Fig. 8). In fact, the results with knockouts suggest that neither the pH dependent transporter ChaA nor the putative Na^+/Ca^{2+} exchanger YrbG are significant mechanisms for moving Ca^{2+} in these experiments. This is supported by the report that radioactive Ca^{2+} fluxes in membrane vesicles are not affected when prepared from *chaA* knockouts [37].

Our results in this and previous studies show clearly that *E. coli* maintains sub-micromolar or micromolar levels of intracellular free Ca^{2+} in the presence of 0.1–10 mM external Ca^{2+}. Therefore, there must be an effective efflux pathway in *E. coli* (Figs. 4–7). Since ChaA and YrbG appear not to be major regulators of intracellular Ca^{2+}, the question now arises what channels, transporters or exchangers are actually responsible for regulating cytosolic free Ca^{2+}. Furthermore, the energy source for Ca^{2+} efflux, which occurs against an electrochemical gradient, needs to be identified. Electrophysiological analysis has revealed that mechanosensitive channels MscL, MscS, MscK and MscM, transiently create large pores when activated by increases in tension in the lipid bilayer of the cytoplasmic membrane [40,41]. In eukaryotic cells, activation of mechanoreceptors is often associated with Ca^{2+} influx, and the effect of knockouts of such genes on cytosolic free Ca^{2+} in *E. coli* now needs to be investigated.

No Ca^{2+} channels equivalent to those found in eukaryotic cells have been identified so far in *E. coli*. The best candidate for a Ca^{2+} transporter is in fact the complex of polyhydroxybutyrate–polyphosphate, PHB–PP, [21–23,29,42], which *in vitro* is La^{3+} sensitive and appears to have a conductance for Ca^{2+} 18 times that of Na^+. The conductance of PHB/polyP channels, and their selectivity for Ca^{2+}, is very sensitive to variations in pH. Thus, lowering the pH decreases Ca^{2+} conductance and also abolishes the preference for Ca^{2+} over Na^+ [22]. This suggests that the PHB/polyP channels may become less efficient in binding and transporting Ca^{2+} at lower pH values, and the high influx rate which we observed at pH 9.0 could include a role for these PHB–PP channels. Indeed, the carboxyl group of butyrate would be fully ionised at pH 9, but not at pH 5.5, reducing Ca^{2+} binding at acid pH. The effects of pH might therefore be best explained by direct effects on Ca^{2+} influx through the PHB–PP channel. In addition, we also need to consider that the PHB-polyP channel could allow Ca^{2+} to pass in and out of the cell reversibly, though Ca^{2+} efflux would require an energy source since this occurs against an electrochemical gradient.

Another possible efflux channel is the product of *pitB*, a potential Ca^{2+}-phosphate symporter, which appears to act primarily as a phosphate influx mechanism. However, all the experiments reported here were carried out in the absence of external phosphate, arguing against the possible involvement of PitB in Ca^{2+} influx under these conditions. Some 354 putative transporter proteins have been identified in *E. coli*, including four P-Type ATPases (www.membranetransport.org). Several P-type ATPases have been described in other bacteria [43], e.g. a Ca^{2+}-MgATPase in *Bacillus subtilis* encoded by the *yloB* gene, similar to the eukaryotic Ca^{2+}-MgATPase of SERCA and Golgi apparatus [44]. BLAST analysis of MgtA in *E. coli*, which encodes a P-type Mg^{2+}-ATPase, shows sequence homology to the human pMr1 Ca^{2+}-MgATPase. Furthermore, there is some similarity between MgtA and YloB in *E. coli* and *B. subtilis* respectively. However, so far there is no evidence that Ca^{2+} can be transported by any of these putative ATPases. Other ion transporters or exchangers have also been identified in the *E. coli* genome. The effect of knockouts of these genes on Ca^{2+} influx and efflux now also needs to be investigated. Interestingly, as indicated in Table 1, it is clear that the measured Ca^{2+} efflux rates do not match those predicted for a Ca^{2+} pump obeying simple Michaelis–Menten kinetics. Furthermore, a plot of 1/cytosolic free Ca^{2+} against time, when external Ca^{2+} was removed or when efflux occurs when Na^+ was added, was linear [13,14]. Thus, intriguingly, the elusive efflux mechanism may transport 2 Ca^{2+} at a time.

An important issue is how many ions have to move across the plasma membrane of each cell to cause the changes in cytosolic free Ca^{2+} that we have observed. Cytosolic free Ca^{2+} in our experiments varied from 0.1–0.5 μM in resting cells when *E. coli* was incubated in the absence of external Ca^{2+} to 10–30 μM in the presence of a stimulus. The calculated absolute free Ca^{2+} ions in each cell was therefore about 10 at 0.1 μM Ca^{2+}, 134 at 1 μM and 1000 at 10 μM (see Table 1). This compares with nearly 400,000 ATP molecules if the cytosolic concentration is 3 mM, and 16 million K^+ ions for a cytosolic K^+ concentration of 120 mM. In addition, since the calculated reversal potential for Ca^{2+} was +45–88 mV (Table 1), it would take nearly 12,000 Ca^{2+} ions to neutralise this, raising cytosolic free Ca^{2+} to nearly 100 μM. Thus, the Ca^{2+} movements reported here are unlikely to have any significant direct effect on membrane potential. From the reported characteristics of the polyhydroxybutyrate-polyphosphate channel [20], we calculate that in *E. coli* a single such channel would be sufficient, for example, to import the number of Ca^{2+} ions measured in this study in a fraction of a second, whereas the time observed to reach peak cytosolic free Ca^{2+} was 2–3 min. Furthermore, just one eukaryote-like Ca^{2+}-MgATPase pump per cell acting in 1–5 s would be sufficient to cause the total Ca^{2+} efflux reported here (Table 1). Yet efflux took some 5 min to reach the resting level of cytosolic free Ca^{2+}, following addition of EGTA. Even if there was some Ca^{2+} buffering inside the *E. coli* cell, these estimates for the absolute numbers of Ca^{2+} ions moving across the cytoplasmic membrane are orders of magnitude away from those normally experienced in eukaryotic cells. These paradoxes may be resolved by a channel that opens only for a very short time with a conductance in the fS range, or the observed transport may be limited by events in a small sub-populations of cells. Alternatively, a different form of mathematical analysis may be required, based first on low probabilities of channel and pumps opening, or a model where Ca^{2+} influx and efflux occur in a quantal manner in each cell [26]. These results are likely to have important implications for *E. coli* adapting to a range of ionic environments in which it survives and grows.

Acknowledgements

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