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Cytosolic Ca^{2+} regulates protein expression in *E. coli* through release from inclusion bodies

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Abstract

The results here are the first clear demonstration of a physiological role for cytosolic Ca^{2+} in *Escherichia coli* by releasing a Ca^{2+} binding protein, apoaequorin, from inclusion bodies. In growth medium LB the cytosolic free Ca^{2+} was 0.1–0.3 μM. Addition of EGTA reduced this to <0.1 μM, whereas addition of Ca^{2+} (10 mM) resulted in a cytosolic free Ca^{2+} of 1–2 μM for at least 2 h. Ca^{2+} caused a 1.5- to 2-fold increase in the level of apoaequorin induced by IPTG. Whereas EGTA induced a 50% decrease. The effect of a Ca^{2+} was explained by release of protein from the inclusion bodies, together with a stabilisation of apoaequorin against degradation. Ca^{2+} also reduced the generation time by 4–5 min. These results have important implications for unravelling the physiological role of cytosolic Ca^{2+} in bacteria, particularly where several species are competing for the same nutrients, such as in the gut.

Keywords: Calcium channels; Inclusion bodies; Bacteria; Polyhydroxybutyrate; Aequorin; Calcium in bacteria

The role of Ca^{2+} as a signal in eukaryotes is well established [1–5], but in bacteria is less well understood [6–11]. The main reasons for this are, first, the mechanisms regulating Ca^{2+} influx and efflux in bacteria are poorly defined. Second, there has been a lack of direct evidence for Ca^{2+} transients in bacteria generated by agents analogous to transmitters, hormones and drugs in eukaryotes. Third, there is little direct evidence for Ca^{2+} regulating physiological events in bacteria. Several EF hand type proteins have been identified from analysis of prokaryotic genomes [12,13]. However, no true homologue of calmodulin has yet been found. The main evidence for a role of Ca^{2+} in bacteria has been indirect [9,14]. Thus, Ca^{2+} may regulate chemotaxis [15], spore formation [16], the cell cycle [8,9], virulence, competence, and the synthesis of specific proteins [14]. We have shown that the photoprotein aequorin [17–21] enables changes in cytosolic free Ca^{2+} to be correlated with events in live bacteria, showing that metabolites of carbohydrate metabolism, such as butane 2,3-diol and methylglyoxal, induce Ca^{2+} transients in *Escherichia coli* [22].

In low Ca^{2+}, free Ca^{2+} inside *E. coli*, like eukaryotes, is submicromolar [17–21]. However, unlike eukaryotes, changes in external free Ca^{2+} have marked effects on cytosolic free Ca^{2+} in bacteria, being increased by chemorepellants, complement activation, interaction with neutrophils, and certain antibiotics [17]. Higher levels of cytosolic free Ca^{2+} occur in stationary phase cultures of *E. coli*, with evidence for La^{3+}-sensitive Ca^{2+} channels. In addition, the periplasmic space can concentrate free Ca^{2+} with respect to external Ca^{2+} [20], determining the Ca^{2+} seen by the inner membrane containing the putative Ca^{2+} channels [21–24]. During these experiments we observed that the growth of *E. coli* with plasmid appeared slower than in cells

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without plasmid, and that cells grown without external Ca^{2+} expressed lower levels of apoaequorin than cells grown with added Ca^{2+}.

Bacteria store a wide range of substances in inclusion bodies [25,26]. These substances include proteins, organic molecules and inorganic ions such as phosphate. It has also been reported that bacteria may regulate the formation and release of substances from inclusion bodies [26,27]. The aim of the work reported therefore was to test whether conditions, under which cytosolic free Ca^{2+} was elevated, caused a decrease in protein in inclusion bodies, using the Ca^{2+} binding protein apoaequorin as a model, thereby enhancing the rate of growth. Apoaequorin is easy to assay in both broken and intact cells through its light emission. We have shown previously that in eukaryotic cells the level of apoaequorin in the cytosol or endoplasmic reticulum was reduced when the free Ca^{2+} was reduced [28,29]. Here, we show that Ca^{2+} induces protein release from inclusion bodies, thereby increasing growth rate by reducing the generation time.

Materials and methods

Chemicals. Coelenterazine was a gift from Bruce Bryan, Prolume, UK. Bacterial media were from Oxoid, Hampshire, UK. $CaCl_2$ (1 M) was from BDH (Merck Ltd., Leicestershire, UK). All other chemicals were of AnalaR grade from Sigma Chemical Co. (Dorset, UK).

Escherichia coli strains and growth conditions. Escherichia coli strain JM109 (Promega) was used with the multi host range expression vector, pMMB66EH (Amp^R), carrying the *tac* promoter as previously described [17–21]. JM109 cells are $recA^-$. However we showed that changes in cytosolic Ca^{2+} are similar in the $recA^+$ strain MG1655. Bacteria 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 with vigorous shaking. Absorbance at 600 nm was used to estimate growth rate. Colony counts were carried out on LB plates incubated at 37°C overnight. Incubation in buffer A (25 mM Hepes, 125 mM NaCl, 1 mM $MgCl_2$, pH 7.5) with or without Ca^{2+} or EGTA had no effects on the viability of *E. coli*.

Induction of apoaequorin and reconstitution to aequorin. Aequorin cDNA was cloned into the multi-host-range expression vector (pMMB66EH) as previously described [17–19]. The cells were resuspended in 5 ml of buffer A (25 mM Hepes, 125 mM NaCl, 1 mM $MgCl_2$, pH 7.5) containing 2 μM coelenterazine and incubated in the dark at room temperature for 1 h to form active aequorin. Incubation of cells under these conditions had no effect on cell viability.

Luminescence measurements and estimation of cytosolic free Ca^{2+}. Light emission was measured as previously described [17–21,30]. The rate constants (k s^{-1}) at each time point were then estimated as (counts s^{-1})/(total counts remaining at each time point). The rate constants were then converted to free Ca^{2+} concentrations using a standard curve. Multiple measurements under different conditions simultaneously were carried out using a photon counting imaging camera (Photek Ltd., Hastings) as previously described [19,20,31]. Cells (10 μl) were added to 100 μl medium LB in microtitre wells (Fig. 1A), and placed in front of the camera (resolution 640 × 480 pixels, 50 Hz). Ca^{2+} or EGTA were then added and the light emission recorded for 2 h (Fig. 1B). Fifty microlitres of medium containing 4% NP40, 50 mM $CaCl_2$, and 100 mM Tris, pH 7.0 was then added to expose the remaining active aequorin to Ca^{2+}. The light emission was recorded for a further 5 min. The light emission time course was subdivided into a 10-s image sequence, and the light emission measured from each well. The rate constants (k s^{-1}) were calculated and free Ca^{2+} estimated.

Isolation of inclusion bodies from E. coli and extraction of apoaequorin. A single colony of *E. coli* JM109 cells expressing pMM66EH-aequorin plasmid was inoculated into 15 ml LB, containing 100 μg ml^{-1} carbenicillin. The overnight culture (1 ml) was expanded in fresh LB containing carbenicillin and grown to $A_{600} = 0.3–0.6$. Apoaequorin was induced with 1 mM IPTG, IPTG + 10 mM Ca^{2+} or IPTG + 5 mM EGTA for 2 h. Cells were centrifuged at 3000*g* for 10 min, 4 °C and supernatants were removed. Pellets were resuspended in 1/10 of buffer S (50 mM Tris–HCl, pH 7.8, 250 mM NaCl, 5 mM EDTA, 2 mM β-mercaptoethanol) and sonicated for 5 × 15 s. The suspension was then centrifuged at 30,000*g* for 30 min, 4 °C. Supernatants were stored for aequorin reconstitution and pellets resuspended in 1/25 of buffer D (50 mM Tris–HCl, pH 7.8, 8 M

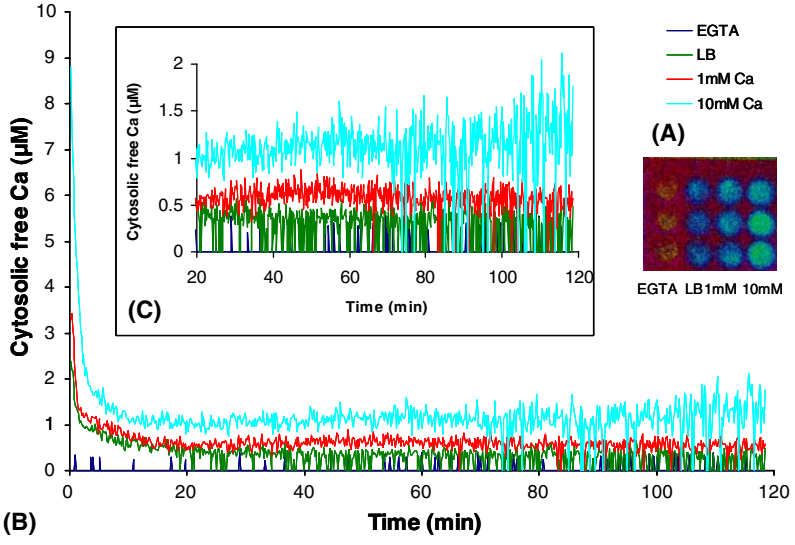

Fig. 1. Effect of external Ca^{2+} on cytosolic free Ca^{2+}. (A) Image of the microtitre (MT) plate containing *E. coli* cells expressing aequorin with or without external Ca^{2+} or EGTA. (B) JM109 *E. coli* cells expressing active aequorin were incubated with LB with or without 5 mM EGTA, 1 mM or 10 mM Ca^{2+}, respectively, and the luminescence counts recorded for 2 h. 12.5 mM $CaCl_2$ + detergent (NP40) was then added for 10 min, to expose the remaining active aequorin. The cytosolic free Ca^{2+} was then estimated as described in Materials and methods. (C) Represents the time course from 20 to 120 min to show the occurrence of Ca^{2+} oscillations. Results represent means of three determinations.

urea, and 5 mM β-mercaptoethanol). The protein was then renatured by 10-fold dilution in buffer R (20 mM Tris–HCl, pH 9.5, 1 mM $CaCl_2$, 5 mM β-Me), and incubated at RT for 20 min. EDTA and NaCl (10 and 250 mM, respectively) were added and centrifuged at 22,000*g* for 30 min, 4 °C. Aequorin was reconstituted in soluble and in insoluble fractions in buffer O (500 mM NaCl, 1 mM Na_2EDTA, 10 mM Tris–HCl, pH 7.5), containing 5 mM β-merceptoethanol, 0.1% gelatine, and 5 μM coelenterazine as 1:1 ratio. The suspension was incubated for 1 h in the dark and the luminescence counts recorded after addition of calcium.

Effect of inhibition of protein synthesis with and without calcium on inclusion bodies. Escherichia coli JM109 cells expressing pMM66EH-aequorin plasmid were grown as described above, and induced with 1 mM IPTG for 2 h. Cells were centrifuged at 3000*g* for 10 min, the supernatant removed and the pellet resuspended in fresh sterile LB containing antibiotic. Ten millimolar Ca^{2+} or five millimolar EGTA was added with chloramphenicol (150 μg ml^{-1}) and incubated at 37 °C, 200 rpm for 1 h. Cells were centrifuged at 5000 rpm, 10 min and the pellets resuspended in 1/10 buffer S (50 mM Tris–HCl, pH 7.8, 250 mM NaCl, 5 mM EDTA, 2 mM β-mercaptoethanol), sonicated 2 × 10 s, and centrifuged 13,000 rpm 1 h 4 °C. After centrifugation, the supernatant was stored on ice prior to aequorin reconstitution, and the pellets resuspended in 1/25 of buffer D (50 mM Tris–HCl, pH 7.8, 8 M urea, and 5 mM β-mercaptoethanol), incubated at RT for 30 min. Buffer R (20 mM Tris–HCl, pH 9.5, 1 mM $CaCl_2$, 5 mM β-mercaptoethanol) as 10-fold dilution was added and incubated at RT for 20 min. EDTA and NaCl, to the final concentrations of 10 and 250 mM respectively, were added and the suspension centrifuged at 22,000*g* for 30 min at 4 °C. Aequorin was then reconstituted in the soluble and insoluble fractions as described above.

Results

The effect of external Ca^{2+} on cytosolic free Ca^{2+}

To investigate any possible effect of Ca^{2+} on inclusion bodies it was first necessary to establish that, under the conditions used to measure changes in apoaequorin within inclusion bodies, the cytosolic free Ca^{2+} was indeed elevated over a 2-h time period required. Thus, *E. coli* cells in the growth medium LB were placed in front of an imaging camera as described in Materials and methods, and the basal level of light emission estimated. EGTA (5 mM) or Ca^{2+} (1 and 10 mM final concentration) were then added, and the cytosolic free Ca^{2+} measured. In medium LB the resting cytosolic free Ca^{2+} was 0.1–0.3 μM. Addition of EGTA (5 mM) resulted in a rapid decrease in cytosolic free Ca^{2+} to <0.1 μM, where it remained for at least 2 h (Fig. 1). In contrast, addition of 1 or 10 mM Ca^{2+} to the cells resulted in a rapid increase in cytosolic free Ca^{2+} to approximately 3 and 9 μM, respectively, followed by a gradual decrease over the following 20 min, when the cytosolic free Ca^{2+} remained at 0.6–0.8 and 1–2 μM, respectively for up to 2 h (Fig. 1B and C). Some small Ca^{2+} oscillations, with an amplitude of 0.2–1 μM, were also observed under these conditions (Fig. 1C). In control cells in medium LB alone the cytosolic free Ca^{2+} was 0.2–0.4 μM over 2 h.

These results confirmed that addition of 10 mM external Ca^{2+} resulted in an elevated cytosolic free Ca^{2+} in *E. coli* of 1–2 μM for at least 2 h, similar to levels stimulated by agonists in eukaryotic cells. Whereas EGTA resulted in levels of cytosolic free Ca^{2+} well below 0.1 μM over this time period.

Effect of cytosolic free Ca^{2+} on apoaequorin expression

To investigate whether rises in cytosolic free Ca^{2+} might regulate the level of cytosolic apoaequorin in inclusion bodies, it was first necessary to investigate the effect of Ca^{2+} on the total level of apoaequorin expression. Thus the effect of 1 and 10 mM Ca^{2+} on the level of apoaequorin induced by IPTG, assessed as described in Materials and methods, was compared with EGTA (1 and 10 mM) in medium LB over 2 h (Fig. 2). The results showed that EGTA caused a 40–50% decrease in apoaequorin expression compared with medium LB alone. In contrast, the presence of 1 or 10 mM Ca^{2+} in the external medium resulted in a 1.5- to 2-fold increase in apoaeaquorin expression ($p < 0.001$).

Ca^{2+} induced loss of apoaequorin from inclusion bodies

Expression of proteins from plasmid DNA in *E. coli* are known to result in proteins trapped in inclusion bodies.

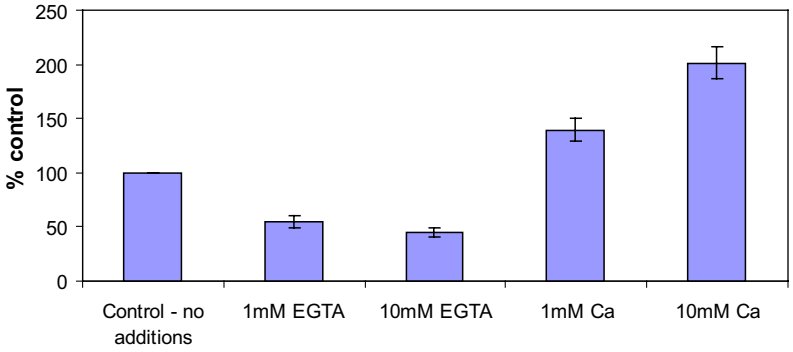

Fig. 2. Effect of external Ca^{2+} on the total level of apoaequorin. JM109 cells expressing apoaequorin were grown in medium LB until the absorbance reached 0.3. The culture was then induced with 1 mM IPTG with or without 1 mM or 10 mM EGTA, or 1 mM or 10 mM Ca^{2+}, respectively. Cells were grown for a further 2 h, washed and resuspended in buffer A (see Materials and methods). 12.5 mM $CaCl_2$ + 1.5% NP40 was then added for 10 min to expose the active aequorin, as described in Materials and methods. Results were expressed (total aequorin) as % of control with no additions, and represent means ± SEM of three determinations.

Inclusion body protein can be several fold higher than that free in the cytosol. The question therefore arose whether the effect of Ca^{2+} on total apoaequorin expression was the result of increased synthesis, decreased degradation, or the amount of apoaequorin in inclusion bodies. Thus, to test whether Ca^{2+} affected the level of apoaequorin in inclusion bodies, apoaequorin expression was induced using IPTG for 2 h in medium LB with or without added Ca^{2+} or EGTA. The cells were then sonicated, and the cytosolic free apoaequorin separated from the inclusion bodies by centrifugation. The inclusion bodies were then solubilised, and the apoaequorin converted into active aequorin, as described in Materials and methods. The results confirmed that the total activity, i.e. cytosolic plus inclusion body apoaequorin, was greatest after the cells had been incubated in Ca^{2+} (Fig. 3A). The total activity after cells had been incubated in 10 mM Ca^{2+} was approximately twice that after cells had been incubated in EGTA (5 mM). Similarly, under these conditions, the apoaequorin isolated from the cytosolic fraction of cells incubated in Ca^{2+} (10 mM) for 2 h, was also approximately twice that compared with apoaequorin isolated from cells incubated in EGTA. Furthermore, the apoaequorin in the inclusion bodies, measured as a ratio of that in the cytosol, in cells incubated in EGTA was 2–3 times that in cells incubated in Ca^{2+}. The total amount of apoaequorin in the inclusion bodies varied considerably between different experiments, reaching a maximum of 20–100 times that in the cytosol in some experiments. These results were consistent with an elevated cytosolic Ca^{2+} reducing the amount of apoaequorin in inclusion bodies. Similar results were obtained when Ca^{2+} or EGTA were added to cells during the exponential growth phase prior to induction with IPTG (results not shown).

Ca^{2+} induced release of apoaequorin from inclusion bodies

Since we have shown previously that apoaequorin in eukaryotic cells is stabilised by Ca^{2+}, the degradation rate being enhanced in low Ca^{2+} [27,28], the lower level of total apoaequorin (i.e. cytosolic + inclusion bodies) in cells incubated in EGTA compared with cells incubated in Ca^{2+} could have been explained either by an effect of Ca^{2+} on protein expression or degradation. In order to distinguish between these two possibilities, and to investigate whether the effect on inclusion bodies was caused by formation or release of protein from these bodies, the effect of Ca^{2+} on cytosolic versus inclusion body apoaequorin was measured by including in the incubation a potent inhibitor of protein synthesis, chloramphenicol (150 μg/ml), in the presence or absence of external Ca^{2+}.

In the presence of chloramphenical and external Ca^{2+} (10 mM), the total apoaequorin (cytosolic + inclusion bodies) was 2–3 times that compared with medium LB alone or EGTA (5 mM). This showed that the increase in total apoaequorin induced by Ca^{2+} was not the result of increased protein or RNA synthesis. Rather differences in *total* activity could be explained entirely by effects on the rate of apoaequorin degradation, Ca^{2+} stabilising the apoaequorin. In contrast, effects of Ca^{2+} on the *ratio* of apoaequorin in the cytosol relative to inclusion bodies (Fig. 4) was explained by release of apoaequorin from inclusion bodies, the ratio of supernatant to pellet being 4–5 times

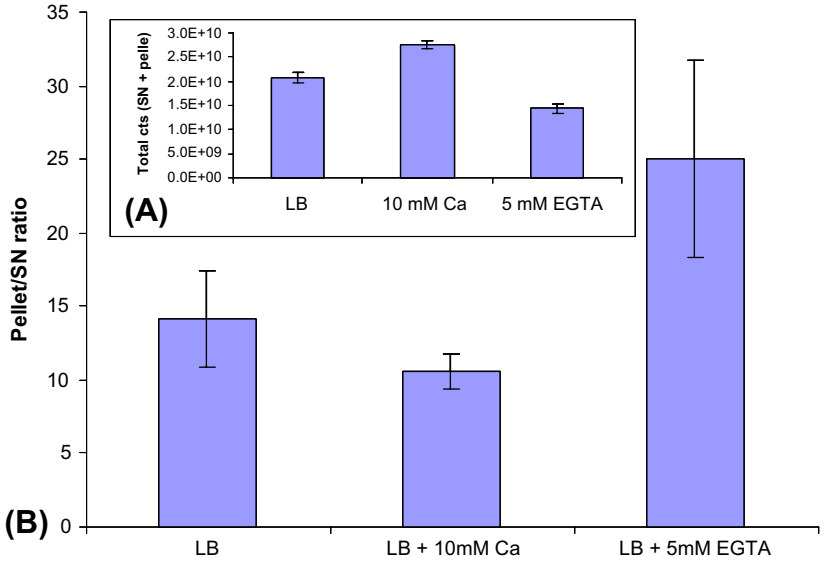

Fig. 3. Effect of external Ca^{2+} on the level of apoaequorin in inclusion bodies versus cytsolic. JM109 cells expressing apoaequorin plasmid were grown in LB at 37 °C until the absorbance reached 0.3. The cells were then induced with 1 mM IPTG for 2 h, in the presence of 10 mM Ca^{2+} or 5 mM EGTA, respectively. The cells were then isolated by centrifugation, and lysed for determination of apoaequorin in the soluble (SN (supernatant), cytosol) and insoluble (pellet, inclusion body) fractions as described in Materials and methods. (A) Total aequorin; (B) ratio of aequorin in pellet/SN. SN, cytosolic aequorin; pellet, inclusion body aequorin. Results represent means ± SEM of three determinations.

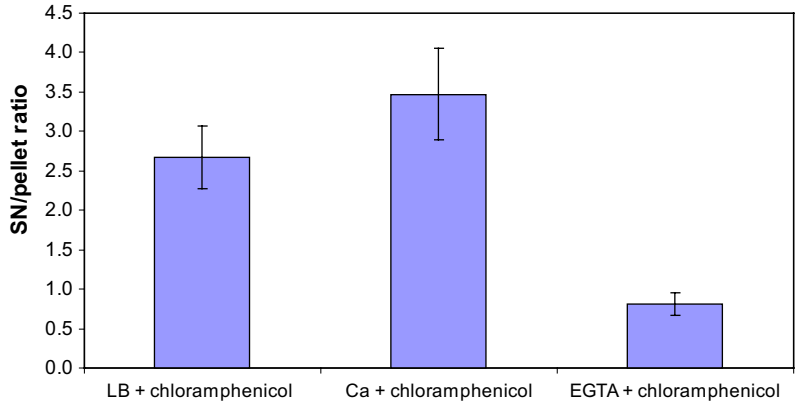

Fig. 4. Effect of Ca^{2+} on the release of apoaequorin from inclusion bodies. JM109 cells expressing aequorin plasmid were grown in medium LB at 37 °C until the absorbance reached 0.3. The cells were then induced with 1 mM IPTG for 2 h. The cells were then centrifuged to remove IPTG, and 10 mM Ca^{2+} or 5 mM EGTA, respectively, added with 150 μg/ml chloramphenicol. The cells were then grown for a further 1 h. Cells were then isolated by centrifugation, and lysed for determination of apoaequorin in the soluble (SN (supernatant), cytosol) and insoluble (pellet, inclusion body) fractions as described in Materials and methods. Results are SN/pellet ratios and represent means ± SEM of three determinations.

in cells incubated in Ca^{2+} (10 mM) compared with cells incubated in EGTA (5 mM).

Ca^{2+} increased the rate of cell growth

Bacteria containing reproducible plasmids grow more slowly than normal cells. In our experiments *E. coli* JM109 grew more slowly when transformed compared with cells without plasmid (data not shown). Furthermore, cells expressing apoaequorin grew slightly faster in Ca^{2+} (10 mM) than in EGTA (5 mM). JM109 cells with expression plasmid grown in Ca^{2+} (10 mM) had a generation time of 50.6 ± 0.9 min (mean ± SEM), whereas cells grown in EGTA (5 mM) had a significantly increased generation time of 54.6 ± 1.3 min (mean ± SEM) $p < 0.01$. These generation times were similar to other reports for JM109 cells, since this strain is known to have a longer generation time than $recA^+$ strains such as MG1655. If this apparently small effect on generation time occurred in exponentially growing cells over 24 h, then the cells with the shorter generation time, i.e. 4–5 min shorter, would dominate the cell population by a ratio of 20,000 to one, a classic example of Darwin–Wallace natural selection.

Discussion

The results reported here show for the first time that elevated cytosolic free Ca^{2+} in *E. coli* can release protein into the cytosol from inclusion bodies. Ca^{2+} also significantly inhibited apoaequorin degradation, consistent with our previous data in eukaryotic cells [28,29]. The results also showed that aequorin can monitor cytosolic free Ca^{2+} at 1–2 μM for >2 h (Fig. 1). We have recently reported that carbohydrate metabolites, such as butane 2,3-diol and methyl glyoxal, generate free Ca^{2+} transients rising to >10 μM over a 5- to 10-min period [22]. In order to investigate whether cytosolic free Ca^{2+} >10 μM persists for 2 h, our aequorin mutant with a reduced affinity for Ca^{2+} will be required [32].

Inclusion bodies are refractile, ovoid or cylindrical bodies, 0.2- to 1-μm wide and 1 μm^3 in volume, found in bacteria and Archea [25,26]. The two most studied consist of misfolded protein [25–27] or polymers of polyhydroxyalkanoic acids (PHA), such as polyβ-hydroxybutyrate [33]. PHA inclusion bodies consist of 98% PHA with an outer coat of protein and lipid, and provide a reserve of carbon and energy. Polyhydroxybutyrate inclusion bodies may also provide a reserve of Ca^{2+} channels. Protein inclusion bodies are common when recombinant proteins are highly expressed, and are an obstacle to efficient recombinant protein production in bacteria. These inclusion bodies may also contain small amounts of the heat shock proteins IbpA and IbpB [26]. Particles containing luciferase have been observed in the luminous bacterium *Vibrio harveyii* [34]. Originally, it was thought that the inclusion body protein was inaccessible to chaperones and proteases [26,27]. However, our results show clearly that active protein can be recovered *in vivo* from inclusion bodies, supporting other reports that inclusion body formation is reversible [35]. Our results also show that some ten times more active recombinant protein can be recovered from inclusion bodies isolated from cells following sonication, centrifugation, and solubilisation at high pH, urea and detergent.

Apoaequorin is a 21-kDa protein, with three EF Ca^{2+} binding sites sequence similar to those in calmodulin, and a C-terminal proline essential for fully active photoprotein [36]. It will be interesting to investigate whether elevated cytosolic free Ca^{2+} can release other proteins from inclusion bodies, and in particular other EF hand proteins in the *E. coli* genome [12,13], and in other bacteria. The results reported here thus are the first report of a signalling mechanism in bacteria for regulating cytosolic proteins post-translationally. The question now arises whether the

mechanism for release of proteins from inclusion bodies we have revealed has important significance in regulating the level of key proteins, ion channels, and thus bacterial generation times. Interestingly our results show a 5 min reduction in generation time in cells containing expressed plasmid when grown in Ca^{2+}, when the cytosolic free Ca^{2+} was >1 μM and release of protein from inclusion bodies was maximal.

In conclusion, we report here the first description of cytosolic Ca^{2+} increasing soluble protein in a bacterium through release from inclusion bodies. This has important implications for maximising cytosolic aequorin when studying free Ca^{2+} in live bacteria [17–21]. Furthermore, our results highlight a Darwinian–Wallace mechanism, whereby changes in bacterial generation time of <10% could have vital consequences where several species compete for the same nutrients, such in the gut [37,38], and in quorum sensing [39,40].

Acknowledgments

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