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Escherichia coli lacking the AcrAB multidrug efflux pump also lacks nonproteinaceous, PHB–polyphosphate Ca^{2+} channels in the membrane

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Abstract

PHB(polyP) complexes bind calcium and form calcium channels in the cytoplasmic membrane in *Escherichia coli* and are likely to be important in Ca^{2+} homeostasis in this organism. *E. coli* N43, which lacks the AcrA component of a major multidrug resistance pump, was shown to be defective in calcium handling, with an inability to maintain submicromolar levels of free Ca^{2+} in the cytoplasm. Therefore, using an *N*-phenyl-1-napthylamine (NPN)-dependent fluorescence assay, we measured temperature-dependent phase transitions in the membranes of intact cells. These transitions specifically depend on the presence of PHB(Ca^{2+}polyP) complexes. PHB(Ca^{2+}polyP) channel complexes, particularly in stationary phase cultures, were detected in wild-type strains; however, in contrast, isogenic *acrA*$^-$ strains had greatly reduced amounts of the complexes. This indicates that the AcrAB transporter may have a novel, hitherto undetected physiological role, either directly in the membrane assembly of the PHB complexes or the transport of a component of the membrane, which is essential for assembly of the complexes into the membrane. In other experiments, we showed that the particular defective calcium handling detected in N43 was not due to the absence of AcrA but to other unknown factors in this strain.

Keywords: Ca^{2+} homeostasis; AcrAB multidrug pump; PHB(Ca^{2+}polyP); *Escherichia coli*; Phase transition

1. Introduction

It is well established that Ca^{2+} is an important signalling molecule in eukaryotes [1,2]. A similar important role for calcium has not so far been clearly established in prokaryotes. Nevertheless, several studies have suggested that free intracellular Ca^{2+} may play a role in the regulation of prokaryotic physiological processes such as chemotaxis, cell division and signal transduction [3–6]. Some studies indicate that the level of intracellular Ca^{2+} may regulate the expression of several genes in *Escherichia coli* and *Bacillus subtilis* [7,8]. Consistent with this, Michiels et al. [9] have recently surveyed the many proteins in the bacterial genome database with apparent EF-hand, calcium binding motifs (see also Ref. [10]). Moreover, the PhoQ sensor kinase in *Salmonella typhimurium* appears capable of sensing both calcium and magnesium [11,12] and a number of other proteins have been identified in bacteria involved in Ca^{2+} transport, including pumps and exchangers [13,14]. In a survey of bacterial transporters, Paulsen et al. [15] also identified in *E. coli* and other bacteria, a putative voltage-gated Na^+,K^+,Ca^{2+} ion channel of the VIC superfamily, and putative primary and secondary transporters for Ca^{2+} uptake. Perhaps surprisingly, recent studies, in particular structural studies, have led to the proposal that at least some conserved prokaryotic potassium conduction pores are gated by intracellular calcium like their eukaryotic counterparts [16,17]. On the other hand, in *E. coli*, nonproteinaceous complexes of the lipidic polymer poly-3-hydroxybutyrate (PHB) with inorganic calcium polyphosphate (Ca^{2+}polyP), have been shown to act as voltage-gated Ca^{2+} channels with complex gating kinetics [18–20]. PolyP itself is implicated in many processes in bacteria, including survival in stationary phase [21].

The role of PHB in *E. coli* has been investigated by Reusch et al. [22–24], who clearly showed that complete

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PHB(Ca^{2+}polyP) complexes can be detected in vivo by examining thermotropic phase transitions of bacterial membranes using the fluorescent probe *N*-phenyl-1-napthylamine (NPN). Thus, in intact *E. coli* cells, the specific temperature-dependent phase transitions, which generate enhanced fluorescence of NPN, are exactly duplicated when extracted PHB(Ca^{2+}polyP) complexes are incorporated into liposomes [24]. This fluorescence assay reports phase transitions of *E. coli* membranes in agreement with light scattering and X-ray diffraction [25].

Previous investigations in our laboratories regarding the mechanism of regulation of intracellular Ca^{2+} have used bioluminescence from endogenous aequorin triggered by free Ca^{2+}, to measure cellular Ca^{2+} levels [26,27]. The results showed that *E. coli* strain JM109 growing exponentially maintains tight control over the level of free cytoplasmic Ca^{2+}, in the range 0.3–0.7 μM, independent of the concentration of Ca^{2+} up to at least 0.25 mM in the medium. However, stationary phase cells showed relatively large increases (up to 2 μM or above) in cytoplasmic free Ca^{2+} in the presence of 0.25 mM Ca^{2+} in the medium. Interestingly, this latter fluctuation, resulting from an apparent influx of Ca^{2+}, was abolished by treatment of cells with $LaCl_3$ [26], which inhibits the channel activity of PHB–polyP complexes [18,19]. In contrast to the cytoplasm, the periplasm equilibrates with the medium at high external calcium levels. However, at low (μM) external Ca^{2+}, the cells appeared capable of concentrating the level of free Ca^{2+} in the periplasm over that of the medium [27].

The AcrAB complex [28] in the inner membrane in *E. coli*, together with TolC in the outer membrane [29], constitutes an export pump for a wide range of drugs, detergents and antibiotics [30,31]. The high-resolution structure of AcrB now shows the molecule as a trimer with proposed docking regions for TolC in the periplasm and potential substrate entry points to the transenvelope channel from the periplasm or the cytoplasmic membrane and from the cytosol [32]. In previous studies concerning the possible physiological role of Ca^{2+} in *E. coli*, we have observed that strain N43 (*acrA*$^-$) [33], lacking the AcrA component of the AcrAB multidrug resistance pump, is also sensitive to EGTA ([7,34]; unpublished data). Moreover, using a derivative of strain N43 hypersensitive to EGTA, we demonstrated [7] that the chelator induced the synthesis of three polypeptides that cross-reacted with antibodies to calmodulin and to calerythrin, a bacterial EF-hand protein [35].

Further investigation of the properties of strain N43 in this study revealed a relatively poor control over free cytosolic Ca^{2+} levels compared with a wild-type strain JM109 (*acrA*$^+$). The possible role of the PHB(Ca^{2+}polyP) channel complexes with respect to these observed differences in the regulation of free Ca^{2+} between different K-12 strains, including isogenic strains of N43 and JM109 differing only at the *acrA* locus, was therefore analysed. The results showed that in *acrA* mutants, the amount of PHB complexes in the membrane is greatly reduced. This raises the novel possibility that the AcrAB pump is also involved in some way in the biogenesis of PHB(Ca^{2+}polyP) complexes. In addition, however, we showed that the defect in calcium handling shown by N43 is due to unknown factors not connected with the *acrA* mutation.

2. Methods

2.1. Bacterial strains and plasmids

The bacterial strains JM109 (*acrA*$^+$) and N43 (*acrA*$^+$) and their *acrA*$^-$ derivatives were used in this study. The multihost range vector pMMB66EH (amp^R), which is suitable for use in any Gram-negative bacterial strain [36], was also utilised. The vector was a gift from Dr. Mark Knight (University of Oxford, UK). Bacteria were routinely maintained and cultured in LB broth (1% Tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2) at 37 °C with vigorous shaking. Solid media was prepared by the addition of 1.5% agar (w/v).

2.2. Construction of acrA mutants

Conversion of N43 (*acrA*$^-$/*B*$^+$) to N43 (*acrA*$^+$/*B*$^+$) and JM109 (*acrA*$^+$/*B*$^+$) to JM109 (*acrA*$^-$, *B*$^+$) was achieved by PI-transduction according to Miller [37].

2.3. Expression of aequorin

JM109 and N43 wild-type cells and their respective *acrA* mutants were transformed with the vector pMMB66EH containing aequorin cDNA and apo-aequorin expression was induced exactly as detailed previously [26]. Apo-aequorin was reconstituted to aequorin inside the live bacteria by the addition of the prosthetic group coelenterazine as described before [26].

2.4. Cytosolic free Ca^{2+} measurements ([Ca^{2+}]i)

Using recombinant aequorin as the bioluminescent Ca^{2+} indicator [38], free [Ca^{2+}]i levels were determined in the bacterial strains using photon counting imaging techniques exactly as detailed before [26].The ability of the bacterial strains to regulate their free [Ca^{2+}]i when challenged with 0.25 mM external Ca^{2+} was determined in either LB medium or Buffer A (125 mM NaCl, 1 mM $MgCl_2$ and 25 mM HEPES, pH 7.5) over 60 min.

2.5. Phase-transition measurements

The assessment of the phase-transition profiles could not be performed directly in LB medium due to the high intrinsic fluorescence of this medium; therefore, the cells were pelleted and resuspended in Buffer A. The cells were maintained at 30 °C in order to prevent the formation of

new complexes, which is known to occur following cell resuspension in HEPES buffer at 4 °C [39]. Lipid phase transitions were determined within 1 h of resuspension by the modified version of the fluorescence assay described by Overath and Trauble [25]. An aliquot of the cell suspension was removed and made up to 3 ml with Buffer A, the fluorescent indicator NPN (Molecular Probes, Oregon, USA) was added (10^{-6} M final concentration). At concentrations of less than 10^{-5} M, NPN does not interfere with the transition temperatures and does not significantly interact with protein [25]. The sample was cooled to 14 °C and then heated (approximate increases of 2 °C/min). Changes in fluorescence were monitored with respect to increasing temperature. The fluorescence measurements were performed using an LS50B luminescence spectrophotometer (Perkin Elmer) with excitation and emission wavelengths set at 337 and 426 nm, respectively.

It should be noted that it was necessary to perform the fluorescence assay on samples adjusted to the same A_{600} units in the experiments where the phase-transition profiles were to be compared, e.g. between bacteria taken at different growth stages. This was achieved by adjusting the volume of the original aliquot taken for the phase-transition measurement.

2.6. Estimation of the levels of PHB(Ca^{2+}polyP) complexes

In order to compare the amounts of the PHB(Ca^{2+}polyP) complexes present between the different bacterial strains, the levels of the complexes were estimated qualitatively by determining the ratio of the peak change in fluorescence due to the sharp transition detected between approximately 54 and 58 °C versus the fluorescence measurement immediately prior to this increase (at approximately 50 °C). It should be noted, however, that while this method allows the comparative assessment of the level of complexes in the bacterial cells, it may not reflect absolute in vivo levels of the complexes as the transition profiles were not determined directly in the growth medium.

3. Results

3.1. Measurements of free intracellular Ca^{2+} in $acrA^{-}$ strains

Using the bioluminescent photoprotein aequorin, free intracellular Ca^{2+} was monitored in the wild-type bacteria JM109 *acrA*$^{+}$ and N43 *acrA*$^{-}$ expressing cytoplasmic aequorin. Exponentially growing cells were preincubated for 10 min in LB medium, followed by the addition of 0.25 mM external Ca^{2+}. The ability of the two strains to regulate their free cytosolic Ca^{2+} over this time period and subsequently was determined and compared. All experiments were performed at 30 °C. During the preincubation period (resting levels of free Ca^{2+}) and for the first 10 min following the addition of 0.25 mM external Ca^{2+}, no marked differences were noted between the free intracellular Ca^{2+} values of the two bacterial strains, which, as shown in Fig. 1, were in the range of 180–230 nM, in agreement with previous findings [26,27]. After this point, the JM109 *acrA*$^{+}$ cells showed very little change in their free cytosolic Ca^{2+}

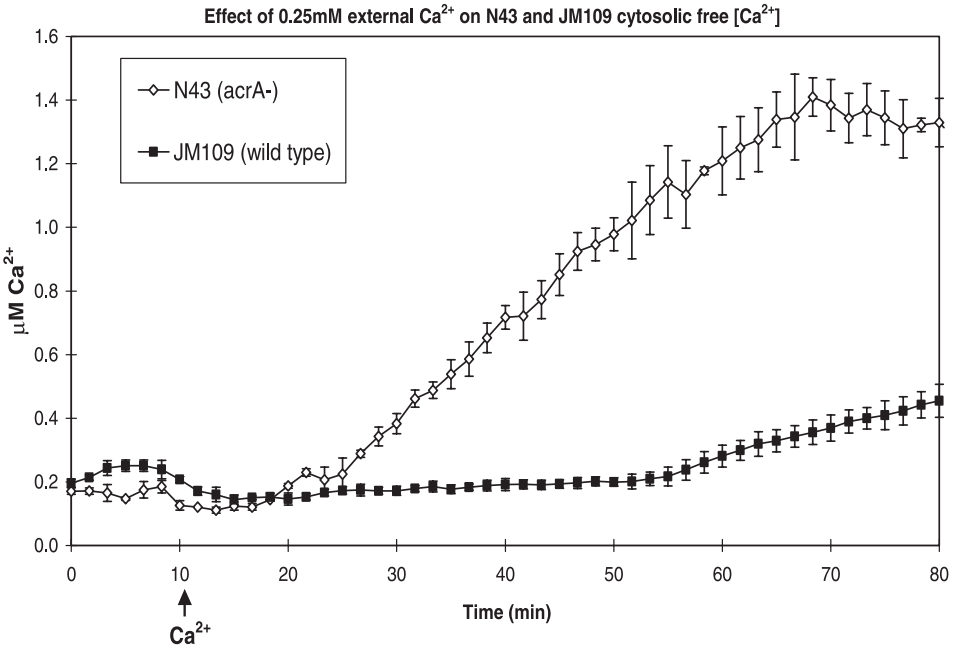

Fig. 1. Regulation of free cytosolic Ca^{2+} in the presence of external Ca^{2+}. Changes in free intracellular Ca^{2+} were monitored in exponential phase JM109 cells (-■-) and N43 (*acrA*$^{-}$) cells (-◇-) in the presence of 0.25 mM external Ca^{2+} over time. The data are expressed as mean ± S.E. per time point of duplicate values. The results are representative of three separate experiments.

over the next 40–50 min. In contrast, N43 (*acrA*$^-$) cells, which lacks a functional AcrAB transporter, demonstrated a substantial increase in free cytosolic Ca^{2+} values, eventually reaching six-fold higher than resting Ca^{2+} values (Fig. 1).

Since the phase-transition profiles described below to measure PHB(Ca^{2+}polyP) channel complexes in the membrane and their possible relationship to the presence of the AcrAB pump were of necessity monitored in Buffer A, the ability of the bacteria to regulate their free [Ca^{2+}]i when placed in this buffer was also determined. Although the efficiency of regulation of intracellular Ca^{2+} levels in both bacterial strains appeared to be reduced when the cells were placed in Buffer A compared with LB (i.e. they were 10% and 20% higher in the two strains, respectively), the same trend in differential regulation of intracellular Ca^{2+} to that observed in LB medium was observed. Thus, N43 cells showed considerable increases in free [Ca^{2+}]i after challenge with 0.25 mM external Ca^{2+} while JM109 cells maintained a tight control on their free [Ca^{2+}]i levels (data not shown).

These results indicated that the AcrAB transporter might be implicated directly or indirectly in the handling of Ca^{2+}. Therefore, since previous studies have shown that the PHB(polyP) complexes, for example, may play a role in Ca^{2+} homeostasis in *E. coli*, we wished to determine whether the level of such complexes was affected in *E. coli* strains such as N43(*acrA*$^-$).

3.2. Phase transitions in E. coli detected with the fluorescent probe NPN

Major phase changes in *E. coli* membranes in whole cells, dependent upon temperature, can be monitored using the fluorescent probe NPN [25]. Since this hydrophobic probe, NPN, partitions into the interior of the bacterial membrane and does not significantly interact with protein [25], it can be used to examine the lipid phase transitions in membranes of whole bacteria, with minimal disturbance to the physiology of the cell. Other studies [22–24] have shown that a major phase transition occurring at approximately 56 °C is primarily associated with the presence of PHB–polyP complexes in the membrane.

The thermotropic lipid phase transitions in the various wild-type and *acrA*$^-$ *E. coli* mutant strains were therefore observed, as described below, at different stages of growth in whole cell cultures by monitoring the changes in fluorescence intensity of the hydrophobic probe NPN with respect to increasing temperatures. Following the criteria established previously [23,24], we anticipated that transitions attributed to phospholipid and free PHB should occur between 12 and 22 °C (low temperatures) and 24 and 50 °C (intermediate temperatures), respectively. The presence of the PHB(Ca^{2+}-polyP) complexes on the other hand is accompanied by a sharp thermotropic transition at approximately 56 °C. This is attributed to an increase in the viscosity of the membrane resulting from the dissociation of the complexes and also indicates the quasi-crystalline structure of the complexes since PHB alone cannot yield this feature [23,24]. For these experiments, the scale of the intensity of the fluorescence was relative and was determined by the amount of NPN that had partitioned into the membranes.

3.2.1. Stability of the PHB(Ca^{2+}polyP) complexes in Buffer A

Due to the high intrinsic fluorescence of LB medium, the phase-transition profiles of the bacterial strains had to be assessed in Buffer A. The stability of the complexes in this buffer over time was therefore first determined. The results indicated that similar levels of the complexes (measured as described in Methods) were detected immediately following resuspension of the cells in Buffer A or after a further 60 min in Buffer A (Fig. 2). Thus, the complexes were stable in Buffer A for at least 60 min. This result also suggests that any differences noted in Ca^{2+} regulation between N43 and JM109 cells in Buffer A (see Fig. 1) were unlikely to result from dissociation or instability of the complexes. In addition, the results in Fig. 2 show that the level of the complexes increases significantly in stationary phase cells.

3.2.2. JM109 cells

Phase transitions displayed by JM109 wild-type (*acrA*$^+$) cells in both exponential and stationary phase, as shown in more detail in Fig. 3a, were largely undetectable at low and intermediate temperatures, indicating little or no uncomplexed, free PHB. In contrast, a sharp and irreversible lipid phase transition was observed at approximately 54–56 °C, in cells from both growth stages, although the transition in stationary phase cells was associated with a higher fluorescence intensity compared with log-phase cells (Fig. 3a). These observations confirmed that strain JM109 contained PHB(Ca^{2+}polyP) complexes, with stationary cells containing the higher levels.

The amounts of the complexes present was estimated by determining the ratio of peak fluorescence due to the phase

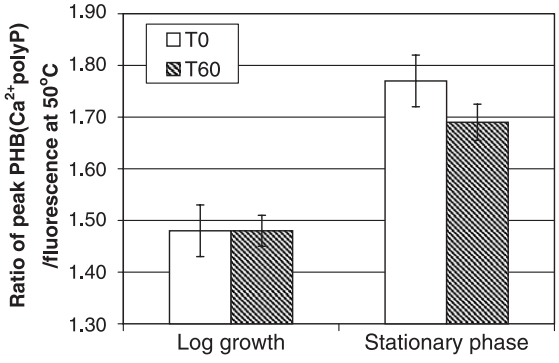

Fig. 2. Phase transitions were performed on JM109 bacterial samples resuspended in Buffer A at different stages of growth, i.e. log growth and stationary phase. The transitions (as shown in Fig. 3a) were analysed and the ratio of the peak fluorescence due to the sharp transition at approximately 56 °C to the fluorescence at 50 °C was determined at time zero (empty box T0) and after 60 min in Buffer A (hatched box T60). The data represents mean ± S.D. of three individual experiments for each point.

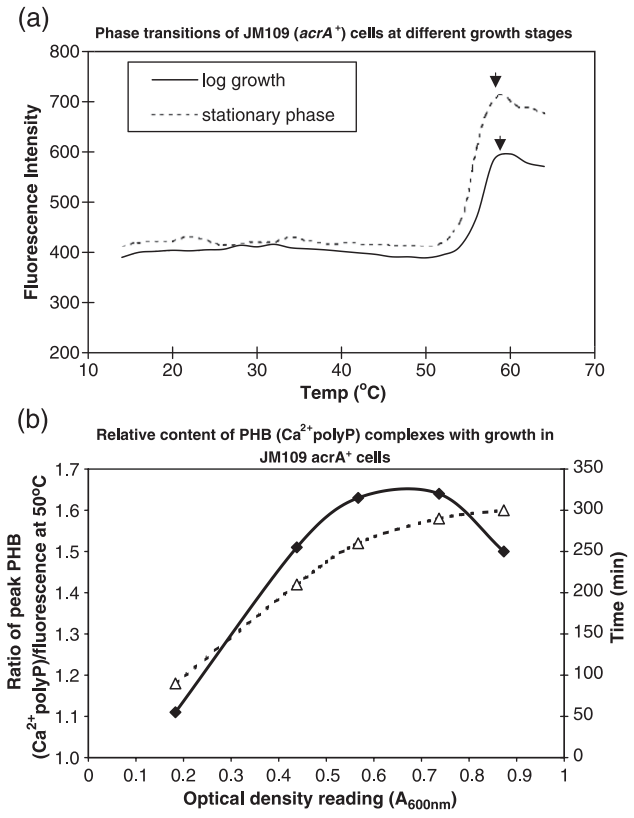

Fig. 3. (a) Fluorescence detection of the thermotropic lipid phase transitions in wild-type JM109 (*acrA*$^+$) bacteria using NPN (10^{-6}M) as an indicator. The phase transitions were monitored in cells in exponential phase (A_{600}, solid line) and cells entering stationary phase (A_{600}, dotted line). The transitions attributed to the dissociation of the PHB(Ca^{2+}polyP) complexes are indicated by arrows. The fluorescence assay was performed on samples adjusted to the same population density, as determined by absorbance at A_{600}. The phase-transition profiles are representative of three separate experiments. (b) Phase transitions were performed on bacterial samples at different stages of growth according to optical density measurements. The transitions were analysed and the ratio of the peak fluorescence due to the sharp transition at approximately 56 °C to the fluorescence at 50 °C was determined and plotted against optical density measurements of the culture. The data is representative of four individual experiments for each point. The broken line shows the optical density of the corresponding culture as this enters stationary phase.

transition occurring at 56 °C, to the fluorescence immediately prior to this increase i.e. at approximately 50 °C (see Methods). As shown in Fig. 3b, the ratio of peak fluorescence/fluorescence at 50 °C increased as the A_{600} of the bacterial culture increased. These findings showed that the membranes of JM109 *acrA*$^+$ cells contained increasing amounts of PHB(Ca^{2+}polyP) channel complexes as growth proceeded.

3.2.3. N43 cells

The lipid phase transitions of N43 (*acrA*$^-$) were also determined at various stages of growth exactly as detailed for JM109. As shown in Fig. 4a, exponential phase N43 cells demonstrated little change in NPN fluorescence up to approximately 62 °C. However, stationary cells of N43 (*acrA*$^-$) showed a dramatic broad phase transition at intermediate temperatures, indicative of high levels of free PHB in the membrane with only a small additional increase in fluorescence at 52–56 °C. These results indicated that N43 (*acrA*$^-$) cells possessed very little PHB(Ca^{2+}polyP) complex in the membrane, even in stationary phase. N43 (*acrA*$^-$) cells nevertheless appeared to contain considerable amounts of free PHB in the membrane in stationary phase, in contrast to cells from the exponential phase (Fig. 4a).

3.3. Phase transitions in JM109 (acrA$^-$) and N43 (acrA$^+$) strains

The results described above indicated that JM109 cells possessed PHB(Ca^{2+}polyP) complexes in the membrane, while N43 (*acrA*$^-$), in exponentially growing cells, produced barely detectable levels of complexes and only small amounts in stationary phase. This suggested that the *acrA/B* gene products may play a role, direct or indirect, in the

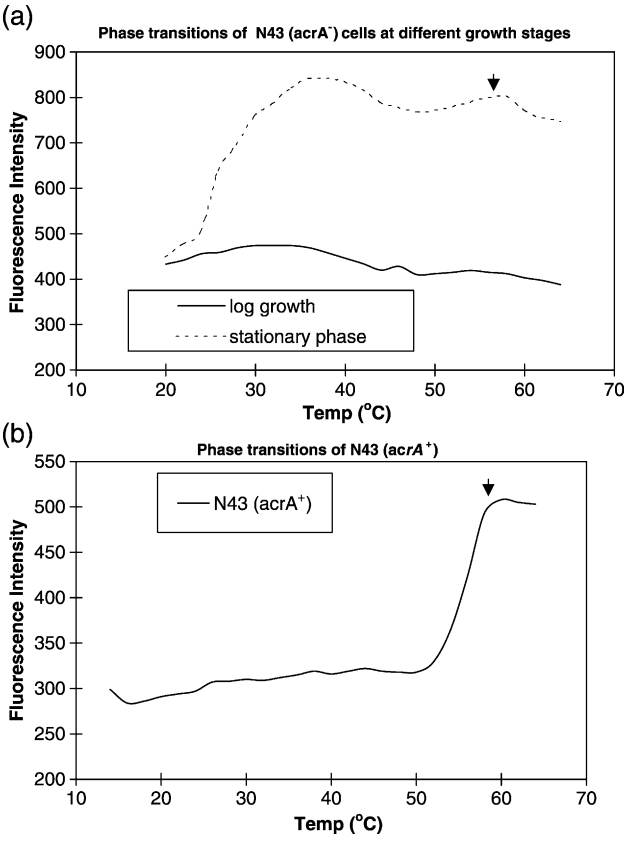

Fig. 4. Phase transitions of N43 wild type (*acrA*$^+$) or *acrA*$^-$. (a) The fluorescence assay was performed on exponentially growing cells of N43 (*acrA*$^-$) (solid line) or entering stationary phase (dotted line). The arrow indicates the phase transition due to the PHB(Ca^{2+}polyP) complexes. Measurements were taken using samples of the same population density (A_{600}) and the phase-transition profiles are representative of three separate experiments. (b) Phase-transition profile of N43 *acrA* in stationary cells. The transition due to the presence of the PHB(Ca^{2+}polyP) complexes is marked by the arrow. The data is representative of three separate experiments.

biogenesis of the complexes. However, importantly, these strains were non-isogenic and therefore for confirmation of these effects, the *acrA* wild-type gene was restored in N43, and conversely *acrA* was deleted from JM109 by phage transduction (see Methods). The thermotropic phase transitions of the mutant JM109 (*acrA*$^-$) and of N43 (*acrA*$^+$) grown into stationary phase were determined in stationary phase cells as before using NPN fluorescence.

The results (Fig. 4b) showed that when, for example, strain N43 now carrying the wild-type *acrA*$^+$ gene was grown into stationary phase, it demonstrated lipid phase transitions identical to that of the JM109 (*acrA*$^+$) strain detected at 52–56 °C, typical of the presence of complexes, with little or no free PHB. This demonstrated that restoration of the *acrA*$^+$ gene in N43 rendered the cells competent for the biogenesis of PHB(Ca^{2+}polyP) complexes, comparable to those found in wild-type JM109 (cf. Fig. 3a).

On the other hand, as shown in Fig. 5, phase-transition measurements comparing wild type and *acrA*$^-$ of stationary phase JM109, demonstrated that the *acrA* mutant unlike its parent was identical to N43 (*acrA*$^-$), i.e. displaying dominant broad phase transitions at low and intermediate temperatures with only a very small increase in fluorescence intensity at 52–56 °C. Thus, JM109 *acrA*$^-$ appeared to show very little PHB(Ca^{2+}polyP) complex formation with considerable amounts of free PHB accumulating in the membrane. Overall, the results clearly indicated a role, direct or indirect, for the AcrAB multidrug resistant pump in generating PHB(Ca^{2+}polyP) complexes.

3.4. JM109 (*acrA*$^-$) is not defective in Ca^{2+} handling

The results described above clearly demonstrated a defect in the biogenesis of PHB(polyP) complexes in strains specifically lacking a functional AcrA protein, including strain N43. As shown in Fig. 1, exponentially growing cells of strain N43, unlike JM109, are also defective in regulating the intracellular free Ca^{2+} concentration. In order to test the possible relationship of these two phenomena, we therefore compared the ability of JM109 *acrA*$^+$ and *acrA*$^-$ strains to control their level of intracellular Ca^{2+} in the face of 0.25 mM Ca^{2+} in the medium. The results obtained in fact demonstrated that the JM109 *acrA*$^-$ strain still displayed tight control of intracellular free Ca^{2+}, essentially as the wild type, despite the much higher level in the medium (data not shown). We conclude therefore that the defect in Ca^{2+} handling displayed by exponentially growing cells of N43 is not due to inactivation of the AcrAB pump with the associated major reduction of PHB(Ca^{2+}polyP) complexes, but to another factor involved in regulating free Ca^{2+} in the N43 background.

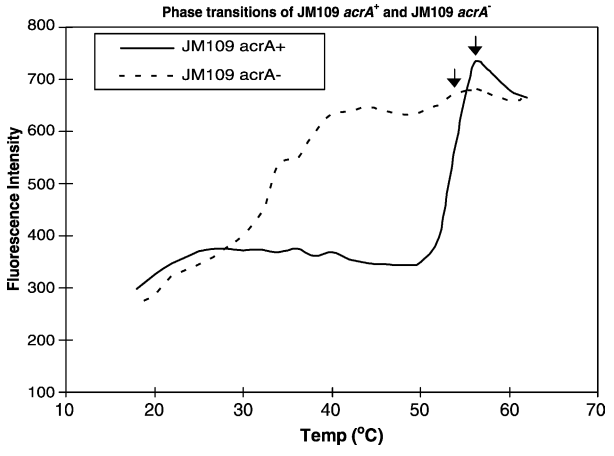

Fig. 5. Lipid phase transitions of stationary phase cells of the mutant JM109 (*acrA*$^-$) (dotted line) and the wild-type JM109 parent strain (*acrA*$^+$) (solid line). Arrows indicate the phase transition due to the PHB(Ca^{2+}polyP) complexes. Measurements were taken using samples of the same population density and the phase-transition profiles are representative of three separate experiments.

4. Discussion

This study demonstrated that a relationship existed between the presence of the *acrA*$^+$ gene and the ability of *E. coli* to form the membrane associated nonproteinaceous Ca^{2+} channels described by Reusch et al. [18,22,24]. These consist of complexes of the lipidic polymer PHB and inorganic calcium polyP giving rise to temperature-dependent membrane changes, detected by the fluorescent probe NPN.

Previous studies have shown that PHB is found in association with many proteins in *E. coli* and comprises approximately 0.4% of the dry weight of log phase cells [39]. Approximately 15% of this PHB is found in the cytoplasmic membrane, of which 30% (0.02% dry weight) is in association with Ca^{2+}polyP, where it forms voltage-activated calcium channels [18,24,39]. PHB is an amphiphilic, flexible polymer capable of forming hydrophobic or hydrophilic bonds with proteins through alternating methyl groups and carbonyl oxygens along the length of the polymer. Presumably, its amphiphilic nature could also allow the integration of PHB into the membrane but the mechanism of this assembly is unclear. On the basis of molecular modelling, the structure of the PHB–polyP channel complexes has been proposed to consist of the hydrophobic PHB forming a helical polar-lined pore that surrounds and solvates the Ca^{2+}polyP in the bacterial cytoplasmic membrane [24]. It can be envisaged that both polymers are formed simultaneously at the membrane: cytoplasm interface and enter the membrane together, or that PHB forms and then partitions existing polyphosphate molecules into the membrane.

Reusch and Sadoff [23,24], using the NPN fluorescence assay to monitor the thermotropic lipid phase transitions of the bacterial membranes, have established that the presence of the complexes could be detected as a sharp phase transition that occurs at approximately 56 °C. Furthermore, any changes seen in the broader phase transitions at inter-

mediate temperatures, i.e. 24–50 °C correlate with the presence of free PHB in the membranes [23,24]. Both types of transition in the wild-type strains were observed in this current study, indicating the presence of free PHB and PHB(Ca^{2+}polyP) complexes, respectively. Reusch et al. [22] showed previously that stationary phase *E. coli* cells contain higher amounts of the PHB(Ca^{2+}polyP) complexes in the plasma membrane when compared with cells in log-phase and this observation, reflected by changes in NPN fluorescence changes was also confirmed in the current study. Importantly, the *acrA*$^-$ mutants of both N43 and JM109, lacking the AcrA component of the multidrug resistant pump, in contrast to their isogenic *acrA*$^+$ derivatives, possessed extremely low levels of the complexes and free PHB in log phase cells, as indicated with the NPN probe. Nevertheless, *acrA*$^-$ cells of both strains, grown into stationary phase, appeared to accumulate significant amounts of free PHB in the membrane at intermediate temperatures (24–50 °C). However, even in stationary phase cells, only low levels of PHB–polyP complexes were detected. Such findings suggest that although the *acrA*$^-$ cells can still accumulate PHB in the membrane, at least under some conditions, the ability to complex it with polyP is apparently substantially reduced.

The AcrA/B system pumps out a very large range of compounds, and detecting common structural features among the substrates can be difficult [30,40]. Many of the substrates, however, carry a net negative charge and other data suggests that the only requirement for the AcrA/B pump is that the hydrophobic domain of the substrate molecule is inserted into the membrane [31]. A possible, direct explanation for the lack of channel complexes in *acrA* mutants could therefore be that components of the complex are themselves normally transported into or across the inner membrane by the Acr pump, for example, the negatively charged inorganic phosphate or even polyphosphate itself. PHB in contrast, still appears to accumulate in the membrane of stationary phase cells even in the absence of the AcrAB pump.

The results in this study indicate that the multidrug pump encoded by the *acrA/B* genes is required in some way for the biogenesis of the PHB(Ca^{2+}polyP) complexes. Considerable evidence has shown that the exclusion of substances harmful to the bacteria is a major function of the AcrA/B multidrug resistant pump [31]. However, although normally implicated in multidrug transport, an additional physiological role for the AcrAB pump in the transport of some endogenous molecules into the cell envelope such as polyphosphate has not been excluded. Alternatively, if the AcrAB pump, like some of its functionally related multidrug transporter counterparts in eukaryotes [41] normally transports some form of lipid into the cytoplasmic membrane, an altered bilayer in *acrA*$^-$ mutants may simply then prevent the assembly of PHB(Ca^{2+}polyP) complexes. Whether the effect on PHB(Ca^{2+}polyP) complexes is direct or indirect, our findings demonstrate an important new phenotype for *acrA* mutants, which has major physiological implications. Finally, our study also clearly demonstrated that exponentially growing cells of JM109, whether wild type or *acrA*$^-$, were apparently able to regulate their levels of free cytosolic Ca^{2+} quite normally, as determined by aequorin-bioluminescence measurements. This was in contrast to N43 *acrA*$^-$, which poorly regulated the free cytosolic Ca^{2+} in response to high external levels of Ca^{2+}. Unfortunately, the N43 *acrA*$^+$ strain for unknown reasons expressed aequorin very poorly and the comparison of free Ca^{2+} levels under different conditions in N43 *acr*$^-$ and *acr*$^+$ strains could not be undertaken. Nevertheless, the results with JM101 and its isogenic *acrA* mutant showed that the presence of PHB(Ca^{2+}polyP) complexes per se is not essential for tight control of free Ca^{2+} levels in the cytoplasm under these growth conditions. Other unknown mutations in the original N43 *acrA*$^-$ strain, for example affecting Ca^{2+} transport, may be responsible for the observed effects on Ca^{2+} homeostasis.

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