Effects of Ca\textsuperscript{2+} and a Protonophore on Growth of an
Escherichia coli L-Form

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(Received 14 August 1987; revised 22 June 1988)

The influence of Ca\textsuperscript{2+} ions on the growth of an L-form (NC7) derived from *Escherichia coli* K12 was investigated. In a medium containing NaCl as osmotic stabilizer 1 mM-Ca\textsuperscript{2+} was required for optimal growth of the L-form, while with KCl as osmotic stabilizer, in a medium containing 0.1 or 1.0 mM-Ca\textsuperscript{2+}, optimum growth was observed at 32 and 37 °C, respectively. When the L-form, growing exponentially at 32 °C in medium containing KCl and 0.1 mM-Ca\textsuperscript{2+}, was shifted to 37 °C growth was strikingly suppressed. In contrast, the suppression of growth in the presence of 1.0 mM-Ca\textsuperscript{2+} at 32 °C was relieved when the culture was shifted to 37 °C. When the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), at a final concentration of 10 μM, was added to a medium containing NaCl and sucrose as osmotic stabilizers, together with 10 mM-glucose, the parent strain could grow exponentially. In contrast, growth of the L-form was completely stopped by 10 μM-CCCP under the same conditions. In the presence of 20 μM-CCCP, the L-form accumulated more than twice as much \textsuperscript{45}Ca as in the absence of the protonophore. Thus, it is suggested that growth of the L-form NC7 is coupled to the protonmotive force. Possible mechanisms for the coupling of calcium to growth of the L-form are discussed.

INTRODUCTION

Ca\textsuperscript{2+} ions play a very important role as a key regulator in eukaryotic organisms, but there is little information on their role in bacterial growth. It is usually assumed that calcium has few, if any, regulatory functions in bacteria. Roles for calcium seem to be restricted to sporulation (Murrell, 1967), to synthesis of cell wall structures by activation of extracellular enzymes (Kojima *et al.*, 1970; Lewis, 1975), and to the assembly of the cell wall (Silver, 1977). A calcium requirement has been demonstrated in a few species only (Charnetzky & Brubaker, 1982; Rosen, 1982). Previous work from our laboratory described the characteristics of morphology and growth in the L-form NC7 derived from *Escherichia coli* K12 (Onoda *et al.*, 1987): in peptone medium containing NaCl as an osmotic stabilizer, growth of L-form NC7, which has a defective cell wall, was stimulated by addition of 1 mM-Ca\textsuperscript{2+}, while in medium containing KCl, the same concentration of Ca\textsuperscript{2+} was inhibitory for growth. Therefore, it was considered to be important to study the physiological function(s) of Ca\textsuperscript{2+} ions on growth of the bacterial L-form. In this paper, we report the influence of Ca\textsuperscript{2+} on the growth of L-form NC7 and on the susceptibility of growth to the protonophore CCCP.

METHODS

Organisms and growth conditions. The L-form NC7 was derived from *E. coli* K12 strain 3301 as described previously (Onoda *et al.*, 1987). Bacteria were grown at 32 °C without shaking on the following complex media. NaPY medium contained (l-1) 10 g peptone, 5 g yeast extract, 2 g glucose and 0.34 M-NaCl as osmotic stabilizer.

Abbreviation: CCCP, carbonyl cyanide m-chlorophenylhydrazone.
KPY medium was the same as NaPY medium except that KCl was used instead of NaCl. In some experiments, bacteria were grown under the same conditions as above in SM medium containing (1–1) 10 g peptone, 5 g yeast extract, 0.13 m-NaCl, 0.3 m-sucrose and 10 mm-glucose. The pH value was adjusted to 7.2 with NaOH or KOH unless otherwise stated. All cells were harvested in the exponential phase of growth by centrifugation (4000 g, 15 min), washed once with PY medium supplemented with suitable osmotic stabilizer, and inoculated into growth medium. Growth was monitored by measuring OD600 nm (1 cm light path). The initial OD600 was about 0.02. The concentrations of contaminating Na+, K+, and Ca2+ in PY medium were determined using a Hitachi model 170-40 atomic absorption spectrometer. The concentrations of these ions in PY medium were 9.2 mm-Na+, 6.5 mm-K+ and 0.15 mm-Ca2+.

Transport assay. Intact cells (the L-form) were concentrated by centrifugation and suspended in 10 mm-Tris/HCl buffer (pH 7.5) containing 0.34 mM-KCl and 0.2 mM-mannitol. The suspension was centrifuged at 12000 g for 15 min; the pellet was then suspended in a solution containing 10 mM-Tris/HCl buffer (pH 7.5), 0.34 mM-KCl, 0.2 mM-mannitol, 10 mM-glucose and inhibitors as indicated in the Figure legends. The suspension was incubated at 32 °C or 37 °C, and then uptake was initiated by the addition of 44Ca [441 μM; specific activity approximately 493 μCi μmol−1 (18.2 MBq μmol−1)]. At suitable time intervals, samples (100 μl) were filtered through Millipore filters (0.45 μm pore size). Samples on the filter were washed with the same buffer containing 2 mM-MgSO4, dried, and radioactivity was measured by liquid scintillation counting.

Media and reagents. Peptone was purchased from Kyokuto Pharmaceutical Industrial Co. Yeast extract powder was obtained from Oriental Yeast Industrial Co. 44CaCl2 was obtained from Amersham. CCCP was purchased from Sigma; it was prepared as an ethanolic solution (1 mM). Sucrose (RNAase free) was purchased from Nakarai Chemicals. All other reagents were of analytical grade.

RESULTS

Requirement of CaCl2 for growth

Previous results from our laboratory demonstrated that growth of the L-form NC7 in PY medium containing 2% NaCl is calcium-dependent (Onoda et al., 1987). As shown in Table 1, addition of 1 mM-Ca2+ resulted in a marked stimulation of growth when the L-form was cultured in NaPY medium. On the other hand, when KCl replaced NaCl as osmotic stabilizer, the L-form could grow in medium that was not supplemented with Ca2+, and growth was markedly inhibited by addition of 1 mM-CaCl2. Because the PY medium used in this experiment contains about 0.15 mM contaminating Ca2+, external calcium was removed by chelating with 0.2 mM-EGTA. When 0.2 mM-EGTA was added to NaPY medium, growth of the L-form was stimulated by addition of about 1-2 mM-Ca2+ (Table 1). However, when KCl was substituted for NaCl in the presence of 0.2 mM-EGTA, growth was only observed on addition of Ca2+ at a concentration of 0.2 mM. The parent strain grew very well in NaPY or KPY medium, independent of the presence of calcium (Table 1). In addition, we investigated the effects of Ca2+ on growth in KPY medium (Fig. 1). The L-form was incubated in medium with or without

Table 1. Effects of EGTA and Ca2+ ions on growth of the parent strain and the L-form

<table>
<thead>
<tr>
<th>Addition(s) to medium</th>
<th>NaPY</th>
<th>KPY</th>
<th>NaPY</th>
<th>KPY</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.60</td>
<td>0.60</td>
<td>0.03</td>
<td>0.37</td>
</tr>
<tr>
<td>CaCl2 (1-0 mM)</td>
<td>0.60</td>
<td>0.59</td>
<td>0.38</td>
<td>0.03</td>
</tr>
<tr>
<td>EGTA (0-2 mM)</td>
<td>0.61</td>
<td>0.61</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>EGTA (0-2 mM) + CaCl2 (0-2 mM)</td>
<td>0.61</td>
<td>0.03</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>EGTA (0-2 mM) + CaCl2 (1-2 mM)</td>
<td>0.60</td>
<td>0.39</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>EGTA (1-0 mM)</td>
<td>0.60</td>
<td>0.60</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>EGTA (1-0 mM) + CaCl2 (1-0 mM)</td>
<td>0.59</td>
<td>0.02</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>EGTA (1-0 mM) + CaCl2 (2-0 mM)</td>
<td>0.58</td>
<td>0.57</td>
<td>0.40</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Fig. 1. Effect of Ca\textsuperscript{2+} concentration on growth of the L-form. Cells were grown at 32°C without shaking in PY medium containing 0.34 M (about 2%) KCl and added Ca\textsuperscript{2+} at various concentrations. Half the cultures were treated with 0.2 mM-EGTA. The OD\textsubscript{600} was measured after 48 h incubation. ●, No EGTA; ○, 0.2 mM-EGTA.

Fig. 2. Temperature-dependence of Ca\textsuperscript{2+} concentration required for growth. The L-form was grown in the temperature range 25 to 40°C without shaking. EGTA was added to all cultures at a final concentration of 0.2 mM. (a) L-form grown in NaPY medium containing 0.1 mM-Ca\textsuperscript{2+} (○) or 1.0 mM-Ca\textsuperscript{2+} (●). (b) L-form grown in KPY medium containing 0.1 mM-Ca\textsuperscript{2+} (○) or 1.0 mM-Ca\textsuperscript{2+} (●). OD\textsubscript{600} was determined after 24 h incubation. These growth experiments were done using a temperature gradient incubator (model TN3, Toyo Kagaku Sangyo Co.). Data are from one representative experiment and are means of at least three determinations.

0.2 mM-EGTA and with various concentrations of CaCl\textsubscript{2} added to the medium. In the medium with EGTA, growth was stimulated by increasing concentrations of CaCl\textsubscript{2} up to 0.1 mM, but Ca\textsuperscript{2+} concentrations in excess of 0.2 mM were inhibitory. In the absence of EGTA, growth decreased with increasing concentrations of CaCl\textsubscript{2}.

Effects of Ca\textsuperscript{2+} on growth at various incubation temperatures

During our study, we found that the stimulatory effect of Ca\textsuperscript{2+} on growth was temperature dependent. The effects of Ca\textsuperscript{2+} on growth were compared within the temperature range 25 to 40°C. A typical result is shown in Fig. 2. The optimal temperature for growth in NaPY medium with Ca\textsuperscript{2+} (1.0 mM) was about 32 to 33°C (Fig. 2a), while in the presence of Ca\textsuperscript{2+} (0.1 mM) no growth was observed throughout the temperature range 25 to 40°C. On the other hand, when the L-form was grown in KPY medium containing 0.1 or 1.0 mM-Ca\textsuperscript{2+}, optimum growth was observed at 32 and 37°C, respectively. As shown in Fig. 2(b), when cells were incubated in the presence of Ca\textsuperscript{2+} at a final concentration of 0.1 mM, growth was markedly affected by temperature with an optimum at about 32°C. At higher temperatures inhibition of growth was observed. In contrast, when Ca\textsuperscript{2+} was added to a final concentration of 1.0 mM, the effect on growth was maximal between 36 and 37°C, with little growth below 32°C or above 39°C.
When the L-form growing exponentially in KPY medium containing 0.1 mM-Ca\(^{2+}\) at 32°C was shifted to 37°C, growth was strikingly suppressed (Fig. 3a). In contrast to 0.1 mM-Ca\(^{2+}\), which stimulated growth at 32°C, 1.0 mM-Ca\(^{2+}\) has a profound inhibitory effect at 32°C, while the inhibition of growth induced by 1.0 mM-Ca\(^{2+}\) was reversed when the culture was shifted to 37°C. In parallel, the opposite process was also examined. When a culture was grown at 37°C in the presence of 1.0 mM-Ca\(^{2+}\) and shifted to 32°C, an abrupt cessation of growth was observed. As shown in Fig. 3(b) growth, which ceased in the presence of 0.1 mM-Ca\(^{2+}\) at 37°C, markedly increased after a shift from 37 to 32°C.

Calcium uptake by intact cells

\(^{45}\)Ca uptake by L-form cells is shown in Fig. 4. In the presence of 20 μM-CCCP, the cells accumulated more than twice as much \(^{45}\)Ca as in the absence of the uncoupler. The initial rate of \(^{45}\)Ca uptake was about the same in the presence or absence of uncoupler, while the rate of \(^{45}\)Ca uptake was more rapid at 37°C than at 32°C.
Ca\textsuperscript{2+}, CCCP and growth of an \textit{E. coli} L-form

Fig. 5. Effect of CCCP on growth of the parent and L-form in SM medium. Both strains were grown without shaking at 32 °C. (a) L-form grown in SM medium (pH 7.5) containing 30 mM-glucose and 1 mM-Ca\textsuperscript{2+}. (b) Parent strain grown in SM medium (pH 7.5) containing 30 mM-glucose. ○, 10 μM-CCCP added at the point indicated by the arrow; ●, control cultures (only ethanol added).

Fig. 6. Effect of CCCP on growth of the parent and L-form in NaPY medium and KPY medium. Both strains were grown without shaking at 32 °C. (a) L-form grown in NaPY medium (pH 3.5) containing 30 mM-glucose and 1 mM-Ca\textsuperscript{2+} (●, ○) or KPY medium containing 30 mM-glucose (△, △). (b) Parent strain grown in NaPY medium (pH 7.5) containing 30 mM-glucose. Open symbols, CCCP added as in Fig. 5; closed symbols, control cultures.

Effect of CCCP on growth of parent and L-form strains

It has been suggested that \textit{E. coli} can grow in the absence of a protonmotive force when glucose is used as an energy source at pH 7.5 (Kinoshita \textit{et al.}, 1984). We examined whether the L-form can grow in the presence of a protonophore in medium containing glucose at pH 7.5. To evaluate the effects of protonmotive force on growth of the L-form, the parent and L-form strains were grown in SM medium containing 1 mM-CaCl\textsubscript{2}, and effects of the protonophore were compared (Fig. 5). The parent strain was able to grow in the presence of 10 μM-CCCP (Fig. 5b), while the L-form did not grow under the same conditions (Fig. 5a). In addition, when CCCP at a final concentration of 10 μM was added to NaPY (or KPY) medium containing CaCl\textsubscript{2} and glucose as energy source, growth of the L-form was completely inhibited (Fig. 6a). On the other hand, when parent cells were cultured under the same conditions as above, growth was also completely inhibited by CCCP (Fig. 6b). Thus, we suggest that although \textit{E. coli} K12 is able to
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grown in the absence of a protonmotive force even when glucose is used as an energy source at pH 7.5, a protonmotive force is obligatory for growth of the L-form.

DISCUSSION
In this paper, we have shown that Ca\(^{2+}\) is required for the growth of the *E. coli* L-form NC7 in growth medium containing NaCl or KCl as osmotic stabilizer. The parent strain could maintain balanced growth in these media over a temperature range from 25 to 45 °C, independent of addition of Ca\(^{2+}\) (data not shown). In a previous paper (Onoda *et al.*, 1987) we reported that when sucrose was used as an osmotic stabilizer, no growth of L-form NC7 was observed even in the presence of Ca\(^{2+}\). These facts suggest that salts such as Na\(^+\) or K\(^+\) at high concentration and in combination with Ca\(^{2+}\) are necessary for growth of the L-form as well as having a protective role. In this study, temperature shifts of only a few degrees resulted in marked changes in the rates of growth. When the growth temperature of the L-form was abruptly increased from 32 to 37 °C, growth stopped; in contrast, the suppression of growth by 1.0 mM-Ca\(^{2+}\) at 32 °C was relieved by a temperature shift to 37 °C. These results suggest that L-form NC7 may have two different transport systems for Ca\(^{2+}\) which are dependent on the incubation temperature.

A high internal potassium to sodium ratio is characteristic of metabolizing prokaryotes as well as eukaryotes. In many bacterial cells, Na\(^+\) is extruded via the Na\(^+-\)H\(^+\) antiporter (West & Mitchell, 1974; Krulwich, 1983), and the driving force for Na\(^+\) extrusion is the protonmotive force in this system. Kinoshita *et al.* (1984) demonstrated that *E. coli* can grow in the presence of CCCP when glucose is used as an energy source at about pH 7.5. In this study, the same conclusion was reached from our experiments with glycolysing *E. coli* cells. Further we found that the L-form required a protonmotive force for growth, and when parent cells were transferred to a Na\(^+\) (or K\(^+\))-rich environment, growth was inhibited by 10 μM-CCCP at the same pH value, indicating that in this case, a protonmotive force is required for growth. The Na\(^+-\)H\(^+\) antiporter may play an essential role in the removal of Na\(^+\) from cytoplasm and for the regulation of intracellular pH.

A general characteristic of bacterial intracellular Ca\(^{2+}\) ions is that the concentration remains low during growth. It has been shown that calcium is transported into everted membrane vesicles of *E. coli* by a secondary transport system which utilizes the protonmotive force established by either of the two primary proton pumps: the respiratory chain or the H\(^+-\)ATPase (Rosen & McClees, 1974; Tsuchiya & Rosen, 1975; Brey & Rosen, 1979). Tsujibo & Rosen (1983) reported that calcium efflux from intact cells of *E. coli* is coupled to the protonmotive force via secondary calcium–proton exchange. Silver *et al.* (1975) reported that non-energy-dependent uptake of \(^{45}\)Ca by exponential-phase cells of *E. coli* occurs under two conditions: at 0 °C or in the presence of CCCP. In this paper, we investigated the uptake of \(^{45}\)Ca in relation to the presence of CCCP and as a function of the incubation temperature. We found that accumulation of \(^{45}\)Ca was greater in the presence of 20 μM-CCCP than in the absence of uncoupler at 32 °C as well as 37 °C. Our results are similar in this respect to those of Silver *et al.* (1975). As a possible explanation for increased accumulation of calcium in the presence of 20 μM-CCCP, we propose that the effect of the uncoupler is to stop the active calcium efflux coupling to the protonmotive force. A further possibility that cannot be ruled out is that accumulation of calcium results from binding to the outside of the cell.

Chang *et al.* (1986) reported that the calcium content was high in *E. coli* envelopes and that cytoplasmic Ca\(^{2+}\) was increased in dividing cells. Unlike other divalent cations, Ca\(^{2+}\) is actively exported from growing bacteria (Silver, 1975) and therefore is found normally in only trace levels, presumably within the cell envelope (Beveridge, 1976). Ordal (1977) reported that Ca\(^{2+}\) is required for the chemotactic response of *Bacillus subtilis*. However, little correlation with the function of Ca\(^{2+}\) in other metabolic systems is evident. We suggest that calcium may play an essential role in the growth of the L-form. Recently, we isolated a variant, Cig-1 (calcium-independent growth), from the L-form NC7. Cig-1 was able to grow regardless of the presence of Ca\(^{2+}\). Comparative studies of the L-form NC7 and Cig-1 strains will provide an approach toward understanding the physiological and biochemical functions of calcium on growth.
Furthermore, it will be interesting to determine whether calcium affects the rigidity of the cell membrane or affects other transport systems and whether or not intracellular calcium plays an initiating or regulatory role in bacterial cell division. In any case, further studies are essential.

We wish to thank Shiori Kawakami for her technical assistance during this work.

REFERENCES


