

## Change in Ca-Requirement on Growth of a Variant, Cig-1, derived from a Stable L-Form of *Escherichia coli* K-12

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A variant, Cig-1 (calcium independent growth) was selected from L-form NC7 culture belonging to the genus *Escherichia coli* K-12. The Cig-1 differed from the parental L-form NC7 strain in growth response to calcium content. Growth of the L-form NC7 was markedly inhibited by addition of EGTA (1 mM), while Cig-1 was able to grow in the presence of EGTA. When the Cig-1 was transferred to growth medium containing 10  $\mu$ M Ca ionophore, A 23187, the cells did not grow, but *E. coli* cells were able to grow under the same conditions. When 10  $\mu$ M of carbonyl cyanide-m-chlorophenylhydrazone (CCCP), a protonophore, was added to growth medium containing glucose at pH 7.5, growth of the Cig-1 was completely blocked, suggesting that proton motive force is required for growth of the Cig-1 strain.

### I. Introduction

The transformation of bacteria into L-forms are accompanied by various morphological, metabolic and antigenic changes (Hijimans, 1962; Reusch and Panos, 1976; Hayami *et al.*, 1979; Das and Banerjee, 1985). Most stable L-forms require the addition of osmotic stabilizers to the culture medium and salts, sugars or serum components are commonly used. Salts used as osmotic stabilizer affect several physiological processes (Linker & Wilson, 1985). The growth of many bacteria, generally, is inhibited in media of high osmolarity. Walter *et al.*, (1987) reported that growth of *Clostridium pasteurianum* was inhibited in media of high solute (KCl and xylitol) content, and glycolysis by the cells was inhibited by these solutes in parallel with growth. Recently, we described isolation of L-form NC7 from *Escherichia coli* K-12, and its biological characters (Onoda, 1968; Onoda *et al.*, 1987). It was noticed that growth response of this L-form NC7 differed markedly among osmotic stabilizers. A stimulated growth of the L-form was observed when calcium was added to peptone (P) medium containing 0.34 M NaCl. Aranha *et al.*, (1987) demonstrated that the steady-state growth yield of *Streptococcus mutans* was doubled by addition of 0.63  $\mu$ M calcium, and subsequent increase in calcium concentration to 1.3  $\mu$ M and 2.5  $\mu$ M lowered steady-state growth yield below the level with no added calcium, suggesting that calcium has dose-dependent stimulatory and inhibitory effects on *S. mutans*. However, there is little information on the effects of calcium on bacterial growth. During our investigations on several characters of the L-form NC7, a variant, Cig-1 (calcium-independent growth) which is

able to grow even in P-NaCl 0.34 M medium without added calcium was isolated from the L-form NC7 culture. The comparative studies of the L-form NC7 and Cig-1 L-form provide a method of approach toward understanding the physiological and biochemical functions of calcium on growth. This paper describes the growth, morphology, and some physiological feature of Cig-1 L-form.

### Materials and Methods

#### *Bacterial strains and growth conditions*

*Escherichia coli* K-12, stable L-form NC7 derived from *E. coli* K-12, and Cig-1 (L-form) were used in this experiment. Cig-1 was isolated from culture of the L-form NC7 grown frequently in BHI (Brain Heart Infusion Broth) medium supplemented with 0.34 M NaCl without added calcium during prolong incubation (3–5 days). Bacteria were grown at 32°C without shaking on the following complex media. Medium P-NaCl 0.34 M contained (per liter) 10 g of peptone, 5 g of yeast extract, 2 g of glucose and 0.34 M NaCl. Medium P-KCl 0.34 M was the same as medium P-NaCl 0.34 M, except that KCl was used instead of NaCl. The pH value was adjusted to 7.2 with NaOH or KOH. The parental strain was maintained in BHI medium in some experiments, the bacteria were grown in BHI medium with or without osmotic stabilizer under the same conditions as above. All cells were harvested in the exponential phase of growth by centrifugation (4000 g×15 min), washed once with P medium supplemented with suitable osmotic stabilizer, and inoculated into growth medium. Growth was monitored by measuring by optical density at 600 nm (1 cm path length). The initial OD<sub>600</sub> was about 0.02 unless otherwise stated.

#### *Materials*

Peptone and BHI were purchased from Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan. Yeast extract Powder was obtained from Oriental Yeast Industrial Co., Tokyo, Japan. Carbonyl cyanide m-chlorophenylhydrazone, CCCD were purchased from Sigma Chemical Co., St. Louis, USA. Ionophore, A 23187, was from Boehringer Mannheim GmbH, Germany. All other reagents used were analytical grade.

### Results

#### *Effect of calcium on growth*

In P medium supplemented with NaCl and calcium, the L-form NC7 grew exponentially with a generation time of 5–6 h, and the OD<sub>600</sub> reached to a maximum within 2 days. No growth was observed in the absence of added Ca<sup>2+</sup> during culture for 2 days. In addition, to examine effect of calcium on growth, 1 mM calcium was added to P medium at an interval and OD<sub>600</sub> was monitored. Fig. 1 shows that the cell growth was resumed by addition of calcium. On the other hand, to see whether cell growth was

suppressed when calcium was removed, EGTA as chelating agent was added to the P medium containing 1 mM  $\text{Ca}^+$ . A final concentration of added EGTA was 1.2 mM, because concentration of contaminating  $\text{Ca}^+$  in P medium was found to be about 0.15 mM  $\text{Ca}^+$ . The L-form in the culture with calcium continued to grow, but the growth ceased immediately when EGTA was added to the culture (data not shown). Thus, these results show that the inhibition of the cell division by calcium-deficient medium was reversible.

Furthermore, the L-form NC7 strain was cultured in P-NaCl 0.34 M medium without calcium, and then a variant, Cig-1, was selected from its cultures. In this experiment, we examined the physiological characters of Cig-1 cells. Fig. 2 indicated that growth pattern of the Cig-1 strain was similar to those of parental type in P-NaCl 0.34 M medium with or without calcium.

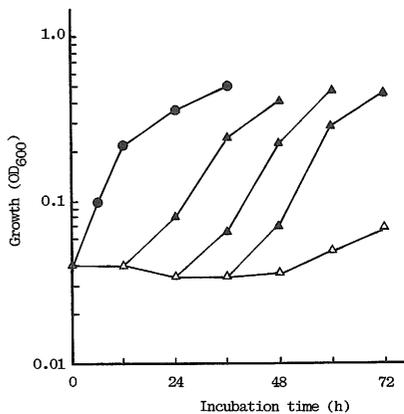


Fig. 1. Recovery of growth of the L-form NC7 on adding calcium. Cells were incubated in P medium containing 0.34 M NaCl at 32°C without shaking. Calcium (1 mM) was added from the start of the experiment (●). Culture was incubated in P medium without added calcium (△), 1 mM calcium were added to cultures at indicated intervals (▲). The  $\text{OD}_{600}$  were measured. Data are from one representative experiment.

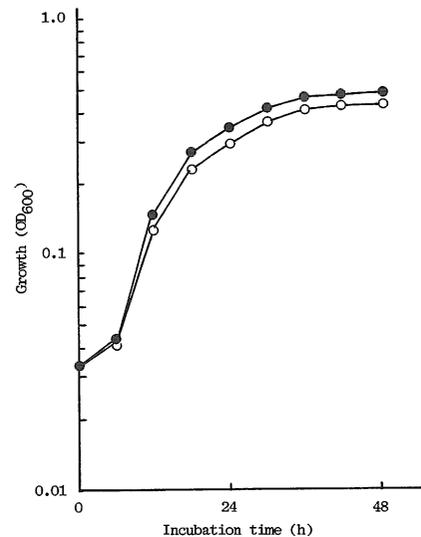


Fig. 2. Growth of Cig-1 strain in P-NaCl 0.34 M medium with or without added calcium. Cig-1 strain which was maintained in P-NaCl 0.34 M medium without calcium was sequentially transferred into the medium with calcium (1 mM) 15 times at 3 day intervals, and then were inoculated in the medium with added calcium (○) or without (●). Growth was monitored by assessing the increase optical density at 600 nm.

#### *Effects of calcium and EGTA concentrations on growth*

Fig. 3 showed the effects of various concentrations of  $\text{Ca}^+$  on growth of the L-form NC7 and Cig-1 strains. The optimum concentration of  $\text{Ca}^+$  for growth of the L-form

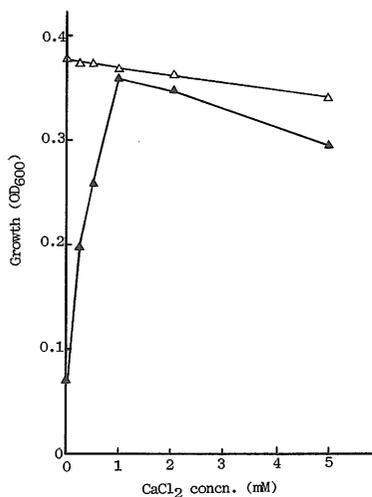


Fig. 3. Effects of calcium concentrations on growth of the Cig-1 and L-form NC7. Both the cells were grown for 48 h in P-NaCl 0.34 M medium and various concentrations of calcium. Symbols:  $\triangle$ , Cig-1;  $\blacktriangle$ , L-form NC7.

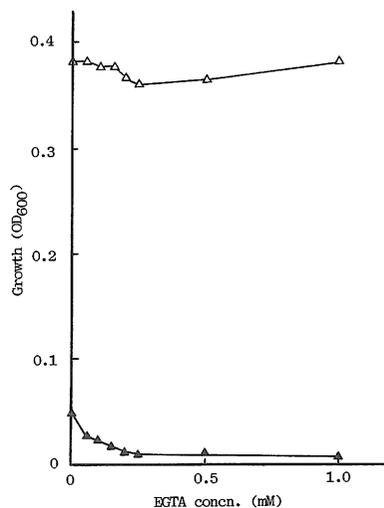


Fig. 4. Effects of EGTA concentration on growth of Cig-1 and L-form NC7 strains. Both the cells were inoculated in P-NaCl 0.34 M medium at 32°C. Various concentrations of EGTA were added to the cell suspension and the OD<sub>600</sub> was measured at 48 h after inoculation. Symbols:  $\triangle$ , Cig-1;  $\blacktriangle$ , L-form NC7.

NC7 laid between 1 and 2 mM, whereas the OD<sub>600</sub> of the Cig-1 culture remained almost over a range of calcium concentrations from 0 to 5 mM.

Further, in order to see whether calcium is required for growth of Cig-1 strain, EGTA at various concentrations were added to the culture of each strain, respectively. Fig. 4 indicated that growth of the L-form NC7 was markedly inhibited by addition of EGTA up to 1.0 mM, while that of the Cig-1 was not blocked by EGTA at same concentration.

#### *Morphology and sensitivity to SDS*

Phase-contrast micrographs of cultures of the Cig-1 were shown in Fig. 5. In liquid P medium supplemented with 0.34 M NaCl, the L-form culture consisted mainly of spherical bodies of varying sizes. On the other hand, the Cig-1 showed marked morphological heterogeneity, including not only spherical bodies but bizarre forms and irregular shape.

In addition, the parental strain was highly sensitive to penicillin, while the Cig-1 was resistant to more than 500 units/ml of penicillin, like as the L-form NC7. Addition of distilled water or sodium dodecyl sulfate, SDS, (0.02%) resulted in rapid lysis of the

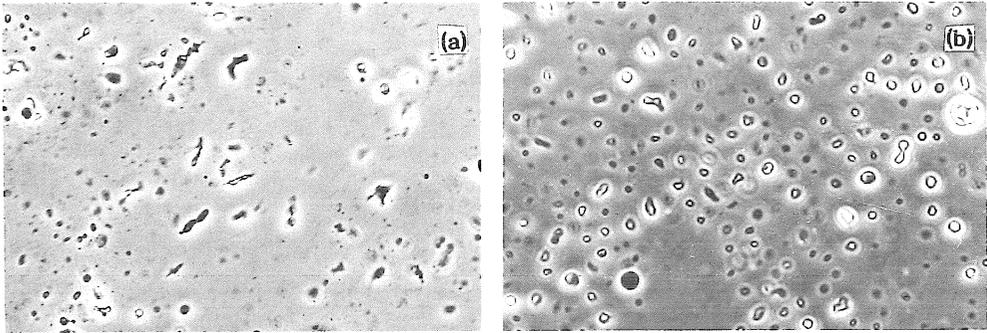


Fig. 5. Phase-contrast micrographs of the Cig-1 and L-form NC7 strains, all incubated at 32°C without shaking. (a) The Cig-1 strain and (b) the L-form NC7 strain (24 h after incubation).

L-form, but had no effect on the parent strain. When SDS (0.02%) was added to the Cig-1 culture grown in P medium containing 0.34 mM NaCl, the  $OD_{600}$  of the culture markedly decreased and the cell lysis was clearly observed by phase-contrast microscopy.

#### *Effect of CCCP and A 23187 on growth*

We examined the effects of a protonophore, CCCP, on growth. When 10  $\mu$ M CCCP was added to P-NaCl 0.34 M medium containing 1 mM calcium and 30 mM glucose as energy source, the growth of Cig-1 was completely blocked by the protonophore, in similar manner as that of the L-form NC7 (data not shown), suggesting that the proton motive force is obligatory for the growth of the Cig-1 and L-form NC7.

Table 1. Effects of Ca ionophore on growth of parent, Cig-1, and L-form NC7.

Medium supplemented with	Growth ( $OD_{600}$ )					
	Parent		L-form		Cig-1	
	NaCl	KCl	NaCl	KCl	NaCl	KCl
None	0.60	0.61	0.05	0.36	0.40	9.36
CaCl <sub>2</sub> (1 mM)	0.59	0.60	0.38	0.06	0.38	0.35
A 23187 (5 $\mu$ M)	0.60	0.59	0.02	0.04	0.04	0.04
CaCl <sub>2</sub> (1 mM) + A 23187 (5 $\mu$ M)	0.60	0.60	0.03	0.02	0.02	0.02
A 23187 (10 $\mu$ M)	0.60	0.61	0.02	0.02	0.02	0.02
CaCl <sub>2</sub> (1 mM) + A 23187 (10 $\mu$ M)	0.59	0.58	0.02	0.02	0.02	0.02

All the cells were grown in P medium containing NaCl- or KCl-0.34 M, as osmotic stabilizer, and then Ca<sup>2+</sup> ionophore, A 23187 alone or combination with A 23187 and calcium was added to each the culture at indicated concentrations. The  $OD_{600}$  was measured after 18 h incubation for the parent strain and 48 h for Cig-1 and L-form NC7. The ionophore was added as an dimethyl sulfoxide solution, and all assays were adjusted to the same final concentration of dimethyl sulfoxide. The values are means from three separate experiments.

Effect of Ca ionophore, A 23187, was also examined. It is known that the ionophore functions as a  $\text{Ca-H}^+$  exchange system. Table 1 showed that growth of the parent strain was not inhibited by A 23187, but strong inhibition on growth of the Cig-1 and L-form NC7 were observed by addition of this ionophore.

### Discussion

L-forms are considered to be fragile and to require osmotic stabilization for growth. L-form NC7 derived from *E. coli* K-12 has been subcultured in BHI medium containing 0.34 M NaCl and 1 mM calcium (Onoda *et al.*, 1987). Optimal growth of the L-form was obtained when 1 mM calcium was added to peptone medium supplemented with 0.34 M NaCl. However, no growth occurred unless calcium was added to the medium. We also isolated Cig-1 (L-form) from culture of L-form NC7. Unlike the L-form NC7, Cig-1 cells were able to grow in P-NaCl 0.34 M medium even without added calcium. In addition, the remarkable resistance to penicillin in the Cig-1 indicated a change in the cell wall, as found in most of bacterial L-forms (Ward, 1975). The Cig-1 was sensitive to sodium dodecyl sulfate (0.02%). Addition of distilled water or SDS (0.02%) resulted in rapid lysis of the Cig-1 cells. These results suggest that Cig-1 does not contain rigid peptidoglycan layer on its surface but do not rule out the possibility of more osmotically protective polymers within or exterior to the cytoplasmic membrane than the L-form NC7.

The study of  $\text{Ca}^+$  transport systems in bacterial cells has shown that  $\text{Ca}^+/\text{H}^+$  and  $\text{Ca}^+/\text{Na}^+$  antiporters are responsible for extruding  $\text{Ca}^+$  (Brey *et al.*, 1978; Brey and Rosen, 1979). Tsujibo and Rosen (1983) reported that calcium efflux in intact cells of *E. coli* is coupled to the proton motive force via secondary calcium-proton exchange. In our study, when 10  $\mu\text{M}$  CCCP was added to P-NaCl 0.34 M medium containing glucose at pH 7.5, no growth of the Cig-1 was observed, suggesting that the proton motive force is obligatory for the growth of the Cig-1 cells. In this paper, effects of calcium ionophore, A 23187, on growth was examined. Growth of the parental cells was not affected by addition of 5 to 10  $\mu\text{M}$  the ionophore, A 23187, while growth of the Cig-1 cells was strongly inhibited by same concentration of the ionophore, A 23187, suggesting that calcium may play a important role on ion transport systems in membrane of the Cig-1. Experiments to study this are now in progress.

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