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Isolation and its characters of *Escherichia coli* K–12, L-form

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Suspension of *Escherichia coli* K–12 was sequentially treated with N-methyl-N-nitro-N-nitrosoguanidine and lysozyme,and its L-forms were selected on brain heart infusion (BHI) agar medium. Of those clones,L-form NC7 was adapted to grow in a liquid cultue with osmotic stabilizer. The L-form NC7 was stable in penicillin-free medium. Morphology of this L-form NC7 agreed with that described for other bacterial L-forms in many respects. The L-form NC7 multiplied in soft agar (0.5%, W/V), but did not in hard agar (1.5%, W/V). In osmotic stabilizers tested,NaCl was most effective in the range of 2 to 3% (W/V). In BHI medium containing NaCl $CaCl_2$ was required for growth of the L-form NC7. Studies of the growth characteristics for L-form NC7 gave the following results:an optimal temperature of 32°C, an optimal pH of 7 to 8,and no requirement for serum. This L-form NC7 also retained many of the physiological characters of the parent strain.

Introduction

Since discovery of L-forms of *Streptobacillus moniliformis* by Klieneberger (1935), a number of bacterial L-forms have been isolated. However,there is a paucity of information on structure and functions of plasma membranes of L-forms. Stable bacterial L-forms which can grow with loss of cell walls are expected to differ largely from their parental bacteria in the structure and function of their cytoplasmic membranes. In L-forms the cytoplasmic membranes are the only barrier of the cells towards its environment. Most stable L-forms are osmotically fragile and usually require osmotic stabilizer in the growth medium. Bacterial L-forms have to maintain cell integrity and regulation of transport proccess in order to carry out all essential metabolic proccess. The study of L-forms provides a method of approach toward understanding the physiological and biochemical functions of bacterial cytoplasmic membranes. Specisl attention was paied to the physiological role of osmotic stabilizers against bacterial L-form.

In this study,L-form NC7 derived from *E. coli* K–12 was adapted to grow in liquid medium with an osmotic stabilizer. Growth response of this L-form differed markedly among osmotic stabilizers. This report describe the morphology,growth and some physiological features of this L-form.

Matrials and Methods

Strains

Strains used included *Escherichia coli* K–12 and its stable L-form NC7.

Induction of L-forms

The *E. coli* K–12 was grown for 3 hr on a rotary shaker (250 rev/min) in nutrient broth at 37°C. The cells were centrifuged,washed three times with distilled water and suspended in the same medium containing 50 μg of N-methyl-N′-nitro-N-nitrosoguanidine per ml. The suspension was incubated for 30 min at 37°C. The cells were harvested,washed once with brain heart infulsion (BHI) broth containing 0.5 M sucrose,and suspended in 15 ml of the same medium containing lysozyme (200 μg/ml) and Na-EDTA(0.05%). After 60 min at 30°C,the protoplasts were plated on BHI agar medium containing 0.5 M sucrose, 1% horse serum, and 750 units of penicillin G per ml. The surviving L-colonies were subcultured by serial transfers on BHI agar medium containing 1.0% horse serum and 750 units of penicillin G per ml. After 4 months of serial passage at a suitable intervals,the horse serum was omitted.

Adaptation in liquid medium

Well-developed L-form colonies were used for adaptation to liquid medium. Agar blockes containing L-form colonies were inoculated into BHI medium supplemented with 3% NaCl as osmotic stabilizer, 5% horse serum, 0.5% yeast extract, 1 mM $CaCl_2$, and 100 units of penicillin G. They were incubated without saking at 32°C. Thereafter, the horse serum was omitted. Under these conditions,the L-form grew as separate cells without visible evidence of lysis and revertant.

Growth conditions

The L-form NC7 was grown without shaking in BHI medium containing 2.5% NaCl, 1 mM $CaCl_2$, 0.5% yeast extract,and 100 units of penicillin G at 32°C. As an osmotic stabilizer,sucrose(0.4 M), and $MgCl_2$ (2.5%) except NaCl also was used in this study. Peptone (P) medium contained (per liter) 10 g of peptone,5 g of yeast extract, and 2 g of glucose. The pH was adjusted to 7.2 with NaOH or KOH. The parent strain was grown in BHI medium. All the bacterial strains were harvested in the logarithmic phase of growth by centrifugation (4000 g$\times$15 min), washed once with BHI or P medium containing suitable osmotic stabilizer,and inoculated to growth medium. The initial cell density (A_{600}) was about 0.02 cm^{-1}. The suspensions then were incubated at 32°C without shaking. Growth was monitored by optical density at 600 nm(1-cm path length).

Chemicals

Brain heart infusion broth and peptone were obtained from Kyokuto Phar-

maceutical Industrial Co.,Tokyo. Yeast extract powder was obtained from Oriental Yeast Industrial Co.,Tokyo. Sucrose (Specially prepared reagent) was purchased from NaKarai Chemicals,LTD Japan. All other reagents used were of analytical grade.

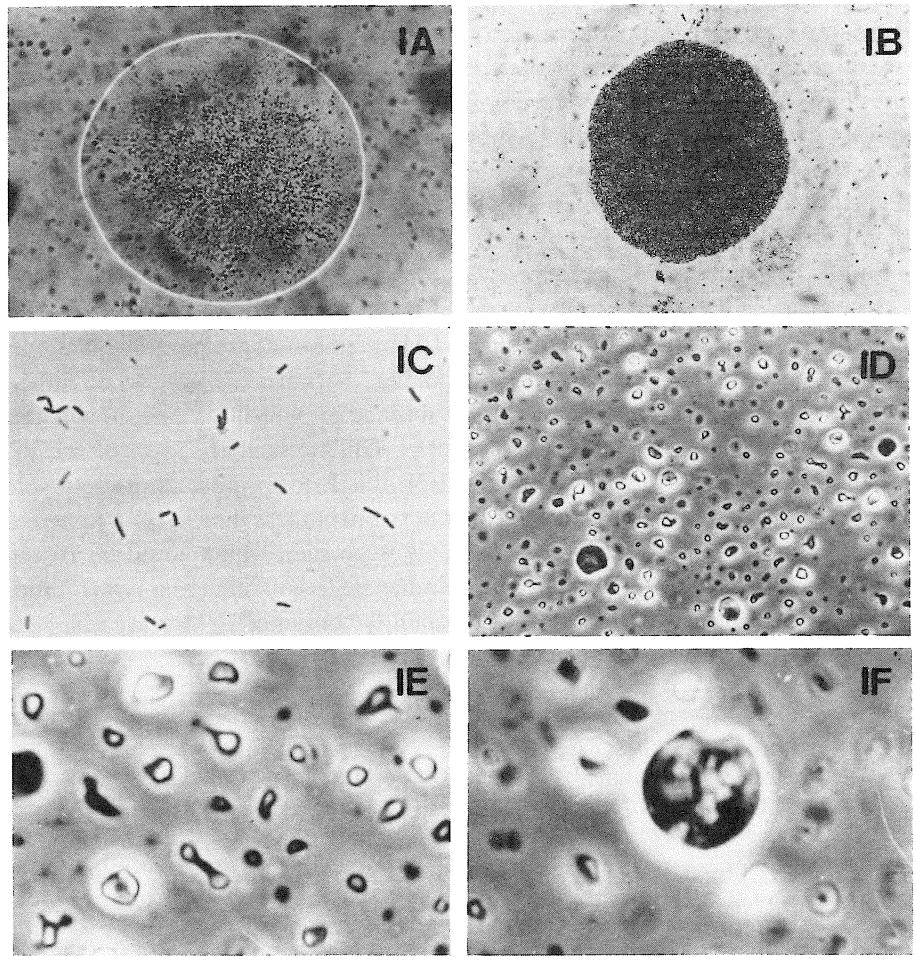

Fig. 1A. L-form colony of E. coli K–12 propagated on BHI-medium with 1.0% agar. This picture was taken with light microscopy.

Fig. 1B. L-form colony growing in BHI-medium with 0.4% agar. The BHI-medium contained 2% NaCl and 1 mM $CaCl_2$, respectively. Light microscopy.

Fig. 1C. Phase micrography of the parent strain growing in BHI-medium at 32°C without shaking.

Fig. 1D. Spherical L-form growing in BHI-medium with 2% NaCl and 1 mM $CaCl_2$ at 32°C without shaking. Phase micrography.

Fig. 1E. L-form cells engaged in a type of budding process and fission. Phase micrography.

Fig. 1F. A large body of the L-form growing in BHI-medium with 2% NaCl and 1 mM $CaCl_2$. Large body contained lighter areas suggestive of vacuoles. Phase micrography.

Results

Morphology of L-form NC7

Typical fried egg L-form colonies,consisting of a granular central core surrounded by a light periphery,were observed on BHI agar medium (Fig. 1A). The colonies are convex and mucoid on account of aboundant production of extracellular slime. On the other hand,one of the L-form colonies appearing in soft agar (0.4%) medium was shown in Fig. 1B. The colonies were composed of more large bodies and granulars. To extend the growth-supportive capacity of agar medium,different concentration of agar were added to the BHI medium. The effect of varing the agar concentration on colony formation was shown in Table 1. Variation in agar concentration produced quantitative differences in the colony count of the L-form. It was found that agar concentration greater than 0.4% were totally inhibitory for the L-form growth. In addition,morphology of the L-form in liquid medium also were examined. The L-form was inoculated in BHI medium containing 3% NaCl and 1 mM $CaCl_2$ and cultured at 32°C without shaking. Serum was found to be unnecessary for growth. This strain is stable in the absence of penicillin. The morphology of the L-form was studied by phase microscopy. Unlike bacillary form of the parent strain under same growth condition (Fig. 1C), this L-form grew as discrete spheres (Fig. 1D). The spheres varied considerably in size,from less than 0.1 μm up to ca. 3.0 μm in diameter. Many morphological forms were seen which could be related to processes of reproduction; (a) binary or asymmetric fission and (b) a type of budding (Fig. 1E). In addition,the large bodies frequently contained lighter areas suggestive of vacuoles (Fig. 1F).

Table 1. Effects of agar conc. on colony formation of the L-form.

Concentration of agar (%)	CFU/dish x 10^{-3}
0.4	3100
1.0	490
1.5	5

L-form NC7 was harvested at logarithmic phase, and the L-form suspension in hypertonic medium (2.0% NaCl and 1 mM $CaCl_2$), containing 0.4 to 1.5% agar, was layered onto the surface of a 1.5% agar base layer (containing hypertonic medium) in petri dishes. The plates were incubated at 32 °C. At 4 days after incubation, the colonies developmented were counted.
CFU; colony forming unit.

Table 2. Effects of osmotic stabilizers on the L-form growth.

Osmotic stabilizer	Growth (A_{600})	
	$-CaCl_2$	$+CaCl_2$
None	0.01	0.01
Sucrose (0.4 M)	0.27	0.07
NaCl (2.5%)	0.02	0.41
$MgCl_2$ (2.5%)	0.26	0.12

The cells were incubated to BHI medium with or without $CaCl_2$, respectively. As osmotic stabilizer, 0.4 M sucrose, 2.5% NaCl and 2.5% $MgCl_2$ were added to the medium, respectively and incubated at 32 °C without shaking. At 48 hr after incubation, the A_{600} was measured.

Growth

The effect of stabilizing substances on growth of the L-form was show in Fig. 2. Sucrose,$MgCl_2$ and NaCl were used as osmotic stabilizers. These stabilizing substances supported the L-form growth. The L-form reached maximum growth at 48 hr in the medium with NaCl plus $CaCl_2$. Growth of the L-form cultured in BHI medium with $MgCl_2$ as osmotic stabilizer were lower than that cultured in the medium containing NaCl plus $CaCl_2$. Addition of $CaCl_2$ to the medium with $MgCl_2$ as osmotic stabilizer prevented significantly the L-form growth. On the other hand,when NaCl was used as osmotic stabilizer,addition of $CaCl_2$ markedly improved the growth,although the L-form grew hardly in this medium in the sbsence of $CaCl_2$.

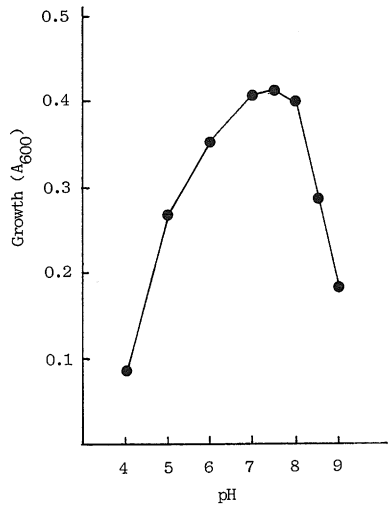

Fig. 2. Effects of pH on growth of the L-form.
The L-form was inoculated to BHI medium with 2% NaCl and 1 mM $CaCl_2$, and incubated at 32°C without shaking. At 48 hr after incubation, the A_{600} were measured. Points are an average of duplicate experiments.

Filtration of the L-form through membrane filters.

In the cells grown at the stationary phase,many photographs such as those shown in Fig. 1 indicated that the L-form varied in size. Large bodies (greater than 2 μm in diameter), medium-sized bodies (0.6–2.0 μm in diameter), and elementary bodies (less than 0.6 μm in diameter) were present. Membrane filters employed for regular filtration of a sample of the broth-grown L-form in stationary phase of growth. The L-form were grown from the 1.2– or the 0.8–μm filtrates (data not shown). However,no the L-form was obtained from the 0.45 μm filtrates.

Effects of pH,temperature,and NaCl conc on growth.

The effect of pH on growth of the L-form was investigated. Figure 2 illustrated

the effect of pH on growth of the L-form. The pH optima of the growth laied between 7 and 8. The effect of temperature for growth of the L-form also was examined. The L-form showed optimum temperature at 32°C (Table 3). The growth at 32°C was faster than that at 37°C, but that of the parent strain at 32°C was much lower than that at 37°C. The effect of varying the NaCl concentration on growth of the L-form was shown in Table 4. The optimal concentration for NaCl in BHI medium was between 2 and 3% NaCl. In P medium,similar result also was obtained.

Evidence for the derivation of the L-form from *E.coli* K–12.

The L-form has a high degree of similarity to *E.coli* K–12 in its physiological characters (Table 4). Nine of the twelve compounds tested as sole carbon sources resulted in similar growth responses for the L-form as the parent strain. Xylose, raffinose,arabinose, and sorbitol were not satisfactory carbon sources for the

Table 3. Effects of various temperature on growth of the parent and L-form.

Temperature (°C)	Growth (A_{600})	
	L-form	Parent
28	0.35	0.96
32	0.39	0.97
37	0.28	1.02
40	0.04	1.03

The L-form and parents were incubated to BHI medium, respectively and then 2.5% NaCl and 1 mM $CaCl_2$ were added to the L-form cultures. At 16 hr (parent strain) and 48 hr (L-form strain) after incubation, the A_{600} were measured.

Table 4. Effects of NaCl concentrations on growth of the L-form

NaCl conc. (%)	Growth (A_{600})
None	0.01
1	0.03
2	0.37
3	0.36
4	0.20
5	0.17

The L-form was inoculated to BHI medium with 2% NaCl and 1 mM $CaCl_2$, and incubated at 32°C without shaking. The A_{600} were measured at 48 hr after incubation.

Table 5. Comparative physiological properties of the parent and L-form

Physiological test	L-form	Parent
Carbohydrates and related C source		
Glucose	+	+
Maltose	+	+
Mannose	+	+
Xylose	−	+
Arabinose	−	−
Sorbitol	−	+
Mannitol	−	+
Galactose	+	+
Fructose	+	+
Lactose	+	+
Citrate	−	−
Malonate	−	−
Other test		
Catalase	+	+
β-galactosidase	+	+
Nitrate reduced	+	+
M-R reaction	+	+
V-P reaction	+	−
IPA reaction	−	−
H_2S from TSI	−	−
Indol	+	+

Symbols: + growth or positive reaction; − no growth or negative reaction.

L-form,but the parent strain grew adequately with these carbon sources. In other tests,the L-form showed positive Voges-Proskauer (V–P) reaction,compared with negative reaction of the parent strain,while other physiological properties of the L-form were similar to those seen in the parent strain.

Discussion

E.cloli K–12 L-form cloned on BHI agar medium was adapted to grow in liquid medium with osmotic stabilizer. The L-form NC7 have been subcultured in BHI medium containing 2 to 3% NaCl,0.5% yeast extract and 1 mM $CaCl_2$ for two years. This strain is stable in the absence of penicillin G and has not been found to revert during laboratory manipulations. When a culture of L-form NC7 is diluted into water,lysis occur immediately.

Stable L-forms are neccessary relatively long proccess. In the course of the adaptation,it is expected that are resistant against environmental changes and are accompanied by various morphologic,metabolic and antigenic changes. The transformation of bacterial cells into stable L-form cells is accompanied by complete loss of the cell walls (Dienes and Bullivant,1968 ; Gumpert et al.,1971 ; Eda et al,.1976), as well as by changes in the cytoplasmic membranes (Gilpin et al.,1973 ; Baykoushava et al.,1980). Hayami et al. (1979) reported that *Staphylococcal* L-forms acquired the ability to synthesize cholesterol as an adaptational change.

In this study,the gross and microscopic morphology of the L-form NC7 agreed in many respects as that described for other bacterial L-forms (Lederberg and Clair, 1957 ; Lapinski and Flakas,1969). However,the optical temperature for growth of the L-form NC7 is lower than that of the parent strain. The differences in the optical growth temperature of the parent bacteria and its derived L-form NC7 may indicate that an alteration in cell metabolism has occurred in the conversion of the bacillary form to the L-form. The L-form NC7 also indicated a close similarity to the parent strain in its physiological characters. On the other hand,this L-form NC7 is unlike those derived from most other bacteria in the growth response to osmotic stabilizers (Montogomerie et al.,1972). When NaCl was used as osmotic stabilizer,the L-form NC7 grew readily if calcium was supplemented to the medium. However,in the presence of other stabilizers,calcium prevented rather the L-form growth. It is interest that calcium can act in two opposite ways on growth of L-form NC7. Those results promoted us to research a role of salts as osmotic stabilizers on physiological functions,involving cation transport systems,as well as osmoregulation. A further study is being made to define the biochemical basis for the physiological mechanism of bacterial plasma membranes more clearly.

Acknowledgments

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