Morphology, Growth and Reversion in a Stable L-form of *Escherichia coli* K12

By T. ONODA,* A. OSHIMA, S. NAKANO AND A. MATSUNO

Department of Biology, Faculty of Science, Shimane University, Matsue 690, Japan

(Received 15 April 1986; revised 16 September 1986)

An L-form isolated from *Escherichia coli* K12 by sequential treatment with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and lysozyme was adapted to grow in hyperosmolar liquid cultures. It was stable in the absence of antibiotic when cultured in brain heart infusion (BHI) broth containing NaCl and CaCl₂, the optimal concentrations being 0.34 M and 1 mM, respectively. No growth of the L-form was observed when CaCl₂ was not added to BHI medium containing 0.34 M-NaCl. On the other hand, when KCl replaced NaCl as the osmotic stabilizer, growth of the L-form was repressed in the presence of CaCl₂. Electron microscopy of the L-form confirmed the absence of a cell wall. A revertant strain derived from the L-form grew as a stable bacillary form in BHI medium without osmotic stabilizer. The growth characteristics of the revertant strain resembled those of the parent strain. The revertant strain produced L-forms in the presence of NaCl.

INTRODUCTION

The transformation of bacteria into L-forms is accompanied by various morphological, metabolic and antigenic changes (Hijimans, 1962; Reusch & Panos, 1976; Hayami *et al.*, 1979). In bacterial L-forms, the cytoplasmic membrane is the only barrier between the cell and its environment. Stable bacterial L-forms, which can grow without cell walls, are expected to differ considerably from their original bacteria in the structure and function of their plasma membrane (Gilpin *et al.*, 1973). Most stable L-forms require the addition of osmotic stabilizers to the culture medium: salts, sugars or serum components are commonly used. NaCl is not only a good osmotic stabilizer for the growth of bacterial L-forms, but it also affects several physiological processes (Linker & Wilson, 1985). Stable bacterial L-forms which have permanently lost their cell walls but multiply in liquid media (Burmeister & Hesseltine, 1968; Young *et al.*, 1970; Gilpin *et al.*, 1973; Eda *et al.*, 1976, 1979), would prove useful in studies of bacterial transport systems and physiological mechanisms of bacterial cell membranes. The present paper describes some investigations concerning growth, morphological features and reversion of a stable L-form of *Escherichia coli*.

METHODS

Strains. The parent strain, *Escherichia coli* K12 strain 3301, the stable L-form NC7 derived from it, and strain RNC7-1, isolated as a revertant of NC7, were used.

Induction of L-forms. *E. coli* was grown for 3 h on a rotary shaker (250 r.p.m.) 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 *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine ml⁻¹. The suspension was incubated for 30 min at 37 °C. The cells were harvested, washed once with brain heart infusion (BHI) broth containing 0.5 M-sucrose, and suspended in 1.5 ml of the same medium containing lysozyme (200 µg ml⁻¹) and Na₂EDTA (0.05%). After 60 min at 30 °C, the protoplasts were plated on BHI agar containing 0.5 M-sucrose, 1% (v/v) horse serum, and 750 units penicillin G ml⁻¹. Of the surviving three L-form colonies, one (L-form NC7) was subcultured by serial transfers on BHI agar

Abbreviation: BHI, brain-heart infusion.

containing 1% horse serum and 750 units penicillin G ml⁻¹. After 4 months of serial passage at suitable intervals, the horse serum was omitted.

Adaptation to liquid medium. Well-developed colonies of L-form NC7 were used. Agar blocks containing L-form colonies were inoculated into BHI medium supplemented with 3% (w/v) NaCl as osmotic stabilizer, 5% (v/v) horse serum, 0.5% yeast extract, 1 mM-CaCl₂ and penicillin G (100 units ml⁻¹). They were incubated without shaking at 32 °C. Thereafter, the horse serum was omitted. Under these conditions, the L-forms grew as separate cells without visible evidence of lysis. Identification of the strains was made by 7.5% (w/v) polyacrylamide gel electrophoresis of cell proteins. Protein was estimated by the Lowry method.

Growth conditions. The L-forms were grown without shaking in BHI medium containing 2% NaCl, 1 mM-CaCl₂, 0.5% yeast extract and penicillin G (100 units ml⁻¹) at 32 °C. P medium contained (l⁻¹) 10 g peptone, 5 g yeast extract and 2 g glucose. The pH value was adjusted to 7.1 with NaOH or KOH. Solidified medium contained BHI medium supplemented with 1% (w/v) agar, 0.5% yeast extract and 100 units penicillin G. The parent strain was grown in BHI medium. All cells were harvested in the exponential phase of growth by centrifugation (4000 g, 15 min), washed once with BHI medium containing osmotic stabilizer, and inoculated into growth medium. The suspensions were incubated at 32 °C without shaking and growth was monitored by measuring optical density at 600 nm (1 cm path length). The initial OD₆₀₀ was about 0.02.

Antibiotic susceptibility tests. The minimal growth inhibitory concentration of antibiotics was determined by the tube dilution method. BHI medium was used as growth medium for the parent strain, and medium supplemented with 2% NaCl and 1 mM-CaCl₂ for the L-form strain. Tubes containing 4 ml medium and serial dilutions of antibiotic were inoculated with 0.1 ml of an exponential-phase culture and incubated at 32 °C for 48 h. The lowest concentration of antibiotic able to inhibit growth was determined visually.

Electron microscopy. Colonies in soft agar were cut out and fixed for 1.5 h in 1.5% (w/v) paraformaldehyde and 1.5% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.3) at room temperature. The prefixed specimens were washed for 30 min on ice in the cacodylate buffer, and fixed in 1% (w/v) osmium tetroxide in 0.1 M-phosphate buffer (pH 7.3). The fixed specimens were dehydrated in a graded ethanol series, and embedded in Epoxy resin after passage through propylene oxide. Sections were cut with glass knives and mounted on Formvar-coated grids. They were stained with saturated aqueous uranyl acetate and lead citrate, and observed in a JEM 100C electron microscope.

Chemicals. Peptone, yeast extract and BHI medium were purchased from Kyokuto Pharmaceutical Industrial Co. Sucrose (RNAase free) was purchased from Nakarai Chemicals. All other reagents used were of analytical grade.

RESULTS

Growth. The L-form NC7 of *E. coli* K12 has been maintained through about 120 transfers in BHI medium containing 3% NaCl and 1 mM-CaCl₂. It is stable under these conditions. The effect of medium components on growth of the L-form is shown in Table 1. Addition of 1 mM-CaCl₂ to BHI medium supplemented with 2% NaCl markedly improved growth and allowed maintenance of the L-form through sequential transfers. Addition of 5% horse serum instead of CaCl₂ gave a similar result. On the other hand, the L-form grew poorly in the medium without NaCl, regardless of whether supplemented with CaCl₂ or serum (Table 1).

In BHI medium supplemented with NaCl and CaCl₂, the L-form grew exponentially with a generation time of 5–6 h, and the OD₆₀₀ reached a maximum within 2 d. No growth was observed in the absence of CaCl₂.

Table 1. *Effect of medium components on growth of the L-form*

The L-form was grown in BHI medium with supplements as indicated. The OD₆₀₀ was measured after 48 h incubation. The values are means from three separate experiments, ± SD.

Supplement(s)	Growth (OD ₆₀₀)	
	– NaCl	+ NaCl
None	0.03 ± 0.01	0.03 ± 0.01
CaCl ₂ (1 mM)	0.03 ± 0.02	0.36 ± 0.03
Serum (5%)	0.03 ± 0.01	0.15 ± 0.01
CaCl ₂ (1 mM) + serum (5%)	0.04 ± 0.02	0.44 ± 0.03

Fig. 1. SDS-polyacrylamide slab gel analysis of whole-cell extracts of the parent strain, the L-form strain and the revertant strain, all harvested in the late-exponential phase at 32 °C. The gels (7.5% SDS polyacrylamide) were stained with Coomassie Brilliant Blue (0.124 mg protein was applied to each lane). A, parent strain grown in unsupplemented BHI medium; B, parent strain grown in BHI medium with 2.5% NaCl plus 1 mM-CaCl₂; C, L-form strain grown in BHI medium with 2.5% NaCl plus 1 mM-CaCl₂; D, revertant strain grown in unsupplemented BHI medium.

Table 2. *Minimal inhibitory concentrations of antibiotics (µg ml⁻¹)*

The highest concentration of methicillin and penicillin G used in this experiment was 1000 µg ml⁻¹; 1 µg penicillin ml⁻¹ is equivalent to 1.7 units ml⁻¹. Data are from duplicate experiments.

Antibiotic	Parent	L-form
Chloramphenicol	5	2
Streptomycin	20	6
Tetracycline	4	1
Kanamycin	30	6
Penicillin	1	1000
D-Cycloserine	100	800
Methicillin	10	1000

Antibiotic susceptibility. The minimal inhibitory concentration of antibiotics known to affect protein or cell wall biosynthesis was determined to see whether the L-form differed in antibiotic susceptibility from the parent strain. Both the L-form and parent strains were sensitive to streptomycin, kanamycin, chloramphenicol and tetracycline. Penicillin, methicillin and D-cycloserine were used in these experiments, as inhibitors of cell wall biosynthesis. As expected, the L-form was resistant to these antibiotics, as compared with the parent (Table 2).

Polyacrylamide gel electrophoresis. To establish the identity of the strains and their relatedness, the electrophoretic patterns of the parent and L-form were studied. Although the gel for the parent showed some bands that were less intense than those present in the L-form patterns, there was a close similarity between the two patterns (Fig. 1), in keeping with the genetic identity of the two morphological forms.

Fig. 2. Phase-contrast micrographs of the parent strain, the L-form and the revertant strain, all incubated at 32 °C without shaking. (a) The parent strain and (c) the revertant strain (each 24 h after inoculation), in unsupplemented BHI medium; (b) the L-form (48 h after inoculation) and (d) the revertant strain (72 h after inoculation), in BHI medium with 2.5% NaCl plus 1 mM-CaCl₂. Bars, 5 μm.

Table 3. *Effects of osmotic stabilizers on growth of the L-form in BHI and P media*

The cells were grown in BHI and P media with or without CaCl₂. As osmotic stabilizer, 2% KCl, 2% NaCl or 0.4 M-sucrose was added. The OD₆₀₀ was measured after 48 h incubation. The values are means from three separate experiments, ± SD.

Osmotic stabilizer	Addition of CaCl ₂	Growth (OD ₆₀₀)	
		BHI medium	P medium
KCl	—	0.47 ± 0.02	0.47 ± 0.02
	+	0.30 ± 0.02	0.04 ± 0.01
NaCl	—	0.01 ± 0.01	0.02 ± 0.01
	+	0.42 ± 0.03	0.47 ± 0.02
Sucrose	—	0.31 ± 0.02	0.01 ± 0.01
	+	0.10 ± 0.01	0.01 ± 0.01

Morphology. Colonies of strain NC7 on BHI agar plates had the fried-egg appearance associated with L-forms. Phase-contrast micrographs of cultures of the parent and L-form are shown in Fig. 2. In liquid BHI medium supplemented with 3% NaCl and 1 mM-CaCl₂, the L-form cultures consisted mainly of spherical bodies of varying sizes. Further, many morphological forms were seen which could be related to processes of propagation: binary or asymmetric fission and a type of budding. Electron microscope examination of ultrathin sections of an L-form colony illustrated that the L-forms were bounded by a single unit membrane (Fig. 3).

Factors influencing growth of the L-form. Optimal growth of the L-form was obtained at 32 °C in the presence of 2% NaCl and 1 mM-CaCl₂ (Fig. 4). MgCl₂ at various concentrations had no

Fig. 3. Electron micrographs of cells of the parent strain and the L-form grown in BHI soft agar (0.5% agar) medium supplemented with 2% NaCl. (a) The parent strain, showing the outer membrane of the cell wall. (b) The L-form: most of the cells are spherical in shape, lack a cell wall, and are surrounded by a single membrane. CM, cytoplasmic membrane; CW, cell wall. Bars, 0.5 μ m.

effect on growth. Sucrose (0.4 M) and KCl (2%, w/v) as osmotic stabilizers, and P medium, were tested as substitutes for 2% NaCl, and BHI medium, respectively. The L-form grew well in BHI or P medium containing 2% KCl only, but was inhibited in both the media when CaCl_2 was added. Addition of 0.4 M-sucrose instead of NaCl to P medium resulted in no growth in the presence or absence of CaCl_2 . However, significant growth in BHI medium containing 0.4 M-sucrose was observed in the absence or presence of CaCl_2 (Table 3). By flame photometry, BHI broth contained approximately 126 mM- Na^+ as NaCl and 52 mM- K^+ as KCl. It appears, therefore, that the growth observed in BHI-sucrose medium without CaCl_2 was supported by the potassium ions present in BHI broth.

Reversion of the L-form. Reversion of the L-form to the bacillary form occurred frequently in BHI medium without added NaCl during prolonged incubation (4–5 d). The revertant strain, RNC7-1, isolated from the L-form NC7 was stably maintained in BHI medium without NaCl. The colonies of strain RNC7-1 on EMB agar were black with a metallic lustre and indistinguishable from the parent strain grown under identical conditions. The morphology and

Fig. 4. Effects of the concentration of CaCl_2 and MgCl_2 on growth of the L-form. The cells were grown for 42 h in BHI medium containing 2% NaCl and various concentration of CaCl_2 (●) or MgCl_2 (▲).

Table 4. *SDS sensitivity of the revertant strain grown in BHI medium with and without NaCl and CaCl_2*

The L-form, revertant and parent strains were inoculated to BHI medium with or without 2.5% NaCl, and CaCl_2 (1 mM) was added to half of the cultures. The OD_{600} was measured after 20 h (revertant and parent strains) or 48 h (L-form) incubation. Part of each culture was treated with SDS (0.05%) for 5 min, and the OD_{600} immediately measured. The bacterial morphology of the cultures was observed by phase-contrast microscopy: (B), bacillary form; (L), L-form; (BL), bacillary form plus L-form. Data are from one representative experiment and are means of at least three determinations.

Addition to BHI medium	SDS (0.05%) treatment	Growth (OD_{600})					
		L-form		Revertant		Parent	
		- CaCl_2	+ CaCl_2	- CaCl_2	+ CaCl_2	- CaCl_2	+ CaCl_2
None	-	0.02	0.02	0.69 (B)	0.58 (B)	2.10 (B)	2.10 (B)
	+	ND	ND	0.63 (B)	0.51 (B)	2.04 (B)	2.03 (B)
NaCl	-	0.02	0.39 (L)	0.52 (BL)	0.44 (BL)	1.04 (B)	1.02 (B)
	+	ND	0.01	0.22 (B)	0.18 (B)	0.96 (B)	0.94 (B)

ND, Not determined.

growth characteristics of the revertant are shown in Fig. 2(c) and Table 4, respectively. The revertant exhibited normal bacillary morphology by phase-contrast microscopy and grew well in liquid medium without osmotic stabilizer when compared with the L-form, but did not grow as rapidly as the parent strain. The revertant and parent strains, maintained in BHI medium, grew with generation times of 72 min and 43 min, respectively. CaCl_2 had equally little effect on growth of the revertant and parent. Furthermore, the revertant was sensitive to penicillin G ($1 \mu\text{g ml}^{-1}$) (data not shown), as was the parent. The morphology, penicillin G susceptibility and growth pattern of revertant RNC7-1 were similar to those of the parent strain, although the revertant's growth rate was lower. When the revertant was transferred to BHI medium with NaCl, it again produced the same spherical bodies as the original L-form (Fig. 2d). The first morphological change noted was the appearance of bulges which subsequently fragmented into

distinct spheres. Production of spherical bodies was stimulated by raising the NaCl concentration. Addition of distilled water or SDS (0.01%) resulted in rapid lysis of the L-form, but had no effect on the revertant and parent strains. When SDS (0.05%) was added to the revertant culture grown in BHI medium containing 2.5% NaCl, the OD₆₀₀ of the culture decreased (Table 4), and lysis of spherical bodies was clearly observed by phase-contrast microscopy. In addition, when the revertant (bacillary form) grown in BHI medium was treated with SDS (0.05%), and transferred into fresh BHI medium with or without added NaCl, the cells were still viable; they grew as bacillary forms in the absence of NaCl, while in the presence of NaCl the culture contained a number of spherical bodies, whose production was stimulated by raising the NaCl concentration (data not shown). These results suggest that reversion of RNC7-1 to the 'parent' bacterium was incomplete.

DISCUSSION

The L-form of *E. coli* has been subcultured in BHI medium containing 0.5% yeast extract, 3% NaCl and 1 mM-CaCl₂ for two years. This strain is stable in the absence of penicillin G, and does not revert under these conditions. The resistance of the L-form to penicillin, D-cycloserine and methicillin demonstrated here was anticipated, since the L-forms lack a rigid cell wall. The absence of a cell wall was confirmed by electron microscopy. Optimal growth of the L-form was obtained when 1 mM-CaCl₂ was added to BHI medium supplemented with 2% NaCl. MgCl₂ had no effect on growth, whereas in the absence of CaCl₂, no growth occurred in BHI medium containing NaCl. Also, the L-form did not grow unless NaCl was added to the medium, regardless of whether supplemented with CaCl₂.

King *et al.* (1969) reported that a polyacrylamide gel electrophoretic method for comparison of whole-cell peptide profiles might prove of value not only in determining or confirming genetic origins of L-forms, but also in clarifying differences among L-forms of a given origin. The results obtained here indicate that the L-form possesses essentially the same patterns as those of the parent strain, thus establishing its genetic identity.

The L-form, which normally required high concentrations of NaCl or sucrose as osmotic stabilizer, reverted occasionally into the bacillary form during prolonged incubation in BHI medium without osmotic stabilizer. The osmotic requirement of L-forms varies markedly with the species and strain of bacterium (Montgomerie *et al.*, 1967). A number of bacterial L-forms can survive without osmotic stabilizer (Gilpin & Patterson, 1976). In the present study, the revertant strain may have resulted from cells which were able to survive without osmotic stabilizer. Although Mg²⁺ can have an effect on reversion (from L-form to bacillary form) (Horwitz & Casida, 1978), no such effect of Mg²⁺ was evident in this study. However, reversion from the bacillary to the spherical form was noted when the NaCl concentration in BHI medium was raised. This may be due to release of protein from the cell envelope. Odgers *et al.* (1985) reported that when exponential-phase cells of *Capnocytophaga ochracea* were washed with 1.0 M-NaCl, agitated in 1.0 M-NaCl for 2 h at 30 °C and exposed to lysozyme in a Tris/salts buffer, this resulted in 98% spheroplast formation with complete removal of the peptidoglycan layer. They indicated that NaCl washes caused the release of protein, possibly from the periplasmic space and/or from an outer periodic macromolecular monolayer.

The growth rate of the revertant strain differed markedly from that of the L-form. In addition, there were differences in the optimal growth temperatures of the parent strain (37–40 °C) and L-form (32 °C). These may indicate that an alteration in cell metabolism has occurred in the conversion of the bacillary form to the L-form. Hayami *et al.* (1979) reported that staphylococcal L-forms differed from their parent bacteria in their membrane lipid composition. Our results suggest that salts such as NaCl or KCl play an important role in physiological functions, involving cation transport systems, as well as osmoregulation. It would be of value to determine the basis for the stimulatory effect of CaCl₂ on growth of the L-form in media containing NaCl, since this may help to explain the functional mechanism involved in the CaCl₂ effect. Cation-transport systems in bacteria are poorly understood. The L-form, which lacks a peptidoglycan layer, may provide an excellent tool for the study of such active transport systems.

We wish to thank Yayoi Sakamoto for her technical assistance during the early phase of this work. We gratefully acknowledge the assistance of Hiroto Kuga in preparing the electron micrographs.

REFERENCES

- BURMEISTER, H. R. & HESSELTINE, C. W. (1968). Induction and propagation of a *Bacillus subtilis* L-form in natural and synthetic media. *Journal of Bacteriology* **95**, 1857-1861.
- EDA, T., KANDA, Y. & KIMURA, S. (1976). Membrane structures in stable L-forms of *Escherichia coli*. *Journal of Bacteriology* **68**, 1564-1567.
- EDA, T., KANDA, Y., MORI, C. & KIMURA, S. (1979). Core-like and microtubular structures in a stable L-form of *Escherichia coli*. *Microbiology and Immunology* **23**, 915-920.
- GILPIN, R. W. & PATTERSON, S. K. (1976). Adaptation of a stable L-form of *Bacillus subtilis* to minimal salts medium without osmotic stabilizers. *Journal of Bacteriology* **125**, 845-849.
- GILPIN, R. W., YOUNG, F. E. & CHATTERJEE, A. N. (1973). Characterization of a stable L-form of *Bacillus subtilis* 168. *Journal of Bacteriology* **113**, 486-499.
- HAYAMI, M., OKABE, A., SASAI, K., HAYASHI, H. & KANE, Y. (1979). Presence and synthesis of cholesterol in stable staphylococcal L-forms. *Journal of Bacteriology* **140**, 859-863.
- HIJIMANS, W. (1962). Absence of the group-specific and the cell-wall polysaccharide antigen in L-phase variants of group D streptococci. *Journal of General Microbiology* **28**, 177-179.
- HORWITZ, A. H. & CASIDA, L. E., JR (1978). Effects of magnesium, calcium, and serum on reversion of stable L-forms. *Journal of Bacteriology* **136**, 565-569.
- KING, J. R., THEODORE, T. S. & COLE, R. M. (1969). Genetic identification of L-forms by polyacrylamide gel electrophoretic comparison of extracts from parent strains and their derived L forms. *Journal of Bacteriology* **100**, 71-77.
- LINKER, C. & WILSON, T. H. (1985). Sodium and proton transport in *Mycoplasma gallisepticum*. *Journal of Bacteriology* **163**, 1250-1257.
- MONTGOMERIE, J. Z., KALMANSON, G. M. & GUZE, L. B. (1967). Effect of osmotic stabilizer on protoplast and bacterial forms of *Streptococcus faecalis*. *Journal of Laboratory and Clinical Medicine* **70**, 539-553.
- REUSCH, V. M., JR & PANOS, C. (1976). Defective synthesis of lipid intermediates for peptidoglycan formation in a stabilized L-form of *Streptococcus pyogenes*. *Journal of Bacteriology* **126**, 300-311.
- ODGERS, J., DYER, J. K., MARTIN, E. L., BOLTON, R. W. & ANG, B. J. (1985). Induction of spheroplasts in *Capnocytophaga ochracea*. *Journal of General Microbiology* **131**, 993-999.
- YOUNG, F. E., HAYWOOD, P. & POLLOCK, M. (1970). Isolation of L-forms of *Bacillus subtilis* which grow in liquid medium. *Journal of Bacteriology* **102**, 867-870.