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Effects of osmotic stabilizers on turbidity changes and growth of *Escherichia coli* L-form NC7

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The effects of osmotic stabilizers on swelling and growth of L-form NC7 derived from *Escherichia coli* K 12 were examined. The presence of impermeable solute, such as NaCl or KCl, in the suspended medium were effective as osmotic stabilizer, while permeable solute, such as glycerol were ineffective. On the other hand, when sucrose was used as osmotic stabilizer, in cells upshocked with 0.2 M sucrose, rapid decrease in optical density and then slow restoration were observed. In the upshocked cells, potassium ion had protective effect against changes of turbidity. From colony forming units at 1 hr after upshock, it was shown that there was no significant different between cells upshocked with 0.2 M sucrose alone and with 0.2 M sucrose plus 0.34 M KCl. Low osmolarity (0.17 M) of cation, such as NaCl or KCl, was not sufficient to protect fragile L-form cells unless 0.2 M sucrose was added in the incubation medium. In combination with NaCl and KCl at high concentration (0.4 M), there are competition between Na and K ions on growth, suggesting that in the combination with calcium (1 mM), Na ion also play a important role on growth of L-form NC7 as well as K ion.

Introduction

In a number of bacteria, the stress resulting from the difference in osmotic value between bacterial cytoplasm and culture medium are kept under control by cell walls. However, bacterial L-forms which have lost a cell wall are osmotically fragile, requiring electrolytes such as NaCl and KCl, and sucrose, or other osmotic stabilizers in the medium to survive (King, 1986). Extremes of medium osmolarity are generally harmful to cell growth of bacteria. However, *Escherichia coli* and other enteric bacteria are capable of adapting to a wide range of external osmotic pressure. Osmotic upshift of growing cells of the enteric bacteria evokes a rapid adaptive response to maintain an osmotic balance. The mechanism of adjustment to rebalancing of internal osmolarity in environments of high osmolarity has been poorly understood.

In this paper, the ability of L-form NC7 from *Escherichia coli* K 12 to survive at different osmolarities and effects of osmotic stabilizers on growth of cells were investigated.

Materials and Methods

Organisms and growth conditions

The parent strain, *Escherichia coli* K 12 strain 3301 and L-form NC7 (Onoda, 1986)

derived from it were used in this experiment. Both the strains were grown at 32°C without shaking on the following complex media. NaPY medium contained (per liter) 10 *g* peptone, 5 *g* of yeast extract, 2 *g* of glucose and 0.34 M NaCl. KPY medium was the same as NaPY medium, except that KCl was used instead of NaCl. The pH value was adjusted to 7.2 with NaOH or KOH. In some experiments, Tris(hydroxymethyl)aminomethans was used as adjustment of the pH. All cells were harvested in the exponential phase of growth by centrifugation (4000×*g*, 15 min), washed once with growth medium supplemented with suitable osmotic stabilizer, and inoculated into culture medium. Growth was monitored by measuring optical density at 600 nm (1 cm path length). The initial OD_{600} was about 0.02. The concentrations of contaminating Na^+, K^+, and Ca^{2+} in PY medium were determined using an EEL model 100 flame photometer. The concentration of these ions in the PY medium were found to be 9.2 mM Na^+, 6.5 mM K^+, and 0.15 mM Ca^{2+}, respectively.

Materials

Peptone was purchased from Kyokuto Pharmaceutical Industrial Co., Tokyo. Yeast extract powder was obtained from Oriental Yeast Industrial Co., Tokyo. Sucrose (RNAase) was obtained from Nacalai Tesque, INC., Kyoto. All other reagents used were of analytical grade.

Results

Effects of osmotic stabilizers on swelling of L-from cells

L-form NC7, lacking of a rigid cell wall, was osmotically fragile and the cells grown in medium of high osmolarity lysed rapidly when suspended in distilled water. Changes in the optical density of no growing cell culture, generally reflect changes of cell volume; an increase in optical density reflects a decrease in cell volume and a decrease in optical density reflects an increase in cell volume (Matts and Knowles, 1971; Alemhammad and Knowles, 1974). To investigate the factors involved concerning the cell volume regulation of the L-form, effects of osmotic stabilizers against swelling or lysis have been examined. Fig. 1 showed effects of different osmotic stabilizers on swelling of the cells. When the cells were suspended in 0.34 M NaCl and KCl solutions, respectively, slight decrease of the turbidity were observed in both the cases. A protective effect of calcium was noticed in their osmotic solutions when it was added at a final concentration of 1 mM. On the other hand, when glycerol was used as osmotic stabilizer, a cell swelling followed by lysis occurred immediately. After upshock with 0.4 M glycerol, the optical density decreased rapidly and never increased during incubation of 24 hr. These results suggest that the presence of impermeable solutes such as NaCl or KCl, in the suspended medium were effective as the osmotic stabilizer, while permeable solutes such as glycerol were ineffective. When sucrose was used as the osmotic stabilizer, however, it contrasted sharply with the response to swelling of glycerol. From the

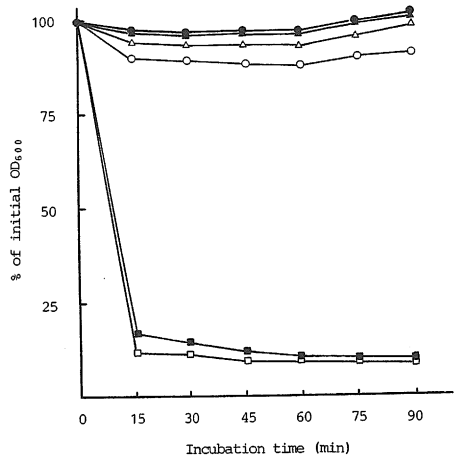

Fig. 1. Effects of Nacl, KCl, and glycerol on changes of turbidity. L-Form NC7 was grown overnight in KPY medium. Then, each 5 ml of the culture was harvested and centrifuged at 2000×g for 15 min. The each pellet was suspended in indicated solutions, respectively and incubated without shaking at 32°C. Glucose (10 mM) and penicillin (100 units/ml) were added to all solutions. The optical densities were followed at 600 nm. Symbols: ○, 0.34 M NaCl; ●, 0.34 M NaCl plus 1 mM $CaCl_2$; △, 0.34 M KCl; ▲, 0.34 M KCl plus 1 mM $CaCl_2$; □, 0.4 M glycerol; ■, 0.4 M glycerol plus 1 mM $CaCl_2$.

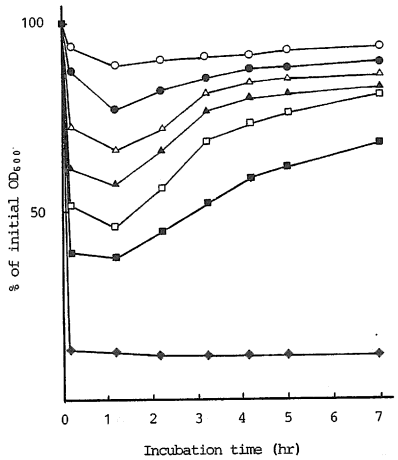

Fig. 2. Effects of KCl and sucrose on changes of turbidity. Assay procedure for changes of turbidity was carried out as described in Fig. 1. Symbols: ○, 0.34 M KCl alone; ●, 0.2 M sucrose plus 0.34 M KCl; △, 0.2 M sucrose plus 0.17 M KCl; ▲, 0.2 M sucrose plus 0.08 M KCl; □, 0.2 M sucrose plus 0.04 M KCl; ■, 0.2 M sucrose alone; ◆, 0.5 M glycerol alone.

optical density changes after upshock with 0.2 M sucrose (Fig. 2), one can see that optical density rapidly decreased, but thereafter gradually increased, suggesting that cell volume increased and then slowly restored. During this experiment, no growth was observed. On the other hand, at 1 hr after addition of the osmotic solutions (0.34 M KCl, 0.34 M KCl plus 0.2 M sucrose, 0.2 M sucrose, and 0.5 M glycerol), colony forming units (CFU)/ml in each osmotic solution were determined as following: approximate 1.2×10^6 CFU/ml in 0.34 M KCl solution, ca. 1.5×10^5 CFU/ml in 0.34 M KCl plus 0.2 M sucrose, ca. 2.2×10^5 CFU/ml in 0.2 M sucrose, and ca. 1.5×10^3 CFU/ml in 0.5 M glycerol. Cells upshocked with 0.2 M sucrose in the presence of potassium showed that cell volume was controlled by concentration of external potassium ion. This ion is known to accumulate in osmotically upshocked cells. Thus, the recovery of cell volume may be affected by an increased uptake of potassium. In addition, when the cells were

incubated in 0.2 M sucrose solution for 24 hr and transfered into KPY growth medium, they retained the ability to grow.

Effects of concentrations of osmotic stabilizers in the medium on growth.

To investigate the effects of osmotic concentrations on growth, various concentrations of Na or K ion were added to PY medium with or without calcium (1 mM). Fig. 3 showed that cell growth was depended on the concentration of NaCl in the medium. The optimum concentration of NaCl for the growth was about 0.34 M. No growth occurred in a medium of low osmolarity of NaCl (0.17 M), while it was observed when sucrose (0.2 M) was added into the medium. In the absence of added calcium, however, no significant growth was observed under the same condition. On the other hand, when KCl was substituted for NaCl as osmotic stabilizer, the similar results were also obtained with KCl alone or combination of KCl plus sucrose as osmotic stabilizer, suggesting that low osmolarity of both the salts (0.17 M) were not sufficient to protect fragile L-form NC7.

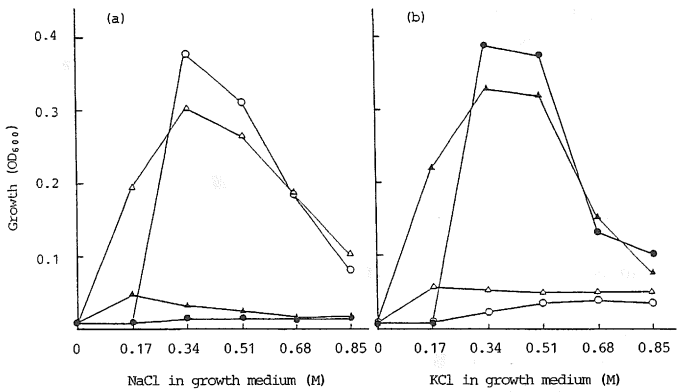

Fig. 3. Effects of concentration of osmotic stabilizers in the medium on growth. The cells were inoculated in PY medium containing various concentrations of NaCl (a) and KCl (b), respectively. Then, $CaCl_2$ (open symbols) and sucrose (triangle symbols) were added in a part of the cultures at a final concentration of 1 mM and 0.2 M, respectively and incubated at 32°C. OD_{600} was measured at 48 hr after incubation. Symbols: ●, control; ○, 1 mM $CaCl_2$, 0.2 M sucrose; △, 1 mM $CaCl_2$ plus 0.2 M sucrose.

Effects of various cations on growth

The following salts were tested: RbCl, LiCl, CsCl, $MnCl_2$, $CoCl_2$, and $MgCl_2$. All the salts investigated were added individually to NaPY or KPY medium at a final concentration of 1 mM, respectively. Table 1 showed effects of different salts on the growth of the L-form. $MnCl_2$ had significant effect on the growth. In contrast, $CoCl_2$ completely inhibited the growth, even in combination with $CaCl_2$. Other salts tested

Table 1. Effects of cations in combination with NaCl or KCl as osmotic stabilizer on growth

Cation supplemented to medium	Growth (OD_{600})	
	Osmotic stabilizers	
	NaCl*	KCl**
None	0.02	0.40
RbCl	0.02	0.39
LiCl	0.02	0.33
CsCl	0.02	0.36
$MnCl_2$	0.40	0.02
$CoCl_2$	0.02	0.02
$MgCl_2$	0.02	0.42
$CaCl_2$	0.43	0.02
$CaCl_2$ plus RbCl	0.44	0.02
$CaCl_2$ plus LiCl	0.44	0.02
$CaCl_2$ plus $MnCl_2$	0.41	0.02
$CaCl_2$ plus $CoCl_2$	0.02	0.02
$CaCl_2$ plus $MgCl_2$	0.42	0.02

*NaCl and **KCl contain 0.34 M at final concentration, respectively.

were ineffective for the growth. Whether other calcium compounds except $CaCl_2$ could support the growth were determined. Following calcium compounds were used in this experiment: $CaCO_3$, $CaHPO_4$, $Ca(NO_3)_2$, and $Ca(CH_3COO)_2$. All the compounds tested stimulated the growth to the same extend as $CaCl_2$ (data not shown). These results indicate that the enhanced growth by addition of CaCl is due to mainly Ca ion.

Effects of different combinations of osmotic stabilizers on growth

The cells were transfered into media with different combinations of NaCl and KCl and incubated in the absence or presence of calcium. In this case, the salt's external osmolarities were constantly kept. Fig. 4 showed that in the absence of calcium the growth rates decreased with decreasing KCl concentration in the growth medium. On the contrary, in the presence of calcium, the rates increased with increasing NaCl concentrations. To examine relationship between Na and K ions on growth, the optical densities were examined in different combinations of NaCl and KCl. NaCl were added at various concentrations up to 0.4 M to the each medium containing constant concentration of KCl (0.4 M). Fig. 5a showed the effects of various combinations of monovalent cations (NaCl and KCl) added to PY medium with or without calcium (1 mM) on growth. In the presence of calcium, the growth rates increased with increasing concentration of NaCl, whereas in the absence of calcium, K-dependent growth was dominant in low concentration of NaCl and the rates inhibited markedly by NaCl concentration

above 0.1 M. On the contrary, KCl was added at different concentrations up to 0.4 M to the each medium containing constant concentration of NaCl (0.4 M) and the growth rates were determined (Fig. 5b). The growth rates, in the medium with calcium, increased with decreasing concentration of KCl, while in the absence of calcium, slight growth was observed over wide ranges of KCl concentrations. As shown in Fig. 5, in combination with NaCl and KCl at high concentration, there are competition between Na and K ions for growth, and in this case, Na ion may have much larger effect for growth than K ion.

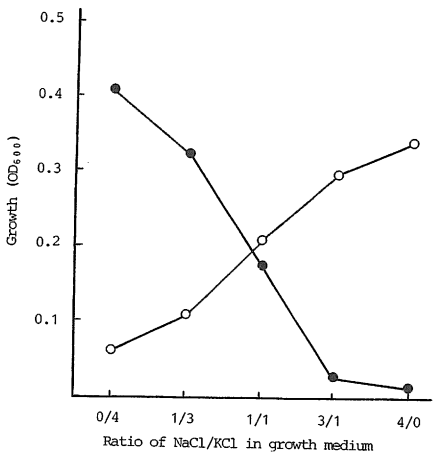

Fig. 4. Effects of ratio of NaCl versus KCl on growth. The cells were inoculated into PY medium containing various combinations with NaCl and KCl such that the final concentrations of added NaCl and KCl were kept up about 0.4 M. In this experiment, 1 mM $CaCl_2$ also was added to a part of the cultures. OD_{600} was measured at 48 hr after incubation at 32°C. Symbols: ●, control; ○, 1 mM $CaCl_2$. Data are from one representative exeriment.

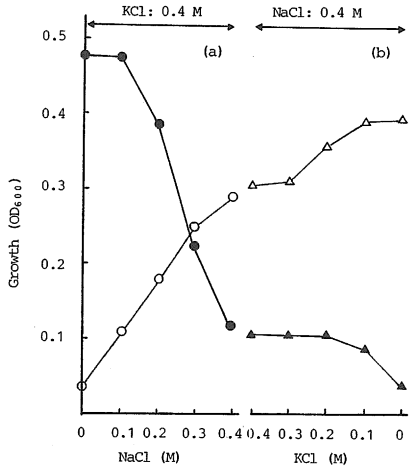

Fig. 5. Effects of various combinations of NaCl and KCl on growth. The cells were inoculated into PY medium containing different combinations with NaCl and KCl as osmotic stabilizer. 1 mM calcium was added to a half of the cultures. OD_{600} was neasured at 48 hr after incubation at 32°C. (a): NaCl was added at different concentrations up to 0.4 M to medium containing KCl (0.4 M) of constant concentration, and (b): in contrast with (a), KCl was added at different concentrations up to 0.4 M to medium containing NaCl (0.4 M) of constant concentration. Symbols: ● and ▲, control; ○and△, 1 mM $CaCl_2$.

Discussion

Most bacteria posses rigid cell wall and are generally assumed to require no additional defenses against changes in osmotic pressure. The osmotic stresses are kept

under control by cell wall. In the L-forms which is able to multiply in the absence of the regidity and shape normally supplied by the intact cell wall, the fluctuation of osmotic pressure must be directly controlled by the cell membrane. The osmotic requirement of L-forms have also varied markedly with the species and strain of bacteria (Montgomerie, 1967). L-form lacking cell wall are especially faced with the problem of volume regulation even in isosmotic environment.

Cellular adaptation to the osmotic stress is serious biological process that protect organisms against the cellular lysis or the lethal effects of dehydration. The osmotic stress such as osmotic upshock produced by an increase in the osmolarity of external medium generally inhibits the growth of bacteria (Costilow, 1981). Control of cell growth and division were also depended upon osmolarity of the growth medium (Baldwin and Kubitscheck, 1984). In the previous papers(Onoda, *et al.*, 1987; Onoda and Oshima, 1988), we reported that calcium ion are always required for growth of L-form NC7 derived from *E. coli* K 12 when sodium or potassium ion was used as the osmotic stabilizer. We also found that when sucrose (0.2 M) was used as the osmotic stabilizer, no growth occurred. Baldwin *et al.*, (1988) showed that *E. coli* cells were flexible and shrank as well as swelled in response to osmotic shocks. Further, we took notice that when the L-form was incubated in the medium containing potassium ion phase-contrast microscopy showed polymorphy varing in shape from spherical to slender branched filaments, whereas when incubated in the medium containing sodium ion, the cells are usually spherical, although filamentous elements have occasionally been seen. These results suggest that cell morphology is mutually changeable by cation used as the osmotic stabilizer (unpublished data).

Raven and Smith (1976) proposed that the two principal factors causing perturbation of internal pH are likely to be passive movement of protons across the cytoplasmic membrane and the production of acids and bases in the cytoplasm. Many kinds of transport system are required for an environmental adaptation of bacteria, that is, the optimum conditions for the bacterial cytoplasm are maintained by the aid of the transport systems when bacteria are growing in harsh environments. For an organism such as *Escherichia coli,* the pH of the cytosole is maintained at a relatively constant value regardless of the external pH (Padan, et al., 1976; Booth, 1985). The cation-proton antiporter systems have been characterized by an essential role for regulation of cytosolic pH (Brey *et al.*, 1978; Beck and Rosen, 1979; Brey and Rosen, 1979; Plack and Rosen, 1980; Krulwith *et al.*, 1981; Krulwich, 1985). Harold and van Brunt (1978) established that rapid growth of *Streptococcus faecalis* was depended upon maintaince of an alkaline intracellular pH value. Roth *et al.*, (1985a) reported that the osmotic stress drastically inhibited active transport of carbohydrate by *Escherichia* coli and that this transport inhibition was able to account for the inhibition of the growth caused by osmotic stress. They described that the deformation of membrane by osmotic stress results in conversion of a membrane component of the transport system to a less functional conformation, which results in the inhibition of transport and the consequent

inhibition of growth (Roth *et al.*, 1985b). Recently, we proposed a evidence that on the hyposalts medium, a proton motive force (pmf) is not necessary for the growth of *Escherichia coli* K 12, but on the hypersalts medium, pmf is obligatory the growth (Nakano and Onoda, 1989). In this paper, we suggest that sodium and potassium ions may play a important role not only in osmotic stabilizer, but in regulation of cell volume. The study of solute transport across the membrane on the L-form may provide some insight into this fundamental process in bacteria.

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