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TEMPERATURE-SENSITIVE MUTANTS OF *ESCHERICHIA COLI* B WHICH CAN GROW IN HIGH-OSMOTIC MEDIUM AT THE NONPERMISSIVE TEMPERATURE

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SUMMARY Two temperature sensitive (TS) mutants (C4 tos and D2 tos) were isolated after mutagenesis of *E. coli* B/SM by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMNG), which can grow even at 42 C in high-osmotic medium supplemented by addition of sucrose, NaCl or other compounds.

Neither of the mutants lyzed when transferred to low-osmotic medium after growning at the nonpermissive temperature in high osmotic medium.

One of these mutants, C4 tos, grew at 42 C in a long filamentous form. When bacteria growing exponentially at 30 C were shifted to 42 C, they continued to grow at a reduced rate even in low-osmotic medium. This strain could also grow or start to grow in low-osmotic medium when supplied with a factor or factors secreted from growing bacteria of another strain. This mutant strain could grow in low-osmotic medium at 42 C when it was cultured anaerobically.

The other mutant strain obtained (D2 tos) displayed normal morphology even when grown at 42 C. When it was shifted from 30 to 42 C in low-osmotic medium, increase of mass, measured as optical density, continued for a while, but viability, measured as the number of colony-formers, stopped increasing and then decreased rapidly.

INTRODUCTION

Matsuzawa et al. (1969) isolated temperature-sensitive (TS) mutants of *Escherichia coli* K-12, which lyzed in normal medium at 42 C but could grow at this temperature on addition of 20% sucrose to the medium. Recently, similar mutants were reported and some of them were not fragile at the nonpermissive temperature even without protection by high osmolality (Ricard and Hirota, 1969; Bilsky and Armstrong, 1973; Eagan and Russell, 1973).

During studies on the immune bactericidal reaction we became interested in the action of complement on a mutant of *E. coli* which has some defect in its cell envelope. We isolated

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two mutant strains (C4 tos and D2 tos), which can grow normally at 30 C but not at 42 C unless protected in high-osmotic medium, by treating *E. coli* B with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMNG). Neither of these mutants was fragile when transferred to medium of low osmolality after culture at the nonpermissive temperature in medium of high-osmolality. However, these mutants differed greatly from each other in many respects.

MATERIALS AND METHODS

1. *Bacteria and Bacteriophages*

E. coli B/SM strain 1-1 (parent strain) was isolated by selecting spontaneous mutants resistant to streptomycin from *E. coli* B, strain Hershey.

Bacteria were grown in trypticase soy broth (TSB) (BBL, Cockeysville, Md.) or Tris-glucose medium (Echols et al., 1961, Otsuji and Aono, 1968) containing 0.1% instead of 0.2% glucose and 6.4×10^{-4} M KH_2PO_4 (high phosphorus medium). For inducing alkaline phosphatase, bacteria were grown in Tris-gulose medium containing 3.2×10^{-5} M KH_2PO_4 (low phosphorus medium) as described by Otsuji and Aono (1968).

In this paper old cultures mean cultures which have been grown at 30 C overnight and then been kept at room temperature for 5 to 7 days. Starved bacteria were those which had been taken from cultures in the exponential growth phase, washed with Tris-buffered saline, pH 7.3 and incubated at 30 C for 60 min with shaking.

Bacteriophages T1, T2L, T3, T4D, T5, T6 and T7 were provided by courtesy of Dr. A. Matsushiro, Department of Microbial Genetics of this Institute.

2. *Measurement of osmolality*

Osmolality was measured by freezing point depression in a Fiske Osmometer, Fiske Associates, Uxbridge, Mass., by courtesy of Miss Fumiko Hojo, Kan-onji Institute, The Foundation for Microbial Diseases of Osaka University, Kan-onji, Kagawa.

3. *Assay of β-D-galactosidase*

Two ml of test sample were mixed with 1.0 ml of 1.0×10^{-3} M *o*-nitrophenyl-*β*-D-galactoside in M/50 phosphate buffer, pH 7.5. After incubation at 37 C for 15 min, 2.0 ml of 1 M Na_2CO_3 were added. The optical density was measured at 420 nm in a Hitachi Perkin-Elmer, UV-VIS Spectrophotometer, Model 139, Hitachi Ltd., Tokyo. For titration of total intracellular enzyme, bacteria were lyzed by incubation with 100 μg/ml of lysozyme and 2,000 U/ml of colistin (polymyxin E) at 37 C for 15 min, and after centrifugation the supernatant was used for tritration.

4. *Assay of alkaline phosphatase*

One ml of test sample was mixed with 3.0 ml of 3.0×10^{-3} M *p*-nitrophenyl phosphate in 1 M Tris-HCl buffer, pH 8.0. The mixture were incubated at 37 C for 30 min and then 1.0 ml of 12%K_2HPO_4 was added. The optical density was measured at 420 nm in a Hitachi Perkin-Elmer UV-VIS Spectrophotometer, Model 139. For titration of total enzyme the bacteria were lyzed with colistin and lysozyme as described above.

5. *Assay of sensitivity to antibiotics and other compounds*

Sensitivity to antibiotics was examined using TRI-DISCS (Eiken) (Eiken Chemicals, Tokyo). Five ml of 0.7% agar containing TSB with or without 20% sucrose were seeded with a mutant or the parent strain from old cultures. The agar was overlayed over 1.5% agar in the same medium. After the top layer had hardened, discs were placed on the top, and the cultures were incubated either at 30 C or at 42 C for 24 hr. Then the diameter of the zone of growth inhibition, if any, was measured.

Sensitivity to methylene blue, acridine orange, trypsin, phospholipase A, lysozyme, lysozyme plus colistin and sodium deoxycholate was assayed in TSB with or without 20% sucrose with incubation at either 30 C or 42 C for 24 hr.

6. *Materials*

NMNG was purchased from K & K Laboratory, New York, N.Y. *o*-Nitrophenyl-*β*-D-galactoside and *p*-nitrophenyl-phosphate were obtained from Nakarai Chemicals, Kyoto. Methylene blue was obtained from E. Merck AG., Darmstadt, Germany. Acridine orange was from Chroma G, Stuttgart-Untertürkheim, Germany. Sodium deoxycholate was obtained from Difco Laboratories, Detroit, Mich. Phospholipase A purified from "Habu" (*Trimeresurus flavoviridis*) venom was provided through the countesy of Dr. R. Murata, National Institute of Health of Japan, Tokyo. Trypsin (2× crystallized)

was purchased from Worthington Biochemical Co., Freehold, N.J. Egg white lysozyme (3×crystallized) was from Sigma Chemical Co., St. Louis, Mo. Colistin (polymyxin E) was obtained from Banyu Pharmaceutical Co., Tokyo. Streptomycin sulfate was from Takeda Chemical Industries, Osaka. Liquid paraffin (Paraffin dickfluüssig, 1 liter=0.88 kg) was obtained from E. Merck AG, Darmstadt, Germany. Rabbit antiserum against the parent strain was obtained by immunizing rabbits with bacteria killed by heating at 56 C for 1 hr. The antiserum was inactivated by heating it at 56 C for 1 hr and stored at −20 C without addition of any preservative. Millipore membranes, HAWP 047 00, HA (0.45 μ in pore size) were obtained from Millipore Filter Corp., Bedford, Mass.

RESULTS

1. *Isolation of mutants*

To isolate TS mutants bacteria were treated with NMNG (Adelberg et al., 1965). Then the bacteria were washed and grown overnight at 30 C. They were then seeded on trypticase soy agar (TSB agar) plates, and incubated at 30 C. After growth, each plate was replicated on two TSB agar plates and two plates of TSB agar containing 20% sucrose. One of each pair of plates was incubated at 30 C, and the other at 42 C. Bacteria which failed to grow only on TSB agar without sucrose at higher temperature were isolated, retested and purified. Finally two strains (C4 tos and D2 tos) were selected for further studies.

The two mutants both gave the same titer as the parent in the quantitative agglutination test against rabbit antiserum to the parent strain. They could both grow in synthetic medium with glucose as the sole carbon source at 30 C. Moreover, like the parent, they were both sensitive to all the members of the T-series of bacteriophages.

2. *Growth of the mutants in high-osmotic medium at 42 C*

Both mutants could grow at the nonpermissive temperature in TSB containing various concentrations of sucrose, glycerol, poly-

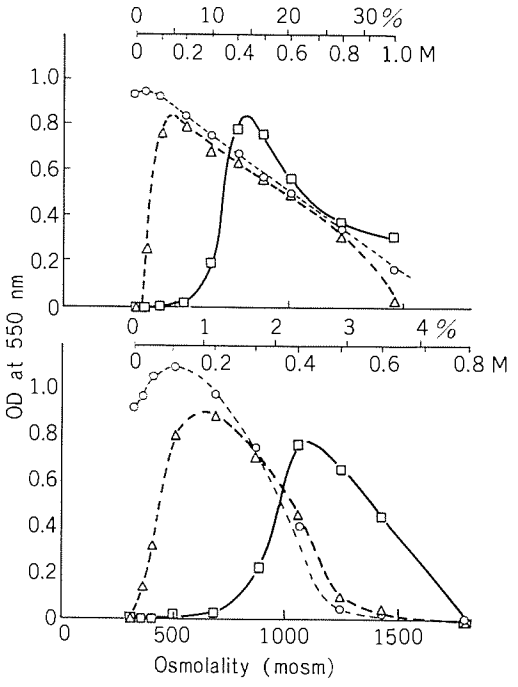

FIGURE 1. *Growth of E. coli B/SM, strain 1-1 (parent) and its TS mutants C4 tos and D2 tos after 800 min of incubation at 42 C in TSB containing various concentrations of sucrose or NaCl.*

Various concentrations of sucrose and NaCl in TSB were prepared by mixing 2.0 M sucrose or 2.0 M NaCl, double-strength TSB and distilled water. Volumes of 3 ml of the media were put into 13×200 mm tubes, with plastic caps, and autoclaved. One set of medium was used for measuring osmolality by freeze-point depression.

The parent and mutant strains were taken from old culture slants, and suspended in Tris-buffered saline, pH 7.3, at a concentration of 1.0×10^8 bacteria/ml. Aliquots of 0.03 ml of each suspension were inoculated into the tubes, and incubated at 42 C with constant shaking. After 800 min of incubation, 0.03 ml of 37% formaldehyde was added to each tube. Optical density at 550 nm was measured in a Hitachi Perkin-Elmer Spectrophotometer.

Top, TSB with sucrose; bottom, TSB with NaCl; ○·····○, *parent strain 1-1;* △----△, *mutant strain C4 tos;* □——□, *mutant strain D2 tos. The scale above each figure represents the concentrations of sucrose or NaCl added.*

ethylene glycol or NaCl. Fig. 1 shows the relationship between the growth at 42 C and the osmolality when TSB was supplemented with sucrose or NaCl. As shown in this figure, the optimal osmolality for growth of D2 tos was higher than for C4 tos.

The efficiency of sucrose for supporting growth of these mutants was the same when the sucrose was sterilized by autoclaving it as a solution in the culture medium as when it is sterilized by filtering it through a Millipore membrane or autoclaving it separately in distilled water and then adding it to concentrated culture medium.

3. *Temperature shift experiments*

When exponentially growing cultures of the mutant strain D2 tos were shifted from 30 to 42 C without protection by high osmolality, the optical density (OD) increased further for a while, remained constant and then decreased, as shown in Fig. 2. However, measurement of viable counts showed that the growth stopped immediately after the shift from 30 to 42 C, remained constant for a while, and then decreased rapidly, as shown in Fig. 3. When the culture was shifted to 30 C after incubation at 42 C without protection the colony-forming ability recovered rapidly, and the bacteria divided at a comparable rate to bacteria maintained continuously at 30 C.

When the OD of the culture incubated at 42 C reached about the maximum, large, plump bacteria were observed microscopically. No lyzed bacteria, ghosts or debris, were observed even when the culture were examined during decrease in the OD.

Fig. 4 shows the growth curves of the mutant strain D2 tos in medium containing various concentrations of sucrose after a shift from 30 to 42 C. With low concentrations of sucrose, maximum or plateau OD values were observed, as in the medium without sucrose. As the sucrose concentration increased, the increase of OD was delayed for a while after the shift, possibly due to temporary plasmolysis of bacteria. However, after this lag period bacteria grew steadily.

The OD of strain C4 tos increased further after shift from 30 to 42 C, as shown in Fig. 2. The viable counts also increased at a somewhat reduced rate.

Microscopic examination of cultures of this mutant growing at 42 C showed many filamentous or chain forms. These filamentous forms were also observed when this mutant was growing at 42 C in medium supplemented with sucrose. This suggests that there might

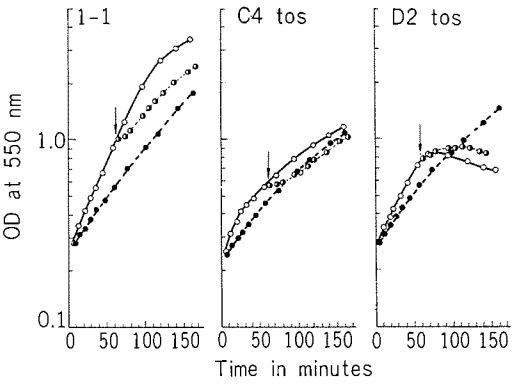

FIGURE 2. *Effects of a temperature shift on the parent and mutant strains (Optical density).*

Three ml of a suspension of each strain grown at 30 C overnight in TSB containing 200 μg/ml streptomycin (SM-TSB) were inoculated into 30 ml of fresh SM-TSB, put in a 200 ml Erlenmeyer flask, and incubated in a water bath at 30 C for 3 hr with constant shaking. Then 10 ml of each culture were inoculated into 50 ml of prewarmed SM-TSB, in a 200 ml Erlenmyer flask, in a water bath at either 42 C or 30 C at time 0, and incubated with constant shaking.

At intervals, 2.0 ml aliquots were removed and mixed with 2.0 ml of cold 4% formaldehyde in Tris-buffered saline, pH 7.3. The mixtures were kept in an ice bath until their optical density at 550 nm was measured in a Hitachi Perkin-Elmer spectrophotometer against SM-TSB diluted with an equal volume of 4% formaldehyde. After incubation for about 60 min, as indicated by arrows, 20 ml of each culture at 42 C were transferred to an Erlenmeyer flask in a water bath at 30 C and treated as above.

The strain is indicated at the top of each figure. ○——○, *growth at 42 C;* ●- - - -●, *growth at 30 C;* ◑·····◑, *growth first at 42 C and then at 30 C.*

be a defect in some step of cell division when this mutant is grown at 42 C. However, it is interesting that when an exponentially growing culture was shifted from 30 to 42 C the mutant continued to grow and even divided, as revealed by the increase in the viable count. On the other hand, bacteria from an old culture or starved bacteria, could not grow at 42 C. Eagan and Russel (1973) reported that some of the mutants which they isolated, continued to grow in liquid medium after shift from 32 to 42 C, although they showed no visible growth on solid media at 42 C.

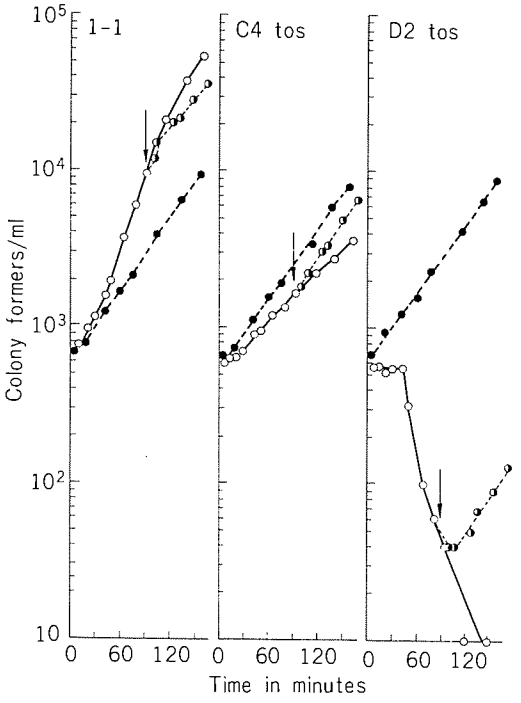

FIGURE 3. *Effect of a temperature shift on the parent and mutant strains (Viable count).*

Each strain was grown at 30 C overnight in TSB containing 200 μg/ml streptomycin (SM-TSB) with constant shaking. Then the cultures were diluted 10^{-4} with SM-TSB, and incubated at 30 C with constant shaking for 90 min. Volumes of 0.2 ml of each culture were inoculated at time 0 into 20 ml of SM-TSB in 125 ml Erlenmeyer flask in a water bath at either 30 or 42 C, and incubated with constant shaking. At intervals, aliquots of 0.1 ml were removed and inoculated onto TSB agar plates containing 200 μg/ml of streptomycin with 5 ml of soft TSB agar also containing 200 μg/ml of streptomycin. After 55 min of incubation, 0.1 ml of a 1/10 dilution of the sample was also added. At 90 min 10 ml of each of the cultures at 42 C were transferred to Erlenmeyer flasks in a 30 C-bath, and incubated in the same way. The plates were incubated at 30 C overnight before counting colonies.

Strains are indicated at the top of the figure. ●----●, incubated at 30 C; ○·····○, incubated at 42 C; ◑·····◑, incubated first 42 C and then at 30 C.

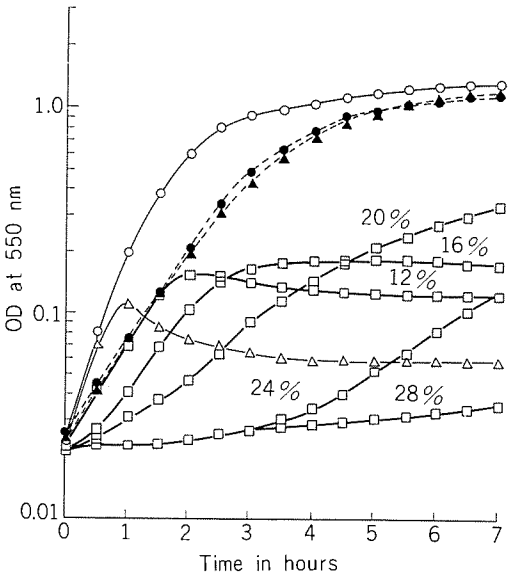

FIGURE 4. *Growth of the mutant strain D2 tos in TSB containing various concentrations of sucrose at 42 C.*

The experimental procedure as for Fig. 2. Bacteria growing exponentially in TSB at 30 C, were transferred at time 0 to TSB containing various concentrations of sucrose and incubated at the temperature indicated. The final concentrations of sucrose after the shift are shown.

○·····○, parent strain 1-1 in TSB without sucrose at 42 C; ●----●, strain 1-1 in TSB without sucrose at 30 C; △·····△, strain D2 tos in TSB without sucrose at 42 C; ▲----▲, strain D2 tos in TSB with sucrose at 30 C, □——□, strain D2 tos in TSB with sucrose at the indicated concentration at 42 C.

4. *Studies on a possible defect of the envelope structure of mutants grown at 42 C*

The mutant strains were grown at 30 C in Tris-glucose medium. Then cultures were shifted to 42 C. Aliquots were removed at intervals, and centrifuged. The OD of the supernatants was measured at 260 nm and at 280 nm. No increase in UV-absorbing materials was observed, even in the supernatant from the culture of D2 tos during decrease in the OD, as described above.

Next the mutants were grown at 30 C in Tris medium containing 0.1% lactose instead of glucose to induce the intracellular enzyme, β-D-galactosidase. Then the bacteria were washed and transferred to Tris-glucose medium, and incubated at 42 C. No release of this enzyme from either mutant into the surrounding medium was observed.

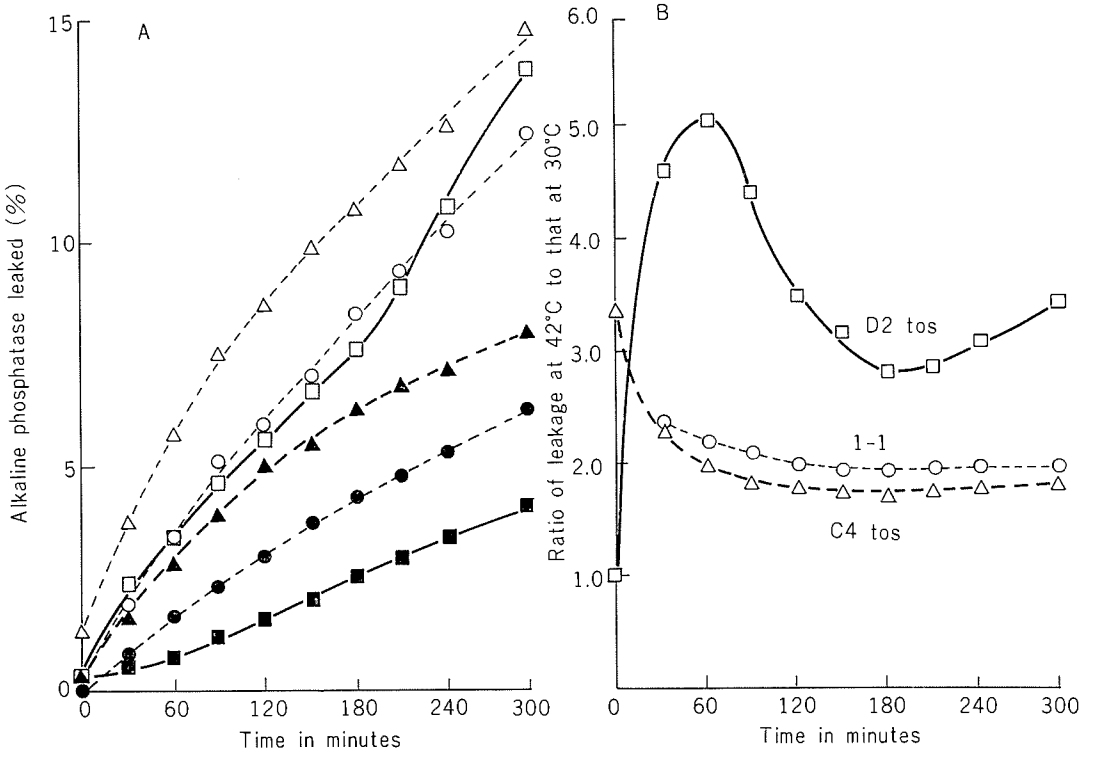

FIGURE 5. *Leakage of alkaline phosphatase from the mutant strains C4 tos and D2 tos and the parent strain 1–1 at 42 C or at 30 C.*

All the strains were grown in low phosphorus Tris-glucose medium. Bacteria were harvested by centrifugation and suspended at a concentration of 8×10^8 *bacteria/ml in Tris-glucose medium without phosphate (TGMS̄P). The suspensions were incubated at 30 C for 30 min and then parts of each suspension were mixed with 4 volumes of TGMS̄P prewarmed at either 30 or 42 C and incubated at these temperatures with constant shaking. At intervals, aliquots were removed and divided into two portions. One was centrifuged and the other was treated with lysozyme and colistin, as described in the Materials and Methods.*

A: percent leakage of enzyme. Open symbols, at 42 C; closed symbols, at 30 C. ○·····○ *and* ●·····●, *strain 1–1;* △----△ *and* ▲----▲, *strain C4 tos;* □·····□ *and* ■·····■, *strain D2 tos.*

B: Ratio of enzyme leakages at 42 C and 30 C. ○·····○, *strain 1–1;* △----△, *strain C4 tos;* □·····□, *strain D2 tos.*

Next the mutants were cultivated in Tris-lactose medium supplemented with 20% sucrose, and each culture was divided into two parts and centrifuged. The precipitated bacteria were suspended in either distilled water or 20% sucrose in Tris-buffered saline, pH 7.3. The OD of these suspensions were the same, showing that there was no lysis of either mutant. A part of each suspension was then centrifuged and β-D-galactosidase activity in the supernatant was measured. Less than 0.35% of the total intracellular enzyme of either mutant leaked out into the distilled water.

Both strains were grown at 30 C in Tris-glucose medium of low phosphorus concentration to induce alkaline phosphatase, which is a peripheral enzyme, known to be located outside the cytoplasmic membrane and inside the outermost layer of the cell envelope. The cultures were then shifted to 42 C.

As shown in Fig. 5, some enzyme leaked out from the mutants. The enzyme leaked out of the parent strain to a similar extent. The ratios of enzyme leakage at 42 to that at 30 of strains C4 tos and 1–1 were similar while the ratio with strain D2 tos was a little higher.

Therefore, these mutants do not seem to be lyzed when cultured at 42 C. However, it is unknown why cultures of the strain D2 tos showed decrease in OD after reaching a maximum value when they were shifted from 30 to 42 C.

5. *Sensitivities of the mutants to various antibiotics and other agents*

The osmotic reversal of temperature sensitivity of these mutants suggests that there is some defect in their cell envelope. Therefore, their sensitivities to some antibiotics, dyes, detergents and enzymes at 30 and at 42 C were compared. Little or no difference was found in their sensitivities at the two temperatures with any of the agents listed in Table 1.

6. *Growth of strain C4 tos at 42 C*

As shown in Figs. 2 and 3, growing cells of strain C4 tos could continue to grow when shifted from 30 to 42 C, but starved bacteria or cells from old cultures could not grow at 42 C.

TABLE 1 *E. coli B/SM, strains 1-1, C4 tos and D2 tos did not show enhanced sensitivities to the following agents when grown on TSB agar with 20% sucrose at 42 C*

ANTIBIOTICS AND CHEMOTHERAPEUTIC AGENTS	Leucomycin
Penicillin (R)[a]	Spiramycin (R)
Aminobenzylpenicillin	Lincomycin (R)
Carboxybenzylpenicillin	Nalidixic acid
Methylphenylisoxazolylpenicillin (R)	Sulfisoxazol
Methylchlorophenylisoxazolylpenicillin (R)	Panfuran
Cephaloridine	DYES
Novobiocin (R)	Methylene blue (R to 0.025%)
Polymyxin B	Acridine orange (R to 0.05%)
Colistin (polymyxin E)	ENZYMES
Chloramphenicol	Trypsin (R to 1 mg/ml)
Tetracycline	Phospholipase A ("Habu" venom) (R to 500 μg/ml)
Streptomycin (R)	Lysozyme (R to 10 mg/ml)
Kanamycin	Lysozyme+Colistin
Gentamicin	DETERGENT
Erythromycin	Sodium deoxycholate (R to 1%)

[a] (R) indicates that all the strains are similarly resistant to the agent.

TABLE 2. *Growth of C4 tos mutant in isotonic Tris-glucose medium at 42 C after inoculated from the culture growing at 30 C*

A) Growth and Inoculum size

Inoculum size		0.1	0.01	0.001
OD at 550 nm	425 min	0.320	0.035	0.020
	1450 min	0.423	0.058	0.021

B) Washed and unwashed inoculum (Inoculum size : 1/25)

Inoculum		Unwashed	Washed
OD at 550 nm	300 min	0.167	0.150
	1150 min	0.343	0.301

Growth was also related to the inoculum size, especially when cells were grown in simple synthetic medium.

A small inoculum of the mutant, which had been grown in Tris-glucose medium at 30 C overnight, could not grow when inoculated into the same medium and incubated at 42 C, as shown in Table 2A.

The growth observed with a larger inoculum was not due to the carry-over of some extracellular substance(s), which promoted the growth of this strain at the restrictive temperature, as will be described later. A similar overnight culture of this mutant in Tris-glucose medium was divided into two parts. One part was centrifuged and the bacteria were washed twice with fresh medium and then resuspended in the medium at the same OD as that of the unwashed culture. The washed and unwashed bacteria were then inoculated into 25 volumes of new medium and incubated at 42 C. As shown in the Table 2B both inocula grew well.

It is well known that *E. coli* shows biphasic growth when it is transferred from one synthetic medium to another containing a different carbon source, which is metabolized only after induction of some adaptive enzymes. After transfer to the new medium, the bacteria continue to grow at reduced rate until they have used up the first carbon source, carried over with the inoculum. Then after a lag period they resume growth on the new substrate using the newly induced enzyme system.

TABLE 3. *Adaptive growth of C4 tos mutant in Tris lactose medium at 42 C after transferred from the culture grown at 30 C in Tris glucose medium*[a]

	30 C	42 C
0.1% glucose Tris medium	0.295	0.323
0.1% lactose Tris medium	0.375	0.329
Tris medium without carbon source	0.048	0.047

[a] C4 tos mutant was grown in 0.1% glucose Tris medium at 30 C for 380 min. 0.5 ml of the culture were inoculated into 10 ml of each medium described above, and incubated further for 960 min either at 30 C or at 42 C. After the incubation, OD at 550 nm was measured by a Coleman Jr. II spectrophotometer.

The experiment shown in Table 3 was to test whether new enzyme was induced in mutant C4 tos after shift to 42 C. As shown in this table, after growth in Tris-glucose medium, the mutant could grow at 42 C in Tris-lactose medium. If a new enzyme system for lactose metabolism could not be induced at 42 C, growth would have been the same as in medium without a carbon source and due only to the glucose carried over with the inoculum. Therefore, this mutant can adapt to the new carbon source and produce a new enzyme

system for metabolizing it even after shift to the nonpermissive temperature.

7. *Growth-promoting factor for strain C4 tos*

E. coli B, strain Hershey, which is sensitive to streptomycin, was streaked on six TSB agar plates, and incubated at 30 C for 6 hr. After incubation, pairs of plates were overlayed with soft TSB agar containing 500 μg/ml of streptomycin and strain 1–1, C4 tos or D2 tos from old cultures. One of each pair was incubated at 30 C and the other at 42 C. The parent strain 1–1 grew over the entire surface of the plate at either 30 or 42 C. Similar growth was observed in the plate overlayed with strain C4 tos and incubated at 30 C as shown in Fig. 6. D2 tos grew well at 30 C. However, it did not grow at all at 42 C, as shown in Fig. 6, and only

FIGURE 6. *Promotion of growth of strain C4 tos by killed E. coli B, strain Hershey. See text for details.*

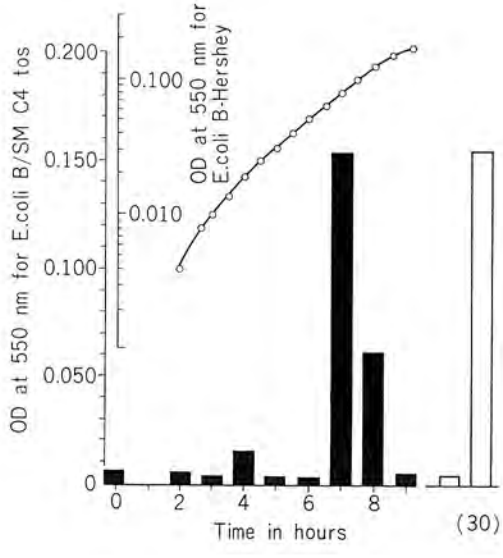

FIGURE 7. *Support of growth of the mutant strain C4 tos at 42 C by the culture filtrate of E. coli B, strain Hershey.*

E. coli, strain Hershey was grown in Tris-glucose medium at 30 C as shown by the curve (○ ○) in the upper part of the figure. At intervals, aliquots were removed, mixed with a final concentration of 200 μg/ml of streptomycin, and filtered through Millipore membranes. One volume of each filtrate was mixed with 9 volumes of fresh Tris-glucose medium, and inoculated with 0.2 volume of the starved mutant suspended in Tris-buffered saline at a concentration of 2×10^8 bacteria/ml. The mixtures were incubated at 42 C overnight. Then the OD was read in a Coleman Junior II spectrophtometer (black bars). As controls, two tubes were incubated without filtrate (white bars), one at 42 C, but the other at 30 C.

faint growth of the streak inoculum of strain Hershey, killed with streptomycin, was observed. Strain C4 tos did not grow at 42 C, except in the area over the streak inoculum of killed strain Hershey, as shown in the center of Fig. 6. This suggests that this mutant strain can grow or start to grow at 42 C by utilizing some factor(s) secreted from strain Hershey.

The growth-promoting factor seems to be secreted from the growing bacteria during about the middle of the exponential growth phase, as shown in Fig. 7.

8. *Anaerobic growth of strain C4 tos at 42 C*

It was found that the TS mutant C4 tos can grow at 42 C anaerobically even from inocula of starved cells or old cultures. As shown in Table 4, this strain, like the parent, could grow anaerobically at 42 C in TSB covered by liquid paraffin. The possible capacity of the overlaying paraffin or of some contaminant in the paraffin to promote the growth was eliminated by the fact that the mutant could not grow aerobically in shaking cultures in L-tubes, even in the presence of paraffin.

E. coli is known to degrade glucose anaerobically by mixed-acid fermentation. TSB contains 0.25% (18.9 mM) glucose. Therefore, it seemed interesting to know whether the end products of the mixed-acid fermentation could support the aerobic growth of this mutant at 42 C. The results obtained with the end products were variable, but in most experiments 1 mM lactate or succinate could support the growth of this mutant. Pyruvate also supported growth in many experiments, while ethanol and acetate supported growth in about half the experiments. Formate usually did not support growth.

The reason for the variability in results is unknown, but might be due to the necessity of another factor besides the end product of mixed-acid fermentation, or to the physiological condition of the inoculated bacteria which would affect their response to these. Further investigations are required on this.

DISCUSSION

Recently many TS mutants have been isolated from *E. coli* and extensively analyzed. Some of these mutants were reported to be able to grow when protected by medium of high osmolality (Matsuzawa et al., 1969, Ricard and Hirota, 1969, Bilsky and Armstrong, 1973, Eagan and Russel, 1973). When they were shifted to the restrictive temperature without protection by high osmolality, some mutants were lyzed (Matsuzawa et al., 1969), some stopped growing but were not fragile like strain D2 tos described in this paper, and others continue to grow like C4 tos in this work (Eagan and Russel, 1973).

Some of these mutants were more sensitive to detergent, dye or various antibiotics at the nonpermissive temperature (Bilsky and Armstrong, 1973, Eagan and Russel, 1973). Neither of the TS mutants described in this paper displayed increased sensitivity to the agents tested.

TABLE 4. *Anaerobic growth of E. coli B/SM C4 tos mutant in TSB at the restrictive temperature*

Strain	1-1		C4 tos	
Growth at	30	42	30	42
Aerobic (in an L-tube)	1.445[a]	1.449	0.880	0
Aerobic with liquid paraffin (in an L-tube)	1.210	1.175	0.593	0
Anaerobic under liquid paraffin layer in a screw-capped tube	0.219	0.258	0.241	0.292

[a] OD at 550 nm by a Coleman Jr. II spectrophotometer.

The TS mutant strain C4 tos possesses very peculiar characters. It can grow at the restrictive temperature when cultured anaerobically or by utilizing some factor secreted by another strain. Further investigations are necessary on these peculiar characters. Studies on mutants like this should contribute useful information on the physiology of the growth of bacteria.

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