Effect of Salt on a Thermosensitive Mutant of *Bacillus subtilis* Deficient in Uracil and Cell Division

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A thermosensitive uracil requiring mutant of *Bacillus subtilis* Marburg 168 thy trp₂ ts42 was examined as to the colony forming ability at the permissive and nonpermissive temperatures. The viability of the mutant cells decreased rapidly at the restrictive temperature in the modified Woese's (MW) medium. However, the cells retained viability when sodium succinate or potassium chloride was added to the medium at that temperature although uracil deficiency was unchanged. A little but significant incorporation of adenine-8-¹⁴C into RNA still continued even after the incorporation of N-acetyl-³H-D-glucosamine into acid insoluble fraction of the cells terminated in the MW medium at 48°C. Both incorporations as well as increase of absorbance were slowed down in the presence of sodium succinate at 48°C. This mutant, ts42, was more sensitive to deoxycholate (DOC) than the parent strain. The restoration of colony forming ability after the temperature shift back from 48 to 37°C was suppressed by the addition of DOC to the medium. However, the cell became resistant to DOC when uracil was added to the medium prior to the temperature shift.

In a preceding paper,¹⁾ a thermosensitive mutant of *Bacillus subtilis*, ts42, is shown to form filament and lose viability at 48°C and rapidly restore colony forming ability when temperature is shifted back to 37°C or uracil is added to the medium at 48°C. These observations suggest that uracil deficiency of this mutant at the restrictive temperature causes unbalance of intracellular material and cell surface because uracil participates both metabolic pathways synthesizing protein through RNA and cell envelope through UDP-sugars.^{2~4)} Therefore, it is investigated whether such unbalance is the case in the present mutant and the colony forming ability is restored by the addition of salt which increases osmotic pressure of the medium. Many of the temperature sensitive mutants including division mutants have been reported to be thermoresistant when salts are introduced to the medium at the nonpermissive temperature.^{5~10)} It is found that the loss of viability of the present mutant ts42 at 48°C is completely suppressed by addition of sodium succinate or potassium chloride though filament formation and uracil deficiency are un-

changed.

MATERIALS AND METHODS

Cell growth. A wild-type strain *Bacillus subtilis* Marburg 168 thy trp₂ and its thermosensitive uracil requiring derivative, ts42, were grown in MW (modified Woese's) medium and allowed to form colony on an NBT-agar plate. The constituents of the media have been shown in a previous paper.¹⁾

The cell growth was followed by increase of absorbance at 550 nm determined by a Spectronic 20 photometer. The total cell number was examined by a Coulter Counter as described previously.¹⁾

Syntheses of RNA and envelope fraction. The incorporation of adenine-8-¹⁴C into RNA or N-acetyl-³H-D-glucosamine into acid insoluble fraction was examined at 37 and 48°C with or without addition of sodium succinate. RNA fraction was prepared following the method of Schmidt and Thannhauser¹¹⁾ as described previously.¹²⁾ In the case of N-acetylglucosamine, 5 μ Ci of tritiated compound with 100 μ g of cold one were added to 10 ml of an exponentially growing culture in MW medium at 37°C at a cell density of 4×10^7 /ml. The mixture was divided into three parts. The first one was incubated at 37°C, the second at 48°C and the third was supplemented with 0.3 M of sodium succinate and incubated at 48°C. At time intervals, 0.25 ml of the suspension was poured into 0.25 ml of 10% ice-cold trichloroacetic acid. After one hr at 0°C, the acid

insoluble fraction was collected by centrifugation and washed twice with 5% cold trichloroacetic acid and once with ethanol-ether. The precipitate was dissolved in one ml of 1 N NH_4OH and the radioactivity was determined by a liquid scintillation counter.

Determination of minimum growth inhibitory concentration (MIC). MICs of the drugs including detergents, antibiotics, chemical agents and basic dyes were determined by the method of broth dilution. Overnight cultures of ts42 and the parent strain in MW medium were diluted 1000 fold with fresh MW medium. The cell growth in the presence of serially diluted drugs was examined turbidmetrically after 48 hr of incubation at 37°C.

RESULTS

Effect of sodium succinate on colony forming ability of ts42

An exponentially growing culture of ts42 at 37°C was shifted to 48°C. The number of

FIG. 1. Number of Total Cells and Colony Formers of ts42.

An exponentially growing culture of ts42 at 37°C was shifted to 48°C at a cell density of $4 \times 10^7/\text{ml}$. After 2.5 hr of incubation, uracil ($10 \mu\text{g}/\text{ml}$) was introduced to portions of the culture (closed symbols). At indicated times, cells were withdrawn and allowed to form colonies at 37°C on an NBT-agar plate supplemented with (□, ■) or without (○, ●) 0.5 M sodium succinate. Total cell number (△, ▲) was examined by a Coulter Counter.

colony formers on an NBT-agar plate supplemented with 0.5 M sodium succinate was compared with total cell number counted by a Coulter Counter (Fig. 1). As described in a preceding paper,¹⁾ the viable counts on an NBT-agar plate began to decrease after one hr of incubation at 48°C. However, when the cells were allowed to form colonies on an NBT-agar plate supplemented with sodium succinate,¹³⁾ the number of viable cells increased about three times as many as those before the temperature shift within 1.5 hr and remained constant thereafter. The total cell count nearly coincided with the number of colony formers on an NBT-agar plate containing sodium succinate.

When uracil was introduced to the culture after 2.5 hr of incubation at 48°C, the number of colony formers increased rapidly with little period of lag as described previously.¹⁾

FIG. 2. Effect of Sodium Succinate on Increase of Absorbance and Colony Forming Ability of ts42.

An exponentially growing culture of ts42 at 37°C was shifted to 48°C at a cell density of $4 \times 10^7/\text{ml}$. Sodium succinate was introduced to the medium at the time of the temperature shift. At time intervals, cells were withdrawn and allowed to form colonies on an NBT-agar plate supplemented with (closed symbols) or without (open symbols) 0.5 M sodium succinate. ○, ●, control; △, ▲, 0.1 M sodium succinate was added; □, ■, 0.3 M sodium succinate was added.

However, the increase of total cell number was observed only 60 min after addition of uracil. The results suggested that the rapid increase of viability after the introduction of uracil was mainly resulted from restoration of colony forming ability by the cells already present at the time of uracil addition and not from the rapid production of newborn cells.

It was examined whether sodium succinate could protect ts42 cells in MW medium at 48°C from losing colony forming ability on an NBT-agar plate. As shown in Fig. 2, the increase of absorbance was slowed down in the presence of 0.3 M sodium succinate but the cells could form colony on an NBT-agar plate as well as that supplemented with sodium succinate. The loss of viability was significantly reduced even by addition of only 0.1 M sodium succinate to the MW medium. Therefore, an appropriate concentration of sodium succinate either in MW medium or an NBT-agar plate was effective in making cells of ts42 retain viability. An optimal concentration of sodium succinate in MW medium was

found to be 0.3 M. The increase of absorbance was suppressed or the number of colony formers reduced at either concentrations more or less than 0.3 M.

Similar results were obtained when potassium chloride was added to the MW medium at 48°C in place of sodium succinate although optimal concentration was different. Therefore, it is plausible that the effect of sodium succinate is brought about not by sodium or succinate ion but as a salt which may influence the osmotic condition of the medium.

Syntheses of RNA and materials containing N-acetylglucosamine

The elongation and loss of viability of ts42 at 48°C were suggested to be derived from uracil deficiency in the pathways including formation of cell wall, septum or envelope other than RNA. As described above, loss of colony forming ability of the mutant at 48°C was suppressed by addition of sodium succinate or potassium chloride. Therefore, the syntheses of RNA and envelope fraction were

FIG. 3. Incorporation of Adenine into RNA (A) and N-Acetylglucosamine into Acid Insoluble Fraction (B).

○, 37°C in MW medium; ●, 48°C in MW medium; ×, 48°C in MW medium containing 0.3 M sodium succinate.

examined in the presence and absence of sodium succinate using radioactive adenine and N-acetylglucosamine as indicators (Fig. 3).

The rate of incorporation of adenine-8- ^{14}C into RNA fraction was shown to be identical both at 37 and 48°C in initial 20 min and slowed down thereafter at 48°C in MW medium. However, significant increase of RNA was observed until 160 min. The uptake of adenine-8- ^{14}C was not so rapid in the medium supplemented with sodium succinate and normally proceeded for 160 min at 48°C. In contrast to the case of RNA, the incorporation of N-acetyl- ^3H -glucosamine into acid insoluble fraction of the mutant terminated within 90 min at 48°C in the MW medium. This coincided with the time when the loss of viability of the cells initiated. Gradual increase of N-acetylglucosamine uptake by the cells at 48°C in the medium enriched with sodium succinate was shown until 120 min when it reached the maximal level observed in MW medium.

Drug sensitivity of ts42 and the parent strain

It has been reported that some bacteria defective in peptidoglycan layer are sensitive to detergents.^{9,14)} As the present mutant ts42 elongated and became labile to osmotic conditions at 48°C, some abnormality of cell surface could be expected. Therefore, MIC of drugs against ts42 was compared with the case of the parent strain by the broth dilution method. Among the detergents tested, deoxycholate was found to be two to four times more effective on the mutant than on the parent (Table I). However, the MICs of following agents against the mutant was comparable to that against the parent; inhibitors of DNA synthesis, mitomycin C and bleo-

mycin A₂; inhibitors of RNA synthesis, rifampicin and streptolydigin; inhibitors of protein synthesis, kanamycin, streptomycin and chloramphenicol; drugs affecting cell surface, colistin, polymyxin, tunicamycin, cycloserine and penicillin G; basic dyes, malachite green and methylene blue.

Effect of deoxycholate on colony forming ability of ts42

An exponentially growing culture of ts42 at 37°C was shifted to 48°C and divided into three parts. All of them were shifted back to 37°C after 75 min of incubation at 48°C. Final concentration of 10 $\mu\text{g/ml}$ of uracil was added to the second part 5 min prior to the temperature shift back but not to the third one. Deoxycholate (48 $\mu\text{g/ml}$) was introduced into both second and third parts at the time of temperature shift back. The number of colony formers were examined and the result is presented in Fig. 4. When the culture was shifted back to 37°C without deoxycholate, the first part, rapid increase of viable count was shown after 10 min of lag. On the other

FIG. 4. Effect of Deoxycholate on Cell Viability of ts42.

After incubation at 48°C for 75 min, cells were shifted back to 37°C (time 0), with (●, x) or without (O) addition of deoxycholate (48 $\mu\text{g/ml}$). Uracil (10 $\mu\text{g/ml}$) was introduced to a portion of the culture (x) 5 min prior to the temperature shift back.

TABLE I. MINIMUM GROWTH INHIBITORY CONCENTRATIONS OF DETERGENTS AGAINST THE PARENT STRAIN AND ts42

Detergent	Parent	ts42
Sodium lauryl sulfate	75 $\mu\text{g/ml}$	75 $\mu\text{g/ml}$
Deoxycholate	300	75~150
Triton X-100	150	150

hand, when shifted back to 37°C with addition of deoxycholate, the viable count decreased and then resumed to the value before the shift but failed to increase thereafter. When the culture was shifted to 37°C after addition of uracil, increase of viable count was similar to that without uracil even in the presence of deoxycholate.

DISCUSSION

It has been reported that a mutant of *Bacillus subtilis*, ts42, requires uracil for growth, forms filaments and loses colony forming ability at 48°C and rapidly restores viability when shifted back to 37°C.¹⁾ In the present results, it was shown when the mutant cells were shifted from 37 to 48°C in MW medium, the incorporation of N-acetyl-³H-glucosamine, a typical component of cell envelope,^{2~4)} into acid insoluble fraction terminated earlier than that of adenine-¹⁴C into RNA. The increase of net protein content observed previously¹⁾ should be supported by this persistently synthesized RNA and let the cell volume increase after the cell envelope formation was suppressed. Thus, the unbalance in the ratio of intracellular materials to cell surface made the cell elongate and osmotically fragile and caused loss of viability. Therefore, it is plausible that the addition of salt such as sodium succinate or potassium chloride to the medium at 48°C protected the cell from such lability by increasing the osmotic pressure of the medium. This kind of salt effect has been reported by several investigators.^{5~10)} Moreover, in case of the present mutant, it has been observed that both incorporations of adenine into RNA and N-acetylglucosamine into acid insoluble fraction at 48°C as well as increase of absorbance were slowed down in parallel in the presence of sodium succinate. This slow and balanced increase of both components might be the reason why the mutant cell could retain colony forming ability when spread on an NBT-agar plate without sodium succinate.

The present mutant was a little more sensitive to deoxycholate than the parent strain at 37°C. This suggests that ts42 is still partially defective in some cell surface structure although growth and morphology were normal at the permissive temperature. The increase of viability after temperature shift from 48 to 37°C was suppressed by addition of deoxycholate to the culture. However, when uracil was introduced to the culture 5 min prior to the temperature shift, viable count of the cells increased abruptly in the presence of deoxycholate as well as in the absence of the agent. Therefore, the mutant cell restores resistance to deoxycholate provided that uracil is added to the culture at 48°C.

From above, it is concluded that uracil deficiency of the present mutant, ts42, at the restrictive temperature causes unbalanced growth resulting in filament formation with concomitant loss of viability which can be rescued by the addition of salt to the culture.

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