THE SALINE-SENSITIVE PHENOMENON IN *BACILLUS SUBTILIS*

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Cells of *Bacillus subtilis* grown to the age of middle or late logarithmic phase were sensitive to saline and lost their colony-forming ability rapidly during the liquid holding. The sensitive cells turned to resistant cells when the growth progressed to stationary phase. The sensitive cells were generally competent, that is, able to take up DNA and perform transformation. Release of DNA and transformation-stimulating agents from the sensitive cells were observed and the saline-sensitive phenomenon in *Bacillus subtilis* was compared with the cold-sensitive phenomenon in *Escherichia coli*.

In the transformation experiments with *Bacillus subtilis* Marburg, washing of the recipient cells with sterile water or saline significantly lessens the yield of transformants. The low yield was found to be a result of rapid loss of viability of the recipient cells during the washing. Characteristics of this phenomenon, called saline-sensitive phenomenon, are described.

Cells of *B. subtilis* Marburg are not always sensitive to saline. Cells harvested from early logarithmic phase and stationary phase are saline resistant. This fact suggests that the high sensitivity to saline is a transient phenomenon. The competent phenomenon in *B. subtilis* (1, 2) and the cold-sensitive phenomenon in *E. coli* (3–8) are also transient phenomena that occur in middle or late logarithmic phase. Interrelationship among these phenomena will be discussed.

MATERIALS AND METHODS

Strains. A tryptophan-requiring mutant of *B. subtilis* Marburg (160 *trp*), a methionine-requiring mutant of *E. coli* K12 (W2252, Hfr *met*), and a wild type strain of *B. cereus* T were used. In the transformation experiments, a wild type strain and an adenine-, leucine-, and methionine-requiring mutant (101 *ade leu met*) of *B. subtilis* were also used.

Media. Lennox medium contained, in 1,000 ml, Bacto tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; and glucose, 1 g. When necessary, the medium was solidified with 15 g/liter of agar. Minimum medium

(MM) contained, in 1,000 ml, KH_2PO_4 , 8.47 g; KOH, 2.26 g; $\text{Na}_3\text{-citrate}\cdot 2\text{H}_2\text{O}$, 0.5 g; $(\text{NH}_4)_2\text{SO}_4$, 1 g; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.2 g; and glucose, 5 g. MM-G medium: Minimum medium devoid of glucose. C2-medium: The medium contained, in 1,000 ml, double the amount of the components in MM, Bacto vitamin-free casamino acids (Difco), 100 mg; L-arginine·HCl, 100 mg; and L-tryptophan, 5 mg.

Transformation procedures. Unless otherwise stated, the transformation experiments were carried out as described by BOTT and WILSON (2). DNA was prepared from a wild strain of *B. subtilis* by the method of SAITO and MIURA (9).

RESULTS

Saline-sensitive phenomenon

An example of the saline-sensitive phenomenon is shown in Fig. 1. In these experiments, *B. subtilis* 160 *trp* was incubated at 37° with gentle shaking in Lennox medium for 30 and 90 min, and the cells were, after being washed once with MM-G medium, suspended in 0.15 M NaCl solution or MM-G medium. The suspension contained about 5×10^7 /ml of colony formers (assayed on Lennox agar medium). The curves in Fig. 1 indicate shift in the number of colony formers during the incubation at 37°. The cells suspended in MM-G medium retained the colony-forming ability for 30 min, but the cells suspended in 0.15 M NaCl solution lost the ability markedly.

Conditions for measuring the killing activity of the saline was studied by using the 90'-cells. In the range of starting cell concentrations from 1×10^7 /ml to 1×10^8 /ml (from 0.1 to 0.8 OD units), the cells lost their colony-forming ability, taking the course similar to that in Fig. 1. In later experiments, the initial OD was adjusted to approximately 0.4 units and colony formers after treatment with 0.15 M NaCl at 37° for 15 min were counted on Lennox agar medium.

$$\text{Survival}(\%) = \frac{\text{colony formers in 0.15 M NaCl}}{\text{colony formers in MM-G}} \times 100$$

The survival percentage fluctuated considerably from experiment to experiment (10 ± 9.9 , mean of 10 independent experiments).

Survival percentage of 160 *trp* cells in plain water or water supplemented with an inorganic salt or sodium citrate was studied. As shown in Table 1, the cells lost their colony-forming ability even when suspended in plain water, but the loss was more remarkable when suspended in water supplemented with either NaCl (0.15 M), KCl (0.15 M), $(\text{NH}_4)_2\text{SO}_4$ (0.1%), or $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ (0.002%). The cells suspended in MM, MM-G, or phosphate buffer (0.05 M, pH 7.2) retained the ability almost completely. Sodium citrate (0.05% as $\text{Na}_3\text{-citrate}\cdot 2\text{H}_2\text{O}$) seemed to protect the cells from loss of viability.

Fig. 1. Loss of colony-forming ability during incubation in 0.15 M NaCl at 37°.

Cells harvested from 30- and 90-min incubation in Lennox medium were suspended in MM-G medium or 0.15 M NaCl after being washed with MM-G medium. Initial concentration was adjusted to 5×10^7 cells/ml. The small figure indicates growth curve of the mother culture.

Saline-sensitive phase in bacterial growth

Cells grown in Lennox medium were diluted to 1/50 with fresh medium, and at intervals during cultivation at 37° aliquots were withdrawn to measure saline sensitivity of the cells. At an incubation time of 210 min, the first culture was diluted again with Lennox medium to 1/50 and saline sensitivity of the growing cells was measured. Fig. 2 shows results of three independent experiments; the cells in the early logarithmic phase and the stationary phase are not sensitive to saline, but the cells in the middle and late logarithmic phase are sensitive.

In *B. subtilis* Marburg, the cells in the late logarithmic phase are known to be able to take up DNA molecules into the cells (competence). Relationship between saline sensitivity and competence was studied. Cells of 160 *trp* were cultivated in nutrient broth at 37°. When the competence was to be examined, the cells ($ca. 2 \times 10^7$ /ml) were transferred to C2 medium and exposed to DNA (0.1 μ g/ml) prepared from a wild type strain. The number of *trp*⁺ transformants might be proportional to the number of competent cells. When the saline sensitivity was to be examined, the cells were treated with

Table 1. Percentage survival of saline-sensitive cells in various solutions.

Solutions tested	Survival (%)
Minimum medium	100
Minimum medium minus glucose	100
KH ₂ PO ₄ (8.5 g/1,000 ml)	1
(NH ₄) ₂ SO ₄ (1 g/1,000 ml)	0.1
MgSO ₄ ·7H ₂ O (0.2 g/1,000 ml)	0.2
Na ₃ citrate (0.5 g/1,000 ml)	43
Plain water	3.1
Phosphate buffer (0.05 M, pH 7.2)	100
KCl (0.15 M)	0.04
NaCl (0.15 M)	0.1

Fig. 2. Saline sensitivity of the cells incubated in Lennox medium.

Cells harvested from the stationary phase of the first culture were inoculated to fresh medium for the second cultivation. The results are the mean of three independent experiments.

0.15 M NaCl as described above. As shown in Fig. 3, the transformants began to appear at the time when the cells become saline sensitive and the number reached the maximum at the time when the cells were highly sensitive to saline. One difference observed was that the cells in the stationary phase were not saline sensitive although they yielded a considerable number of transformants.

Morphology of the saline-sensitive cells

Saline-sensitive cells were harvested from a 120-min culture and their

Fig. 3. Saline-sensitive cells and competent cells.

Competent cells (Δ — Δ) are expressed as *trp*⁺ transformants.

○—○ saline sensitivity curve.

morphological change during treatment with 0.15 M NaCl was examined under a microscope. No significant change was found as far as the cells were observed at a magnification of 600. Release of amylase and alkaline phosphatase from the cells was studied by using the cells suspended in 0.15 M NaCl for 15 min. The release was quite small. Moreover, the supernatants exhibited no lytic activity against *B. subtilis* 160 *trp*. The activity was tested by the spot method and the turbidometric method.

Saline-sensitive and cold-sensitive phenomena

B. subtilis 160 *trp* was cultivated in Lennox medium and *E. coli* K12 W2252 in Lennox medium at 37°, and at intervals during the cultivation the cells were harvested and washed with MM-G. Sensitivity to the saline treatment was examined by keeping the suspension in 0.15 M NaCl at 37° for 15 min and sensitivity to the cold treatment by keeping the suspension in MM-G at 0° for 15 min. The treated cells were plated onto Lennox-agar medium and incubated at 37°. As shown in Fig. 4, *B. subtilis* cells are more sensitive to the saline treatment than to the cold treatment and *E. coli* cells are more sensitive to the cold treatment than to the saline treatment. One significant finding was that there was a growth phase when the saline-sensitive cells were not always cold sensitive and *vice versa*. The progression from cold sensitive state to cold resistant state had been reported by SATO and TAKAHASHI (7).

Saline sensitivity of *Bacillus cereus* cells was also examined. The cells exhibited saline sensitivity throughout the logarithmic phase. The survival

Fig. 4. Saline-sensitive phenomenon and cold-sensitive phenomenon in *B. subtilis* and *E. coli*.

Initial concentration was about 5×10^7 cells/ml. $\bigcirc$ — $\bigcirc$ saline sensitivity curve; $\triangle$ — $\triangle$ cold sensitivity curve.

Table 2. Amount of materials released from 10^{10} cells and relative absorbance at $260 \text{ m}\mu$ per 10^{10} cells.

	Dryweight (mg/ 10^{10} cells)	Absorbance at $260 \text{ m}\mu/10^{10}$ cells
30'-cells	2.05	2.2
120'-cells	5.12	10.2
180'-cells	1.03	1.1

% was less than 1.

Release of DNA from the saline-sensitive cells

Cells of 160 *trp* grown in Lennox medium at 37° were harvested at 30, 120, and 180 min of incubation and, after being washed with MM-G, were suspended in distilled water and shaken at 37° for 15 min. The cells were removed by centrifugation and the supernatant was concentrated by lyophilization. Table 2 shows the amount of the materials released from 10^{10} cells and relative absorbance of these materials at $260 \text{ m}\mu$ per 10^{10} cells. The 120'-cells, which were most sensitive to saline, released the largest amount of UV-absorbing materials. Fig. 5 shows absorption spectra of the concentrated supernatants.

Under the expectation that the supernatant might contain transforming DNA, about 10^8 cells of strain 101 *ade leu met* were exposed to a supernatant that had been prepared from 5×10^6 cells of 160 *trp*. Fig. 6 shows the number

Fig. 5. Absorption spectra of the released materials.
The concentration of released materials was adjusted to 0.3 mg/ml.

Fig. 6. Transforming activities of the supernatants.

Using 101 *ade leu met* as the recipient, *ade*⁺ (blank) and *met*⁺ (black) transformants were scored.

of *ade*⁺ and *met*⁺ transformants appearing on selection media. The supernatant from 120'-cells again gave the largest number of transformants. The supernatants from 30', 60', and 180'-cells also gave a considerable number of transformants. According to an unpublished work of Mr. K. Hashimoto of

Fig. 7. Effect of released materials on the transforming activity of wild type DNA.

Using 160 *trp* as the recipient, *trp*⁺ transformants were recorded.

this laboratory, the 160 *trp* cells cultivated under normal condition release DNA into the medium in early and late logarithmic phases. This fact was found by effecting the transformation by addition of culture filtrate.

Release of transformation-stimulating factors

Akrigg *et al.* (10) reported that a transformation-stimulating factor was released from the competent cells of *B. subtilis* when washed with water. The effect of the supernatant from 30'- and 120'-cells of 160 *trp* on transforming activity of wild type DNA was therefore examined. In the experiments shown in Fig. 7, about 5×10^7 cells of 160 *trp*, which were cultivated in Lennox medium for 120 min, were exposed to a mixture of supernatant and wild type DNA (0.1 μ g/ml). These results suggest that a stimulating agent may be contained in the supernatants. Chemical properties of the stimulating factor are under investigation.

DISCUSSION

As stated above, cells of *B. subtilis* in late logarithmic phase are sensitive to saline. It must be mentioned, however, that the cells are sensitive not only to saline but also to a solution containing KCl, MgSO₄, or other inorganic salts. Even when placed in plain water at 37° for 15 min, the cells lose their colony-forming ability and the survivals decrease to 3% of the initial number.

The sensitivity of bacterial cells to saline is a transient phenomenon. Early logarithmic cells and stationary cells are not sensitive to saline. In *B. cereus*, however, early and late logarithmic cells are saline sensitive, and stationary cells are not. Thus, the saline-sensitive period differs from strain to strain. A common principle is that the saline-sensitive cells disappear with the progression of growth. Stationary phase cells are invariably saline resistant.

A good coincidence was found between the saline-sensitive period and the competent period, although one datum indicated that the competent cells might not always be saline sensitive. Saline-sensitive cells differ from cold-sensitive cells in their appearance. In the cultures of *B. subtilis* and *E. coli*, the highest sensitivity appeared at different periods of growth. Meanwhile, there still remains the possibility that the three phenomena stated above are the result of the same cellular physiology viewed from different aspects.

The saline-sensitive cells release DNA and other materials. This observation suggests that the cell membrane may be especially fragile during the saline-sensitive period, but we do not know whether the release of intracellular materials is the direct cause for the loss of colony-forming ability or not. Recently, SATO and TAKAHASHI (11) reported that DNA in the cold-sensitive cells of *E. coli* was nicked and ready to be degraded into small pieces. This mechanism may also be related to the loss of colony-forming ability and the release of DNA from the cells.

It is widely believed that bacterial cells in early and late logarithmic phase are structurally similar but studies on the cold-sensitive phenomenon, competent phenomenon, and saline-sensitive phenomenon suggest that young cells may pass through a sensitive phase prior to arriving at the stationary phase. In the sensitive phase, the cells may reorganize their internal structures to age into old cells.

REFERENCES

- 1) F.E. YOUNG and J. SPIZIZEN, *J. Bacteriol.*, **81**, 823 (1961).
- 2) K.F. BOTT and G.A. WILSON, *J. Bacteriol.*, **94**, 562 (1967).
- 3) G.G. MEYNELL, *J. Gen. Microbiol.*, **19**, 380 (1958).
- 4) R.E. STRANGE and F.A. DARK, *J. Gen. Microbiol.*, **29**, 719 (1962).
- 5) M. SATO and H. TAKAHASHI, *Agr. Biol. Chem. (Tokyo)*, **31**, 1100 (1967).
- 6) J.R. SMEATON and W.H. ELLIOTT, *Biochem. Biophys. Res. Commun.*, **26**, 75 (1967).
- 7) M. SATO and H. TAKAHASHI, *Agr. Biol. Chem. (Tokyo)*, **32**, 259 (1968).
- 8) M. SATO, M. SUZUKI and H. TAKAHASHI, *Agr. Biol. Chem. (Tokyo)*, **32**, 1270 (1968).
- 9) H. SAITO, and K. MIURA, *Biochim. Biophys. Acta*, **72**, 619 (1963).
- 10) A. AKRIGG, S.R. AYED and G.R. BARKER, *Biochem. Biophys. Res. Commun.*, **28**, 1062 (1967).
- 11) M. SATO and H. TAKAHASHI, Abstracts of the Ann. Meet. of the Agr. Chem. Soc. Japan, p. 267 (1969).