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Growth Patterns of Temperature-sensitive Mutants of *Bacillus Thuringiensis*

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*Bacillus thuringiensis*의 Temperature-sensitive Mutants 분리와 특성연구

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Bacillus thuringiensis was mutagenized with UV light irradiation and nitrosoguanidine. Twenty-four tem perature-sensitive ts mutants were isolated at 42°C and classified into two groups by growth on nutrient agar at 42°C. First is the lethal group, which did not grow at the nonpermissive temperature, the second is the reduced group whose growth was restricted from one-half to one-fourth, Thirteen ts mutants belong to the lethal group and eleven ts mutants belong to the reduced group. Auxotrophic mutant, A-N28 required five amino acids as growth factors, A-N65 also five amino acids, A-N92 seven, A-N115 four and A-N156 three. *Bacillus thuringiensis* wild type is resistant to penicillin, ampicillin, and cephalothin. The ts-U171, A-N92 and A-N115 are sensitive to the three antibiotics. The ts-U601, -U603, -U604 and -U171 did not grow at the permissive temperature after temperature-shifting from 42°C. Four auxotrophic mutants (A-N38, A-N65, A-N92 and A-N115) did not form spores in their cells.

Bacillus thuringiensis is a gram positive, endospore-forming bacillus. The bacterium produces exotoxin[2,3,5,11,16] as well as endotoxin[7,10]. The toxins paralyze and kill certain insect larvae by ingestion[1,17]. Therefore the toxins are useful as bacterial pesticides for biological control of certain insect pests[4,6,8,9].

However, the genetic studies of the *Bacillus thuringiensis* have not been reported yet, so our laboratory has undertaken an extensive genetic study of the microorganism. The study of temperature-sensitive mutants of *B. thuringiensis* should better define the role of each essential bacterial gene in the production of bacterial pesticides. We now report the isolation and the characterization of twenty-one temperature-sensitive (ts) mutants and six auxotrophic mutants of *B. thuringiensis*.

Materials and Methods

Bacterial strains

Bacillus thuringiensis 3ab K-3 was used as for isolation of temperaturesensitive mutants and B. thuringiensis S1 K-2 for isolation of auxotrophic mutants. The strains were maintained on nutrient agar at 4°C for stocks in this laboratory.

Media

Nutrient agar and broth, Muller-Hinton agar and broth (DIFCO), and Spizizen mineral salts media[12] were autoclaved at 121°C to be used.

Amino acids

The following amino acids were used in this experiment; DL-alanine, L-arginine, glycine, L-histidine,

L-leucine, DL-methionine, DL-serine, DL-valine, DL-phenylalanine, DL-aspartic acid and L-glutamic acid (Sigma Co).

Antibiotics

The following antibiotic discs (DIFCO) were used: penicillin (10 units), chloramphenicol (30 μg), tetracycline (30 μg), ampicillin (10 μg), cephalothin (30 μg), erythromycine (15 μg), Kanamycin (30 μg), streptomycin (10μg), genetamycin (10μg) and tobramycin (10 μg).

Isolation of temperature-sensitive mutants

20 ml of sterilized nutrient broth was poured to a 150 ml sterile flask, then inoculated with *B. thuringiensis* 3ab K-3 and incubated at 28°C for 12 hours with shaking. Before they were mutagenized, the number of colony forming units in the 12 hour culture was caculated by plating methods. Then 5 ml of the 12 hour culture was pipetted into sterilized petri-dishes (9 x 2cm) and vertically irradiated with ultra-violet light (253nm, Mitusubishi Co, GL-15W) with gentle shaking at distances of 30cm for 30, 60, 90, and 120 seconds. The UV irradiated cultures were serially diluted and 0.1 ml of each dilution were inoculated on untrient agar plates (actually triple plates per dilution) and then incubated at 28°C for 16 hours. The plates growing about 20 to 30 colonies per plate were duplicated on fresh nutrient plates by replica method and then the one set of plates were incubated at permissive temperature (28°C) and the other at the nonpermissive temperature (42 °C) for 5 days. After 5 days, each numbered colony on the master plates was compared with the replica plates at 42 °C to check the temperature-sensitivity of each colony. If certain colonies showed something different at 42 °C from the characteristics at 28 °C, they may be temperature-sensitive mutants. The selected temperature-sensitive mutants were confirmed twice by the same methods described above.

Growth characteristics of the ts mutants:

20ml of sterized nutrient broth were asceptically transferred into a 100ml flask, then each ts mutant was inoculated into the flasks and cultured at 28°C for 15 hours. After 15 hours, 3ml aliquots of each culture was transferred into two 50ml fresh media, repectively, and the one flask again was cultured at 28°C and the other 42°C with shaking. Then their growth was measured at 60 minute intervals by optical density at 640 nm with Spectronic 20 (Bauch and Lomb Co) and then the data were ploted.

Isolation of auxotrophic mutants of *B. thuringiensis* SI K-2

20ml of nutrient broth was poured into a 150ml flask, and then 4 loops of *B. thuringiensis* S1 K-2 wild type strain were inoculated and incubated with shaking at 28°C for 12 hours. After twelve hours, 200μg of nitrosoguanidine per ml of culture media was added in the 12-hour culture, and cultured 30 minutes more. Then the treated cells were spun down at 3,000rpm for 20 minutes and washed twice with sterile Spizizen media. The pellets were resuspended in 10ml of Spizizen media, serially diluted and plated on nutrient agar plates. Properly diluted bacterial solutions were plated again on the nutrient agar plates and incubated at 28°C for 12 hours. Then the plates were made replica plates both on the Spizizen mineral salts agar and on nutrient agar, and incubated at 28°C for 7 days. After 7 days the growth of colonies on the Spizizen plates were compared with that on the nutrient agar. The isolated auxotrophs were reexamined twice.

The selected auxotrophic mutants were checked for their amino acid reqirements as growth factors. Eleven amino acids were weighed separately and then dissolved in distilled water, filtered with 0.45 μm millipore filters, and put into the separate Spizizen media. The selected auxotrophs were inoculated on the Spizizen agar plates containing different amino acids and their growth observed for 7 days. The amounts (*mg/l*) of amino acids added were as follows; DL-Ala (80), L-Arg (160), Gly (80), L-His (160), L-Leu (100), DL-Met (160), DL-Ser (80), DL-Val (320), DL-Phe (200), DL-Asp (100) and L-Glu (500).

Examination of Antibiotic Resistance

B. thuringiensis S1 K-2 and its auxotrophs and *B. thuringiensis* 3ab K-3 and its ts mutants were isolated and examined for their antibiotic resistance on Muller-hinton agar at 28°C. Antibiotic discs were placed on the Muller-hinton agars plates streaked with the bacteria and incubated for 16 hours. After 16 hours, microbial susceptiblity was examined.

Observation of Endospore-formation in the Auxotrophs of *B. thuringiensis* S1 K-2

The formation of endospores was observed following the Robinow staining method[13] and then rechecked by the boiling method[14].

Results

Characteristics of mutagenesis of *B. thuringiensis* 3ab K-3

Bacillus thuringiensis 3ab K-3 showed sensitivity against ultra-violet light (Table 1). As UV light irradiation time increased, the number of the viable cells decreased. From the 30 second treatment, 342 colonies were isolated and screened, and from them 11 ts mutants were selected. The frequency of occurrence of the ts mutants was 3.2%. From the 60 second treatment, nine ts mutants out of 350 isolates were selected, and the ratio of occurrence of the ts mutants was 2.5%. From the 90 second treatment it was 1.6%, and from the 120 second treatment it was 4.6%. Consequently at 30 and 120 second treatments the high frequency was observed.

Table 1. Characteristics of Mutagenesis of *Bacillus thuringiensis*

UV (second)	No. of viable cells/ml	Total colonies isolated	No. of Mutants found	%
0	8.7×10^{7}	–	–	
30	8.0×10^{7}	342	11	3.2
60	1.9×10^{6}	350	9	2.5
90	1.1×10^{6}	258	4	1.6
120		64	3	4.6

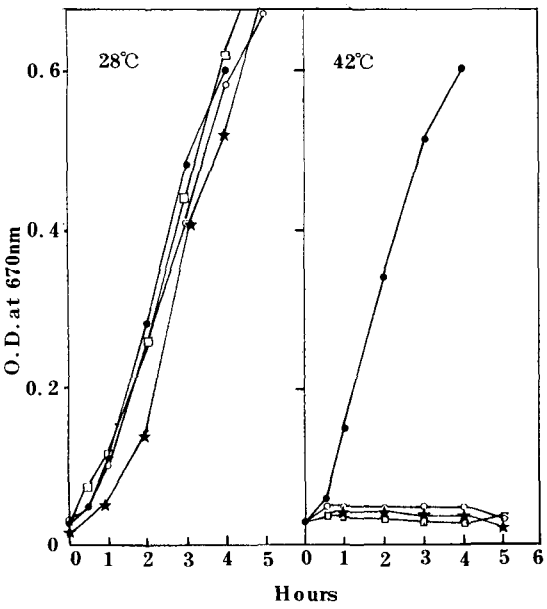

Fig. 1. Comparative Growth Patterns of Temperature-sensitive Mutants of *Bacillus thuringiensis* 3ab K-3 at 28℃ (permissive temperature) and at 42℃ (nonpermissive temperature). (●); wild type, (○); ts-U603, (★); ts-U604, (□); ts-U605.

Table 2. Properties of Temperature-sensitive Mutants of *Bacillus thuringiensis* K-3 on Nutrient agar

Group	Mutants	Growth Characteristics 28℃	42℃
	wild type	G (50)	G (50)
lethal	ts-U23	G (70)	NG
	ts-U31	G (70)	NG
	ts-U71	G (80)	NG
	ts-U26	G (70)	NG
	ts-U73	G (70)	NG
	ts-U74	G (81)	NG
	ts-U91	G (70)	NG
	ts-U171	G (90)	NG
	ts-U601	G (100)	NG
	ts-U602	G (111)	NG
	ts-U603	G (90)	NG
	ts-U604	G (100)	NG
	ts-U605	G (110)	NG
Reduced	ts-U21	G (70)	R (30)
	ts-U32	G (90)	R (24)
	ts-U33	G (70)	R (20)
	ts-U61	G (80)	R (21)
	ts-U72	G (85)	R (20)
	ts-U131	G (80)	R (24)
	ts-U132	G (60)	R (20)
	ts-U151	G (70)	R (20)
	ts-U154	G (70)	R (32)
	ts-U788	G (55)	R (20)
	ts-U1105	G (46)	R (22)

This experiment was carried out by replica plate method. The numbers in paraentheses are colony size measured in mm. G means growth, R, reduced growth, and NG: no growth.

Isolation of temperature-sensitive mutants of *B. thuringiensis* 3ab K-3

Twenty-four temperature-sensitive mutants showing phenotypic differences at 42 °C compared with those at the permissiv temperature of 28 °C were isolated. The mutants may be classified into two phenotypic groups on nutrient agar at 42 °C (Table 2). First is the lethal group which did not grow at the nonpermissive temperature.

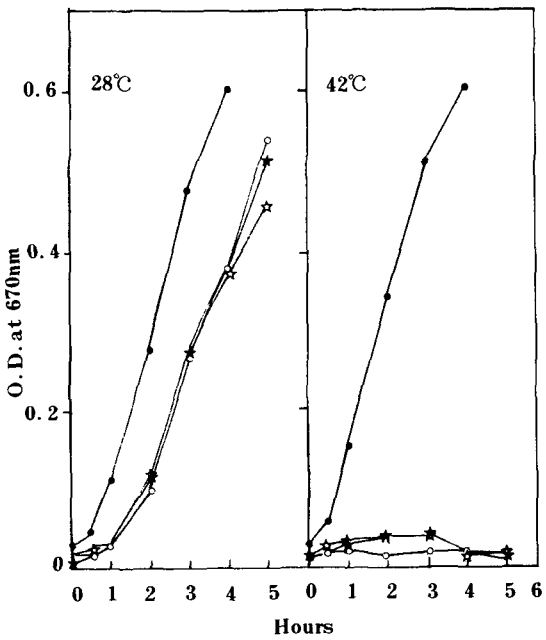

Fig. 2. Compartive Growth Patterns of Temperature-sensitive Mutants of *Bacillus thuringiensis* 3ab K-3 at 28℃ (permissive temperature) and at 42℃ (nonpermissive temperature). (○); ts-U72, (★); ts-U73, and (☆) ts-U74.

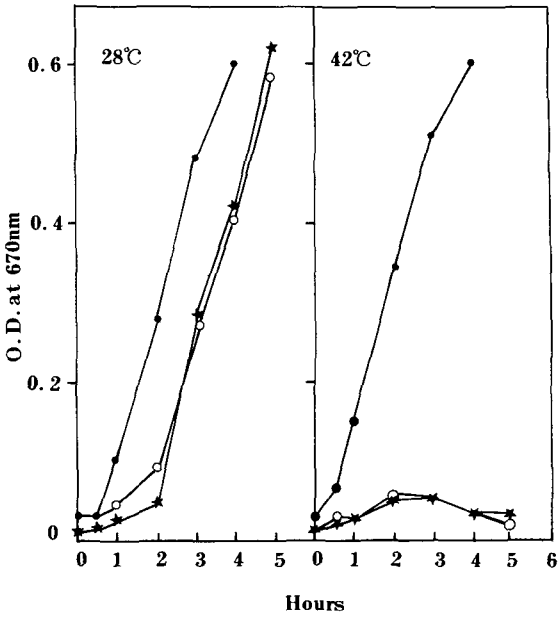

Fig. 3. Comparative Grewth Patterns of Temperature-sensitive Mutants of *Bacillus thuringensis* 3ab K-3 at 28℃ (permissive temperature) and at 42℃ (nonpermissive temperature). (○); ts-U31, and (★); ts-U26.

The second is the reduced group whose growth was restricted from one-half to one-fourth. Thirteen is mutants belong to the lethal group and eleven is mutants belong to the reduced groups (Table 2).

Growth patterns of the twenty-four ts mutants in broth culture

All the temperature-sensitive mutants isolated showed temperature-sensitivity in liquid cultures at the nonpermissive temperature, too. According to the results shown in figures 1 through 8, the twenty-four ts mutants are divided into two groups: lethal growth and reduced growth groups. Eight ts mutants (ts-U603, -U604, -U605, -U72, -U73, -U74, -U32 and -U26) out of the twenty-four did not grow at all in the nutrient broth at 42°C (Fig. 1, 2, and 3). Other nine ts mutants (ts-U171, -U23, -U1105, -U71, -U154, -U601 and -U61) belong to the reduced growth group 1, which grew little in the nutrient broth at the non-permissive temperature (Fig. 4,5, and 6). The remaining seven ts mutants (ts-U131, -U602, -U132, -U32, -U91, -U21 and -U788) belong to the reduced growth group 2, which showed a little more growth than the former nine mutants, however their growths are very poor when compared with those at the permissive

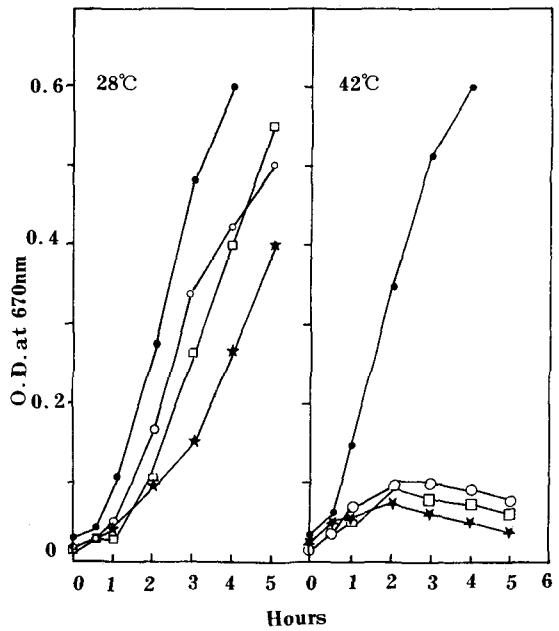

Fig. 4. Compartive Growth Patterns of Temperature-sensitive Mutants of *Bacillus thuringiensis* 3ab K-3 at 28℃ (permissive temperature) and at 42℃ (nonpermissive temperature). (□); ts-U23, (○); ts-U171, and (★) : ts-U1105.

temperatures (Fig. 7 and 8).

The wild type strain grew very well either at the permissive or at the nonpermissive temperatures, and also all the ts mutants above mentioned grew as well as the wild type at the permissive temperature.

Growth requirements of auxotrophic mutants of *B. thuringiensis* SI K-2

Seven auxotrophic mutants of *B. thuringiensis* S1 K-2 were selected and then their requirements of amino acids and carbohydrates for growth were examined. A-N28 mutant required alanine, histidine, phenylalanine, aspartic acid and glutamic acid as growth factors. A-N65 mutant required arginine, histidine, phenylalanine, aspartic acid and glutamic acid as growth factors. A-N92 mutant required seven amino acids, as shown in table 3. Mutant A-N115 required four amino acids, and A-N156 required three amino acids as growth factors (Table 3).

Antibiotic resistance of *B. thuringiensis*

The wild type strains of *B. thuringiensis* SI K-2 and 3ab K-3 showed antibiotic resistance against penicillin, ampicillin, and cephalothin. The ts-U171 gained sensitivity to the three antibiotics. Auxotrophic mutants, A-N92 and

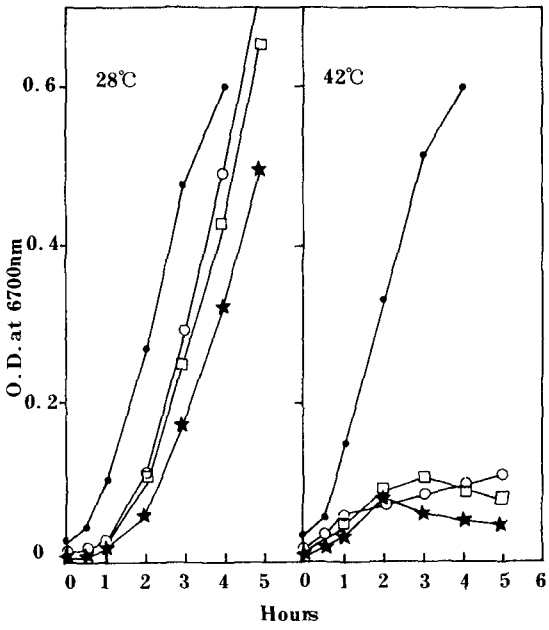

Fig. 5. Comparative Growth Patterns of Temperature-sensitive Mutants of *Bacillus thuringiensis* 3ab K-3 at 28℃ (permissive temperature) and at 42℃ (nonpermissive temperature). (□); ts-U33, (○); ts-U71, and (★); ts U154

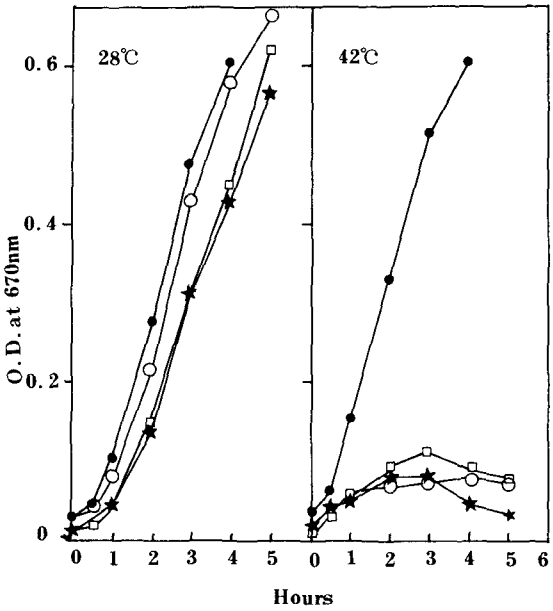

Fig. 6. Compartive Growth Patterns of Temperature-sensitive Mutants of *Bacillus thuringiensis* 3ab K-3 28℃ (permissive temperature) and at 42℃ (nonpermissive temperature). (★); ts-U61, (□); ts-U151, and (○); ts-U601.

-N115 also showed sensitivity to the three antibiotics. The other mutants had resistance against the three antibiotics. The wild type and the mutant strains are sensitive against the other antibiotics tested.

Table 3. Growth Factors of Auxotrophic Mutants of *Bacillus thuringiensis* K-2

auxotrophs / factors	28	38	65	92	115	156
DL-alanine	+	+	−	+	−	−
L-arginine	−	+	+	+	−	−
glycine	−	+	−	−	−	−
L-histidine	+	+	+	−	−	−
L-leucine	−	+	−	−	−	−
DL-methionine	−	+	−	−	−	−
DL-serine	−	+	−	+	+	+
DL-valine	−	+	−	+	−	−
DL-phenylalanine	+	+	+	+	+	+
DL-aspartic acid	+	+	+	+	+	−
DL-glutamic acid	+	+	+	+	+	−

"+" indicates requirements of the amino acid, "−" no requirements.

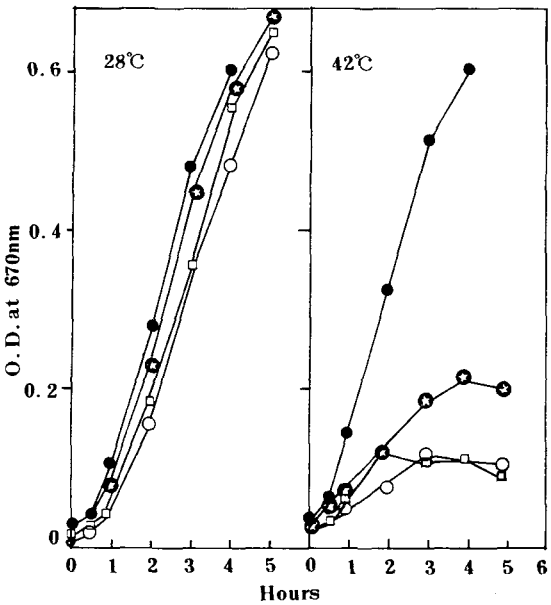

Fig. 7. Compartive Growth Pattems of Temperature-sensitive Mutants of *Bacillus thuringiensis* 3ab K-3 at 28℃ (permissive temperature) and at 42℃ (nonpermissive temperature). (□); ts-U131, (○); ts-U132, and (★) : ts-U 602.

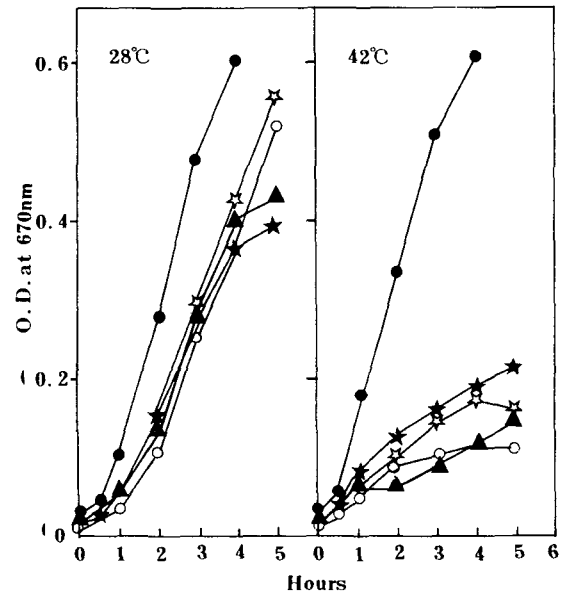

Fig. 8. Compartive Growth Pattems of Tempera ture-sensitive Mutants of *Bacillus thuring iensis* 3ab K-3 28℃ (permissive temperature) and at 42℃ (nonpermissive temperature). (▲); ts-U121, (○); ts-U32, (☆); ts-U91, and (★); ts-U788.

Effects of temperature-shifting culture of the ts mutants

Eight ts mutants were shifted from the 42°C to 28°C during culture. The ts-U601, -U603, -U604 and -U171 did not grow at 28°C, but ts-U602, -U605, -U788 and -U1105 grew at 28°C after temeprature-shifting from 42°C to 28°C.

Asporogeneous mutants of *B. thuringiensis* S1 K-2

Four auxotrophic mutants (A-N38, A-N65, A-N92 and A-N115) did not form spores in their cells.

Disccussions

Twenty-four ts mutants were isolated after UV irradiation. On the solid media, thirteen ts mutants did not grow at the nonpermissive temperature, and the growth of the other eleven ts mutants was reduced. Out of the reduced group, the growth of ts-U72 was one-fourth restricted. However, in the liquid culture, eight ts mutants out of the twenty-four did not grow at all at 42°C, nine of them grew very poorly, and the remaining seven grew a little but when compared with their growth at the permissive temperature, their growth was restricted greatly. Mutant ts-U1105 grew a little on the solid media, but did not grow in the liquid culture at 42°C. Mutant ts-U602 and -U91 did not grow on the solid media, but in the liquid culture they grew a little. Further work on these mutants may be of interest from these properties.

An interesting feature observed with ts-U601, -U604 and -U171 is not to grow further when they were shifted to 28°C after having been cultured at 42°C. The other four mutants, ts-U602, -U605, -U788 and -U1105 grew after temperature-shifting from 42°C to 28°C.

Seven auxotrophic mutants of *B. thuringiensis* S1 K-2 required several amino acids as their growth factor. Mutant A-N38 grew on the media containing all the nine amino acids, but it did not grow on the Spizizen mineral salts medium. A-N115 required Ser, Phe, Asp and Glu as growth factors. A-N156 required three amino acids, namely Ser, Phe and Asp. These characteristics will be useful as markers for future studies.

B. thuringiensis S1 K-2 and 3ab K-3 strains have antibiotic resistance toward penicillin, ampicillin, and cephalothin. These organisms may have antibiotic resis-

tant R-plasmids. These R-plasmid will be useful for ther work in genetic engineering using these microorganisms[15]. Auxotrophic mutants, A-N92 and-N115 might be mutated in R-plasmids, therefore they obtain antibiotic susceptibiltity toward the three antibiotics.

One of the interesting findings is asporogenous auxotrophs of *B. thuringiensis* 3ab K-3. Four auxotrophs, A-N38, -N65, -N92 and -N115 lose their abilities to form of endospores in their cells. This spoproperties are very important for the further genetic studies.

The phenotypes and characteristics of the mutants described herein form a basis for continued genetic analysis. More biochemical and molecular biological works are necessary for further understanding the nature of the mutant defects. Several of the mutants may be of considerable interest in exploring the control of *Bacillus thuringiensis* spore formation. Antibiotic resistant characteristics are useful for further genetic work because they may be utilized as a vector system carrying foreign DNA fragments.

요 약

Bacillus thuringiensis 3ab K-3 균주를 28℃에서 UV광으로 돌연변이를 유도한 다음에 42℃에서 24개의 Temperature-sensitive(ts) 돌연변이 균주를 분리하여 42℃에서의 성장과 항생제 저항성에 대한 성격을 연구하였다. 24개의 ts 돌연변이 균주중에 13개의 ts돌연변이체는 42℃의 제한온도에서 전혀 성장을 하지 않았으며, 나머지11개의 돌연변이체는 성장이 28℃에서 보다 1/2 에서 1/4까지 감축되었다. 이러한 성격에 따라서 ts 돌연변이체는 치사군과 비치사군으로 나눌수 있었다.

또한 *B. thuringiensis* SI K-2을 nitrosoguanidine으로 28℃에서 처리하여 7개의 Auxotrophic mutant을 분리하였다. A-N28은 Arg^-, Gly^-, Leu^-, Met^-, Ser, Val을 요구했고, A-N 65는 Ala^-, Gly^-, Leu, Met, Ser, Val을 요구했으며, A-N92는 Gly^-, Leu, Met을, A-N 115는 7개의 아미노산을, A-N156은 8개를 요구했다.

B. thuringiensis 3ab K-3와 SI K-2는 Penicillin, Ampicillin과 Cephalothin에 저항성을 나타냈고, ts-U171, A-N92와 A-N115는 이 세항생제에 민감성을 나타냈다.

Temperature-sensitive돌연변이균주를 42℃에서 24시간 배양후에 28℃로 옮기어 배양한 결과 ts-U601, -U603, -U604와 -U171은 성장을 더 이상하지 않았다.

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