Kor. J. Appl. Microbiol. Bioeng.
Vol. 13, No. 2, 103 - 107 (1985)

Curing Action of Antibiotic Resistant Factor in *Bacillus subtilis*

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(Received May 16, 1985)

*Bacillus subtilis*의 항생물질 내성에 대한 Curing작용

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(1985년 5월 16일 수리)

A variety of plasmid curing agents such as sodium dodecyl sulfate, acriflavine, ethidium bromide, and mitomycin-C were used to cure *Bacillus subtilis* cells of streptomycin resistant factor. The drug susceptibility was increased by 0.1% sodim dodecyl sulfate at stationary growth phase. The curing frequency was obtained highly at 4 μg/ml of acriflavine, 10 μg/ml of ethidium bromide, and 200 μg/ml of mitomycin-C, respectively. Curing action occurred competitively for the streptomycin and terramycin resistant factors in *B. subtilis*.

For antibiotic resistance in a strain of *B. subtilis*, the correlation between sensitivity and the loss of extrachromosomal DNA nicely complements evidence from curing and infectious transfer that plasmids are involved.[1-4] Bacterial chromosome and plasmid as replicons are associated directly with a segment of membrane.[5] An anionic detergent such as sodium dodecyl sulfate (SDS) may destroy the cell completely or partially which would further result in the lysis of bacteria.[6] R or F factors associated more closely with the cell membrane as small replicons than the chromosome would then be damaged more easily by SDS. Some sorts of repair may then follow, and cells with R or F factors partially or completely eliminated might survive in the culture.[7-9] Meanwhile intercalating acridine dyes[7,10] and phenathridinium dyes such as ethidium bromide[7,11,12] might act on R factor harboring cells via mechanisms of both selective inhibition of plasmid replication and selective toxicity. Mitomycin-C as an antineoplastic drug decomposes DNA by forming a complex and inhibits division of cells by interfering with the biosynthesis of DNA.[7] As a partial work on the construction of competent *B. subtilis*[13], a variety of plasmid curing agents were used to free or cure *B. subtilis* cells of antibiotic resistant factor, and some properties of the curing action were determined.

Materials and Methods

Bacterial Strains

B. subtilis KPM 44 is a met^- mutant derived from the nitrosoguanidine treated IAM 12118 (Str^R). KPM 143 (Str^R, Ter^R, Kan^R, Hg^R, met^-) is a UV treated mutant of KPM 44. KPM 3 (Str^S, spo^-) is a SDS treated mutant of IAM 12118.

Media and Culture Conditions

B. subtilis was held on tryptose agar base overnight prior to use as inocula for the curing of R factor. The cells for the curing test were grown in 10 ml of Penassay broth[14] at 30°C with shaking. For the isolation of antibiotic resistant cells, nutrient agar medium was used with appropriate concentrations of antibiotics.

Curing by SDS

An overnight culture of *B. subtilis* IAM 12118 on tryptose agar containing 50μg/ml of streptomycin was diluted to abou

10^4 cells in 10 ml of Penassay broth with 0.1% SDS, and incubated in shaker.[7-9] Survivors were plated on nutrient agar after dilutions in saline. Colonies on the plate were tested for their drug resistant characters by replica plating or toothpick picking on nurtient agar containing streptomycin (50 μg/ml).

Curing by Acriflavine

An overnight culture of strain KPM 44 was diluted into Penassay broth to give a cell concentration of 10^3 per ml.[7] The cultures with acriflavine were then incubated for 24 hrs at 30°C with shaking, diluted, and plated onto nutrient agar plates. Colonies were tested for the resistant characters by replica plating on streptomycin (50 μg/ml) plates.

Curing by EtBr

Strain KPM 44 and KPM 143 were treated by ethidium bromide with the above method. Colonies were tested for the resistant characters by replica plating on streptomycin (50 μg/ml) or terramycin (200 μg/ml) plates.

Curing by Mitomycin-C

Strain KPM 44 was diluted to 10^4 cells per ml of Penassay broth, and treated by Mitomycin-C with the above method. The cultures were incubated for 40 hrs, and tested for the resistant characters.

Segregation Pattern of Susceptibility

In order to know the frequency of susceptibility, KPM 143 was treated with ethidium bromide. About 10^4 cells were inoculated in 10 ml of Penassay broth cotaining 100 μg of ethidium bromide, and incubated at 30°C overnight.

Results and Discussion

Effect of SDS on Cell Growth

In order to make cured mutants as recipient cells of *B. subtilis*, streptomycin resistant strain IAM 12118 was incubated in Penassay broth containing SDS by inoculating about 10^3 cells per ml. The effect of SDS on the gowth of cells is shown in Fig. 1, which shows SDS at concentrations higher than 0.05% lyses cells efficiently after the culture reached stationary phase. So it could find out that the range of 0.05% made most of the cells have been lysed at this stage, but the survivors were usually enriched for bacteria which had lost the drug resistant factor.

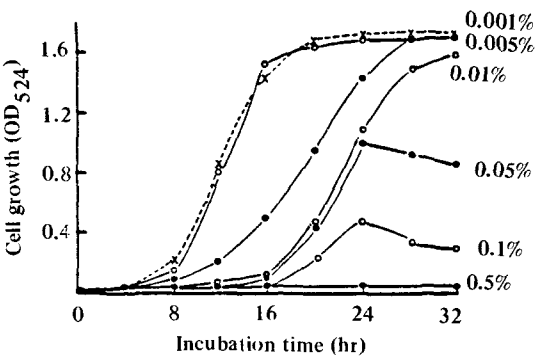

Fig. 1 Effect of sodium dodecyl sulfate on the growth of *B. subtilis* IAM 12118 in penassay broth.
X---X, cell growth in penassay broth without SDS.

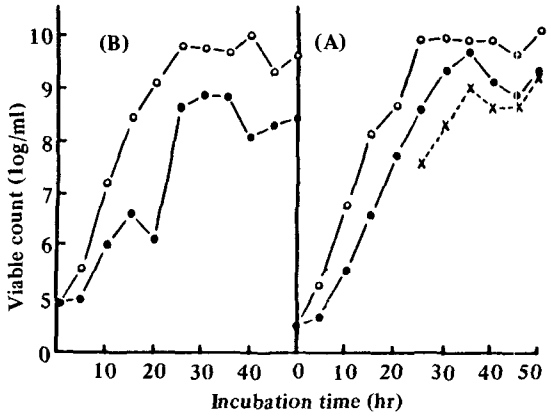

Fig. 2 Kinetics of the sodium dodecyl sulfate treatment on *B. subtilis* IAM 12118 Str^R cells (A) and KPM 3 Str^S cells (B).
IAM 12118 ($3.5x10^5$ cells/ml) and KPM 3 ($7.9x10^5$ cells/ml) in penassay broth with or without 0.1% SDS were incubated at 30°C with shaking. ○ – ○, viable count without SDS; ● – ●, viable count with 0.1% SDS; X -- X, viable count of drug susceptible cells formed in the culture with 0.1% SDS.

Curing Action by SDS

Table 1 shows the elimination of streptomycin resistant factor in *B. subtilis* IAM 12118 by treatment of SDS. It was found that, not as the case of R cells *Proteus mirabilis*[8] and *E. coli*[9]; 48 hrs of incubation time appeared with highest frequencies of drug susceptibility. The effect of inoculum size on susceptibility was not so significant, but a little low frequency of drug susceptibility had been resulted with 10^8 cells were inoculated.

Kinetics of SDS Treatment

For the kinetics of elimination by SDS treatment, IAM 12118 as a streptomycin resistant strain and KPM 3 as a sensitive strain were treated with 0.1% SDS, then the survivors were analyzed at intervals. The results are shown in Fig. 2. Generation times for these strains in the exponential phase, when treated with SDS, were only slightly longer. After the viable count reached maximum, cells began to lyse slowly.

Table 1. Elimination of streptomycin resistant factor in *B. subtilis* IAM 12118 by sodium dodecyl sulfate

A) Inoculum size ; 1.3×10^3 cells/ml

SDS (%)	Incubation (hr)	Cells/ml	Susceptible cell/tested cell*	Frequency (%)
0	24	6.3×10^8	0/304	0
	48	8.4×10^9	0/ 44	0
	72	7.4×10^8	0/ 74	0
0.01	24	3.5×10^8	0/124	0
	48	1.1×10^9	43/ 67	64.2
	72	1.9×10^8	43/ 61	70.5
0.1	24	9.0×10^7	2/ 36	5.6
	48	1.0×10^8	30/ 32	93.8
	72	1.3×10^9	104/116	89.7
1.0	24	5.8×10^4	9/138	6.5
	48	5.3×10^6	52/ 67	77.6
	72	2.9×10^6	184/343	53.6

B) Inoculum size : 1.2×10^8 cells/ml

SDS (%)	Incubation (hr)	Cells/ml	Susceptible cell/tested cell*	Frequency (%)
0	24	2.4×10^{10}	0/220	0
	48	1.1×10^{10}	0/281	0
	72	8.2×10^8	0/ 82	0
0.01	24	8.0×10^8	5/ 71	7.0
	48	4.5×10^9	21/ 39	53.8
	72	2.7×10^8	15/ 27	55.6
0.1	24	3.5×10^8	3/ 33	9.1
	48	4.8×10^8	21/ 25	84.1
	72	7.0×10^7	6/ 21	28.1
1.0	24	1.1×10^6	2/114	1.8
	48	2.0×10^7	58/100	58.0
	72	6.3×10^5	17/ 99	17.2

* Number of colonies showing streptomycin susceptibility per number of colonies tested.

and the net number of drug susceptible cells was increased.

Curing Action by Acriflavine

In order to make one of cured mutants, the elimination of streptomycin resistance in KPM 44 has been done by acriflavine treatment. Numbers of colony from which streptomycin resistance is eliminated are listed in Table 2. A representative strain of drug sensitive mutant was selected as KPM 82.

Curing Action by EtBr

In order to make one of cured mutants, the elimination of streptomycin resistance in KPM 44 has been done by ethidium bromide treatment. The results are listed in Table 3. A representative strain of drug sensitive mutant was selected as KPM 73.

Segregation Patterns

As shown in Table 4, the elimination frequency of streptomycin resistance was found to be less efficient than that of terramycin resistance. With respect to segregation pattern of

Table 2. Elimination of streptomycin resistance in *B. subtilis* KPM 44 by acriflavin

Concentration (μg/ml)	Viable cells /ml	Susceptible cell/tested cell*	Frequency (%)
0	3.7×10^9	0/127	0
1	3.4×10^9	51/ 94	54.3
2	9.1×10^5	59/ 91	64.8
4	9.8×10^4	40/ 47	85.1
6	1.8×10^2	10/ 18	55.6
8	0	–	–

* Number of colonies showing streptomycin susceptibility per number of colonies tested.

Table 3. Elimination of streptomycin resistance in *B. subtilis* KPM 44 by ethidium bromide

Concentration (μg/ml)	Viable cells /ml	Susceptible cell/tested cell*	Frequency (%)
0	3.8×10^9	0/136	0
5	2.1×10^6	24/ 87	27.6
10	1.0×10^5	25/ 81	30.9
20	0	–	–

* Number of colonies showing streptomycin susceptibility per number of colonies tested.

susceptible cells thus obtained, almost all susceptible cells have resistance to the other drug. Namely both of the streptomycin and terramycin resistant factors in KPM 143 were not eliminated simultaneously by the ethidium bromide treatment. It was thereby found that the curing action occurred competitively.

Curing Action by Mitomycin-C

In order to make one of cured mutants, the elimination of streptomycin resistance in KPM 44 has been done by mytomycin-C treatment. The results are shown in Table 5, and a representative mutant of cured bacilli is named as KPM 78.

요 약

*B. subtilis*의 항생물질 내성균주에 대하여 여러 plasmid curing시약인 sodium dodecyl sulfate, acriflavine, ethidium bromide, mitomycin-C등을 처리하여 streptomycin감수성 변이주를 만들었다. Sodium dodecyl sulfate처리의 경우 0.1%농도에서 균체성장이 극대기에 도달한 후부터 항생물질 감수성 비율이 증가되었으며, acriflavine처리의 경우는 4 μg/ml 농도에서, ethidium bromide처리의 경우에는 10 μg/ml농도에서, 그리고 mitomycin-C 처리의 경우에는 200μg/ml 농도에서 가장 curing빈도가 높았다. 또 이들 curing 작용은 *B. subtilis*의 streptomycin과 과 terramycin내성균주에서 보는 바와같이 경쟁적 작용으로 일어났다.

Table 4. Segregation pattern of drug susceptible mutants obtained by ethidium bromide

Selected marker*	Susceptible cell/tested cell**	Frequency (%)
Str^S	19/49	38.8
Ter^S	45/49	91.8
Str^R Ter^S	30/49	61.2
Str^S Ter^R	4/49	8.2
Str^R Ter^R	0/49	0

* Str^S, susceptible to streptomycin; Ter^S, susceptible to terramycin; Str^R, resistant to streptomycin; Ter^R, resistant to terramycin.

** Number of colonies showing drug susceptibility.

Table 5. Elimination of streptomycin resistance in *B. subtilis* KPM 44 by mitomycin-C

Concentration (μg/ml)	Viable cells /ml	Susceptible cell/tested cell *	Frequency (%)
0	6.8×10^9	0/73	0
25	7.0×10^9	0/51	0
50	6.2×10^9	7/48	14.6
100	6.3×10^8	21/62	33.9
200	1.5×10^8	39/78	50.0
400	3.2×10^6	0/64	0
800	0	–	–

* Number of colonies showing streptomycin susceptibility per number of colonies tested.

Reference

1. Hopwood, D.A. : *Annu. Rev. Microbiol.* 32, 373 (1978)
2. Chater, K.F.: *Genetics of Industrial Micro-*

organisms (Sebek, O.K., A.I. Lanskin, ed.) American Society for Microbiology, Washington, D.C., 123 (1979)
3. Okanishi, M.: *ibid,* 134
4. Carton, B.C., B.J.Brown: *Manual of Methods for General Bacteriology,* (Gerhart, P. ed.) American Society for Microbiology, Washington, D.C., 228 (1981)
5. Stanier, R.Y., E.D. Adelberg, J.L. Ingraham: *The Microbial World,* Prentice Hall, N.J., 4th ed., 357 (1976)
6. Schnaitman, C.A.: *Manual of Methods for General Bacteriology,* (Gerhart, P. ed.) American Society for Microbiology, Washington, D.C., 57 (1981)
7. Davies, J.: *Methods in Enzymology,* (Hash, J.H., ed.) Academic Press, N.Y., **43,** 41 (1975)
8. Tomoeda, M., M. Inuzuka, S. Anto, M. Konish: *J. Bacteriol.* **120,** 1158 (1974)
9. Tomoeda, M., M. Inuzuka, N. Kubo, S. Nakamura: *J. Bacteriol.* **95,** 1078 (1968)
10. Boronin, A.M., L.G. Sadovnikova: *Genetika* **8,** 174 (1972)
11. Clowes, R.C.: *Bacteriol. Rev.* **36,** 361 (1972)
12. Helinski, D.R. : *Annu. Rev. Microbiol.* **27,** 437 (1973)
13. Hong, Y.K., J.H. Seu : *Kor. J. Appl. Microbiol. Bioeng.* **11,** 75 (1983)
14. Carlton, B.C., B.J. Brown: *Manual of Methods for General Bacteriology,* (Gerhart, P., ed.) American Society for Microbiology, Washington, D.C., 240 (1981)