

Transformation Conditions of *Bacillus subtilis* by *Streptomyces rimosus* Plasmid DNA

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Streptomyces rimosus Plasmid DNA에 의한 *Bacillus subtilis* 의 형질전환 조건

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To exploit a suitable vector and recipient strain for molecular cloning in *Bacillus subtilis*, oxytetracycline-resistant plasmid DNA has been prepared from *Streptomyces rimosus* by phenol-buffer extraction of lysozyme-lysed cells and introduced into *B. subtilis* KPM 60 [Str^R-mutant of RM 125 (*leu* A8, *arg* 15, *hsm* M, *hsr* M)] by transformation. Oxytetracycline-resistant plasmid was well transferred into *B. subtilis* KPM 60 with average frequency of 10^{-4} per μg of DNA. The highest frequency of plasmid transformation was obtained after 3 hours incubation of recipient cells in the growth medium and 30 to 60 minutes incubation in the competence medium, and then 20 minutes contact of DNA and host cells. Optimum pH for competence was 7.5, and optimum temperature for transformation was 20°C.

The application of recombinant DNA technology in Bacilli is on the developmental stages. Moreover industrially-significant *B. subtilis* appeared for numerous reasons to be a very attractive candidate for the development of a cloning system⁽¹⁻⁵⁾. Nevertheless the development of the *B. subtilis* cloning system has been mainly hindered by the absence of suitable vectors. Some Bacilli have cryptic plasmids that do not permit direct selection of the plasmids⁽⁶⁻¹⁰⁾. An alternative methodology has been to isolate antibiotic-resistant plasmids from *Staphylococcus aureus* and introduced them into *B. subtilis* by transformation^(3, 11-13). A hybrid plasmid of *E. coli* RSF 2124 plasmid containing *B. subtilis leu* genes has converted *B. subtilis leu* phenotype to *leu*⁺ phenotype by transformation.

In order to exploit a suitable shuttle plasmid for *B. subtilis* and *Streptomyces*, the antibiotic-resistant plasmid in *St. rimosus* was isolated and introduced into *B. subtilis* in this work. For oxytetracycline resistance in *St. rimosus*, the correlation between antibiotic-sensitivity and loss of extrachromosomal DNA complements nicely the evidence from curing or infectious transfer that plasmids are involved⁽¹⁵⁻¹⁷⁾. It has therefore been initiated to study the transformation conditions of the oxytetracycline-resistant plasmid.

Materials and Methods

Bacterial Strains

Recipient strain of *B. subtilis* KPM 60 is a transformant of RM 125 (*leu* A8, *arg* 15, *hsm* M, *hsr* M) by streptomycin-resistant plasmid derived from *E. coli* KPM 105. RM 125 was obtained from T. Uozumi, Dept. of Agricultural Chemistry, The University of Tokyo^(18,19). *St. rimosus* IFO 0014 was used to extract donor DNA.

Media and Culture Conditions

B. subtilis was held on tryptose agar base overnight prior to use as inocula for the development of competence. The compositions of growth and competence medium were followed by Mahler method⁽²⁰⁾. Spores of *St. rimosus* on Hickey-Tresner medium⁽²¹⁾ was grown on Sucrose-Casamino acids-Glycine medium⁽²²⁾ at 28°C for 70 hrs shakly.

Isolation of *St. rimosus* DNA

Growth and lysis of *St. rimosus* were followed by the method of Schrepf⁽²²⁾. The lysozyme-SDS treated lyzate was extracted DNA by phenol-buffer of Miura method⁽²³⁾, and followed by Marmur procedure⁽²³⁾ to purify DNA completely. The DNA solution was filtered through a millipore

filter (pore size, 0.9 μ m). To keep the DNA for several months the solution was stored over one volume of chloroform at 4°C (T. Uozumi, personal communication).

Transformation Procedure of *B. subtilis*

The preparation of competent cells and transformation procedure of *B. subtilis* were followed by the method of Mahler⁽²⁰⁾. For transformation, 0.2 ml of competent cells were mixed with 0.1 ml of transforming DNA solution to give 1 μ g of DNA. After incubation at 37°C for 30 min, the cells were chilled and plated on appropriate media. Oxytetracycline-resistant transformants were selected on nutrient agar medium with oxytetracycline at 200 μ g per ml. The plates were incubated at 30°C for 5 days and the colonies were counted.

Results

Effect of Incubation Time on Growth Medium and Competence Medium

The correlation of competency development with cell growth was studied for a recipient strain of *B. subtilis* KPM 60. *B. subtilis* KPM 60 was incubated for various intervals in the growth medium, 90 min in the competence medium, and subsequent 30 min for the DNA contact period as appeared in Fig. 1. Viable counts at the termination of the 30 minutes contact were used as an index of total growth. Fig. 1 shows that there is a marked increase in transformation frequency of oxytetracycline resistance between 3 and 4

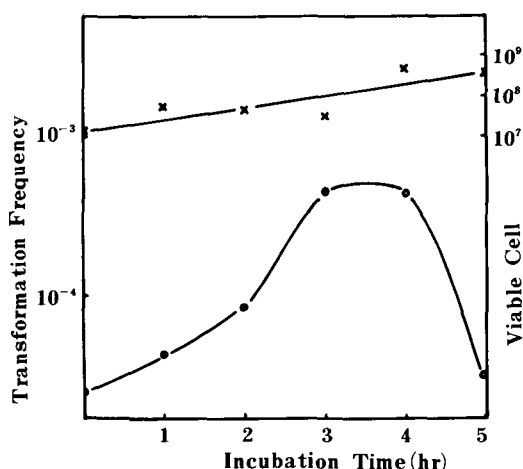


Fig. 1. Effect of Incubation Time on Growth Medium.

O—O, transformation frequency of oxytetracycline resistance; x—x, viable cell count.

hrs culture on growth medium. In order to know the effect of incubation time in competence medium, *B. subtilis* KPM 60 was incubated for 0 to 120 min in the competence medium after 3 hrs growth in the growth medium, and subsequently exposed to DNA for 30 min at 37°C. The cell growth is the sum of these three periods. As elucidated in Fig. 2, there was a clear increase of transformation frequency between 30 and 60 minutes culture on competence medium.

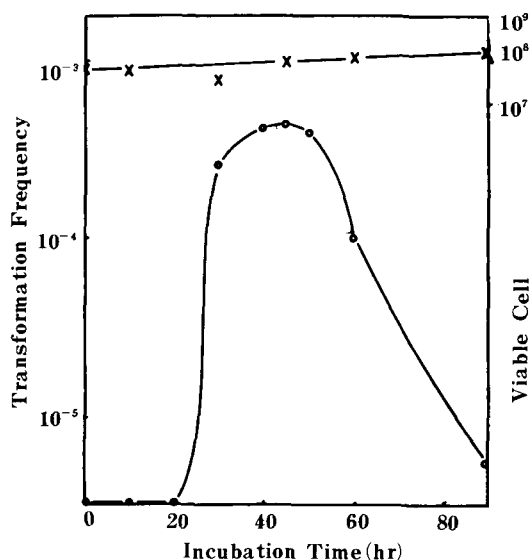


Fig. 2. Effect of Incubation Time on Competence Medium.

O—O, transformation frequency of oxytetracycline resistance; x—x, viable cell count.

Duration of DNA Exposure

A competence population of KPM 60 was incubated at 37°C with *St. rimosus* DNA containing oxytetracycline-resistant plasmid for various intervals of time. The reactions were terminated by addition of 2 μ g of crystalline pancreatic DNase with 5 mM MgCl₂. Transformation reached a maximal level after 20 min of exposure to DNA as shown in Fig. 3.

Effect of *St. rimosus* DNA concentration of Transformation

The relationship between *St. rimosus* DNA concentration and transformation to oxytetracycline resistance was further studied by varying the amount of DNA added to competent cells. Competent cells of *B. subtilis* strain KPM 60 were incubated with DNA for 30 min at 37°C. With the

DNA concentration of 0.05 to 5000 ng per 0.1 ml, the transformation was appeared on a typical dose response curve as shown in Fig. 4.

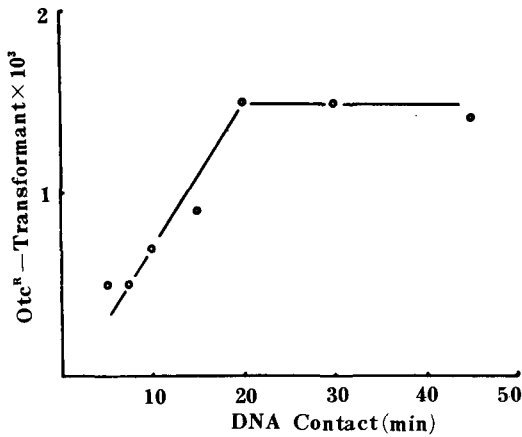


Fig. 3. Effect of DNA Exposure Time on the Formation of Transformants. The reactions were terminated by addition of 2 μ g of DNase.

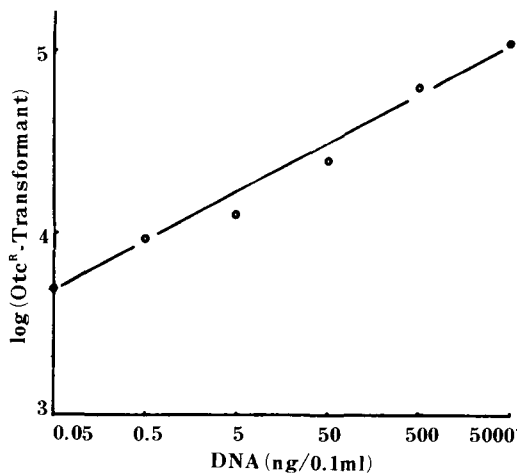


Fig. 4. Effect of *St. rimosus* DNA Concentration on Oxytetracycline-Resistance Transformation. Total viable cells, 1.6×10^8 .

Effect of Competence pH on Transformation

Fig. 5 shows the frequency of transformation as a function of pH of the buffer system used in competence medium. The recipient cells of *B. subtilis* KPM 60 were incubated in the competence medium bufferized with 0.1 M McIlvaine buffer (pH 5-6), potassium phosphate buffer (pH 6-8), or 0.1 M Clark and Lubs buffer (pH 8-9) respectively.

After 30 minutes of contact between cells and DNA, the mixtures were plated routinely on nutrient agar medium containing oxytetracycline. At pH values ranging from 6.0 to 9.0, a peak in the transformation frequency was observed at pH 7.5. It was followed by a shoulder or a lower peak between pH values of 8.0 and 8.5. These results showed that competent cells were transformed maximally at pH 7.5, and then competent factor was made on alkaline which is able to uptake DNA though not optimal pH for transformation. Competent factor was meanwhile not made on acid.

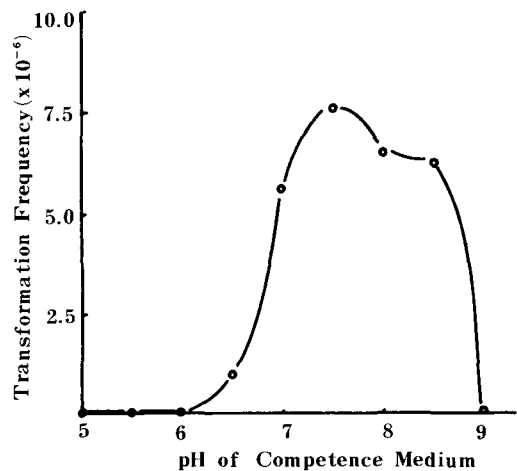


Fig. 5. Effect of pH of the Buffer System Used in Competence Medium on Transformation.

Effect of Temperature on Transformation

Competent cells of KPM 60 were contacted with DNA for 30 minutes at different temperatures, then the cells were plated on agar medium directly from the transformation mixture. The optimal temperature for transformation was 20°C as appeared in Fig. 6. Relatively low level of transformation below 20°C shows that the binding of DNA to competent factor is dependable on a physical contact frequency and the penetration of DNA into cell wall of competent strain is an energy dependent process.

Discussion

A contact period of 20 minutes between recipient cells and DNA is optimal, then treatment of the cells with DNase after attachment of DNA reduces the number of transformants to roughly 26%. The percentage varies in different

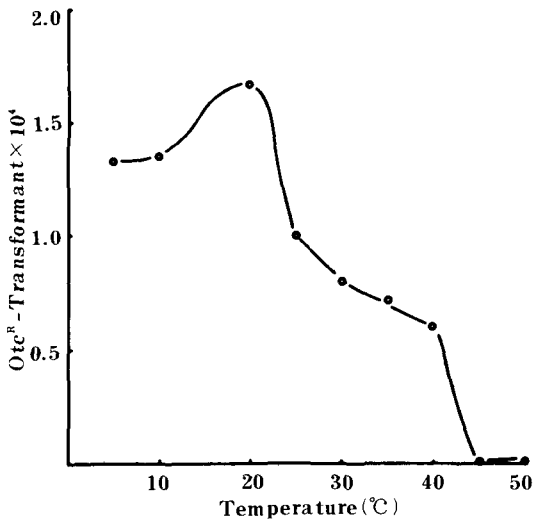


Fig. 6. Effect of Temperature on Transformation. Transformants were plated on nutrient agar containing oxytetracycline (200 μ g/ml) directly from the transformation mixture.

experiments⁽²⁴⁾The DNase may reach and destruct DNA attached to the cell surface and possibly also DNA located in the periplasmic space. The effect of pH on spontaneous competence development in *Streptococcus sanguis*⁽²⁵⁾ cells have been examined and showed a narrow range between 7.2 and 7.8, with a maximum at about 7.4. In the presence of albumine, the range was extended to pH values of 6.8 to 8.2. Tomasz⁽²⁶⁾ found that below pH 6.8 pneumococcal cells did not develop spontaneous competence. Namely competent factor was not synthesized at that condition.

요 약

*Bacillus subtilis*를 유전공학의 숙주 세포로서 이용하기 위해서는 우선 적당한 vector의 개발이 요구된다. Oxytetracycline의 항생물질을 생산하는 방사선균인 *Streptomyces rimosus* IFO 0014로부터 oxytetracycline-resistant plasmid DNA를 pH 9.0의 phenol-buffer system으로 추출하여 *B. subtilis* KPM60 [Str^R-mutant of RM 125 (*leu* A8, *arg* 15, *hsr* M⁻, *hsm* M⁻)] 균주에 형질전환시키면서 이 plasmid DNA가 표현되게 하였다. 이때 형질전환의 조건으로서 KPM60을 growth medium에서 3 시간, competence medium에서는 30분내

지 60분간, 그리고 DNA와는 20분동안 접촉시킴으로서 가장 높은 빈도로 형질전환이 일어났다. (대개 10⁻⁴ 빈도 이상) 또 recipient cell의 competence화에는 pH 7.5에서 그리고 형질전환에는 20°C에서 최적상태를 보였다.

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