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Construction of Protease-defective Mutant of *Bacillus subtilis* by Homologous DNA Recombination

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상동성 유전자재조합을 이용한 단백질분해효소 비생산 바실러스균주의 구축

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Abstract

Competent cell transformation of B. subtilis AC819 was carried out using phenotypic protease-defective (Npr-) DNA of *B. subtilis* MT-2. An obtained transformant, designated *B. subtilis* HL-1, was obtained by homologous DNA recombination. Phenotypes of *B. subtilis* HL-1 were characterized histidine requirement, streptomycin-resistance, tetracyclin resistance and non-producing protease. Protoplast transformation frequency of *B. subtilis* HL-1 by plasmid pUB110 was higher than that of *B. subtilis* MT-2. From this result, *B. subtilis* HL-1 is useful for protease gene transformation and thermostable protease gene cloning as a host.

Key words : *Bacillus subtilis*, transformation, homologous recombination, protoplast, competent cell

Introduction

The representative *Bacillus subtilis* in Bacillus species is different from E. coli and that is much secretion of enzymes. *B. subtilis* is well known as the cells produce many kinds of enzymes. For example, neutral proteases (1,2) are extracellular enzymes of industrial importance. Some structural genes have already been cloned. Expression of these structural genes is controlled by the positive or negative regulators. Fundamentally, it is necessary to get mutants for the cloning experiments that pursue theirs objects. For isolation of a mutant, N-methyl-N'-nitro-N-nitrosoguanidine(NTG) treatment has usually been used(3,4). However, competence concerns the ability of a bacterial cell to bind irreversibly DNA of high molecular weight in such a method that it becomes resistant to deoxyribonuclease. It is generally agreed that the ability to develop competence is genetically controlled-positively by specifying the nature of receptor sites on the cell surface(5). We achieved a non-producing protease mutant, *B. subtilis* HL-1, by the method of DNA-mediated transformation on the competence in *B. subtilis*.

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Materials and Methods

Bacterial strains and plasmid

Bacillus subtilis AC819 (*his H, str A, tet*$^{-1}$, *smo*$^{-1}$) and

B. subtilis MT-2 (*trp C2, leu C7, hsd R, hsd M,* Npr$^-$) (4) were used for competent cell transformation procedure. *B. subtilis* AC819 was used for the recipient cell and the chromosomal DNA of *B. subtilis* MT-2 was used for donor genome. Plasmid pUB110 was used as a Kanamycin-resistant (Km^r) plasmid and prepared as described previously(6).

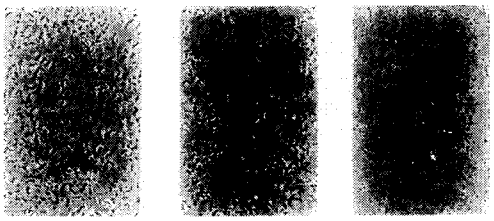
Media and materials

For bacterial growth, Penassay antibiotics medium3 (PAB, Difco) was used. SMM buffer by Wyrick *et al.s'* method(7) contained 0.5 M sucrose, 0.02 M maleic acid (pH 6.5) and 0.02 M $MgCl_2$. For protoplast regeneration, Trypticase Soy Broth(TSB, BBL) medium containing 3% of TSB, 0.02 M $MgCl_2$, 0.3 M sodium succinate(pH 7.3), 0.1 wt% bovine serum albumin(Nacalai Tesque, Kyoto, Japan) and 2% Bacto agar(Difco) was used with or without 100 μg/mL of kanamycin.

L broth was made by 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl in 1 L (pH 7.2) and L agar was consisted with L broth and 2% Bacto agar. LC agar was made by L agar supplemented with 1.0 % (wt/vol) Hammarsten casein (Merck, Darmstadt, Germany).

Simple method for rapid preparation of chromosomal DNA from bacilli

Protoplast cells(Fig. 1) of *B. subtilis* MT-2 was carried out under the same conditions described previously(8,9). A 0.1 mL of preculture of *B. subtilis* MT-2 was inoculated into 25 mL of PAB medium in an Erlenmyer flask to cultivate the bacterial cells at 37℃ for 3hr with shaking(180 strokes/min). The cells were harvested by centrifugation at 3,000xg for 2min at 4℃, and suspended in 4 mL of SMM buffer supplemented with 2.5 mg/mL of lysozyme(Sigma, Co.). The mixture was gently shaken (80 strokes/min) in 50 mL Erlenmyer flask at 40℃ for 20min. The protoplasts(about $2.5x10^9$ cells) were pelleted and resuspended in 4 mL of TNSB buffer containing 0.1 M Tris-HCl(pH 8.0), 0.1 M NaCl, 0.5% bovine serum albumin. The suspension was incubated at 65℃ for 5min with gently shaking. After adding 4 mL of phenol-chloroform-isoamyl alchol(25:24:1, v/v/v), the solution was gently shaked for 5min at 65℃. The upper layer was mixed with 0.5 mL of 3 M potassium acetate and 2.5 mL of isopropanol. Then the mixture was incubated at -80℃ for 15min and centrifuged at 8,000xg for 5min. The obtained pellet was dissolved in water without further purification, for example, pronase treatment, CsCl centrifugation and column chromatography.



Control 2.5μg lysozyme 2.5mg lysozyme

Fig. 1. Conditions of protoplast cells with various lysozyme concentrations.

Competent cell transformation and protoplast transformation

Competent cells were prepared as described by Bott et al. (5) and transformation was carried out by the method of Spizizen *et al.*(10). Protoplast transformation was done as described previously.

Detection of protease-producing or non-producing colonies on LC medium plates

Colonies were transferred by tooth-pickes onto LC agar plates and incubated at 37℃. After incubation overnight, colonies with or without halos were selected as protease producing(Npr$^+$) or protease non-producing(Npr$^-$) strains, respectively.

Assay of protease activity

The bacteria were grown overnight in L broth containing 2 mM of $CaCl_2$ at 37℃ for *B. subtilis*. The supernatant of the culture broth after centrifugation (8,000xg, 10min) was dialyzed against a buffer solution of 50 mM Tris-HCl(pH7.5) that contained 5 mM $CaCl_2$ for at least 2 days at 4℃ before the assay of extracellular protease. Protease was assayed for casein hydrolytic activity, essentially by the method described by Hagihara *et al.*(11). One milililter sample that was diluted with the buffer solution of 50 mM Tris-HCl (pH7.5) containing 5 mM $CaCl_2$ was mixed with 1 mL of Hammarsten casein solution (1% dissolved in the same buffer) and incubated at 37℃ for 15 min. After the

addition of 2 mL of 0.1 M trichloroacetic acid-0.22M sodium acetate-0.33M acetic acid, followed by a vigorous mixing, the mixture was allowed to stand at room temperature for 30min and then filtered on Whatman No. 2 filter paper(Advantec. Co.). The absorbance at 275 nm was measured by UV DU-70 spectrophotometer (Beckman). A blank was prepared by adding 2 mL of the precipitating agent to 1 mL of the casein solution before the addition of the enzyme solution. A unit of protease was defined as the quantity required to liberate the absorbance at 275 nm by an equivalent of 1 μg of tyrosine per min at 37℃(4).

Results and Discussion

Construction of protease-defective transformant

Transfer of phenotypic neutral protease-defective (Npr^-) DNA from *B. subtilis* MT-2 to B. subtilis AC819 by the protoplast DNA-mediated transformation was carried out. As shown in Table 1, the transformation frequency with chromosomal DNA prepared by the method of Saito and Miura(12).

About 10^3 transformants were obtained by competent cell transformation on LC agar containing 5 μg tetracyclin mL^{-1}. Tc^r and Npr^- transformant, designated *Bacillus subtilis* HL-1, was selected. To confirm the Npr^- character of strain HL-1, phenotypes and genotypes of MT-2, AC819 and HL-1 were examined, respectively. The transformant, HL-1, exhibited phenotypes of both histidine-requirement and streptomycin-resistance including Tc^r and Npr^-(Table 1).

Table 1. Characterization of *B. subtilis* strains

Strains	Phenotypes	Halo formation	Protoplast transformation frequency of Km^r
MT-2	Trp^-, Leu^-, Npr^-	-	1.8×10^{-4}
AC819	His^-, Str^r, Tet^r, Smo, Npr^+	+	4.4×10^{-2}
HL-1	Str^r, Tet^r, Smo, Npr^-	-	3.5×10^{-2}

Comparison of protease activity

The extent to which recipient strain, *B. subtilis* AC819, produces the activity could be quickly judged LC agar plate as shown in Fig. 2 and Table 2. Protease-non-producing strains, like as MT-2 and HL-1, didn't form halos. However, protease producing strain, AC819, formed halo. The protease activities of the culture supernatant from these strains were shown as Table 2. *B. subtilis* MT-2 and its representative transformant HL-1 exhibited only a low activity than that of AC819.

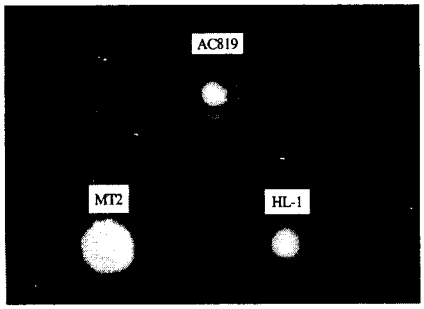


Fig. 2. Detection of producing-protease or not on LC agar plate.

Table 2. Comparison of protease activity

Bacillus subtilis Strains	Cells(O.D.660)	Protease activity (O.D.275)
AC819	3.5	7.2
MT-2	4.2	0.6
HL-1	3.7	1.5

Frequency of protoplast transformation

To examine whether Npr- derivative was convenient as a host, the frequency of both protoplasts and competent cell transformations was compared by using pUB110. The appearance of Km^r transformants of strains AC819 and HL-1 were similar each other, and the frequency was 10^2 higher than that of MT-2. We achieved a mutant, *B. subtilis* HL-1, has the characterizations of high frequency of protoplast transformation and non-forming halos simultaneously. A wide diversity of homology of DNA among species of the genus Bacillus has been reported by several authors(13, 14). And specific transformation among above species were also reported(14). The genotypes of *B. subtilis* AC819 were *hisH*, *strA*, tet^{-1} and smo^{-1}. The phenotypes of *B. subtilis* AC819 were Sm^r and Npr^+. *B. subtilis* MT-2 was charcterized *trpC2*, *leuC7*, Tc^r and Npr^-, respectively. Transfer of the chromosomal DNA of *B. subtilis* MT-2 to competent cells of *B. subtilis* AC819 was carried out. *B. subtilis* MT-2 DNA may be transform into competent cells of *B. subtilis* AC819 through the cell wall randomly. And then,

homologous recombination occurs on the chromosomal DNA of *B. subtilis* AC819. Now, it progress to achieve the cloning of thermostable neutral protease gene of *Bacillus stearothermophilus* into *B. subtilis* HL-1.

요 약

단백질분해효소를 생산하지 않는 균주 *B. subtilis* MT-2의 염색체 DNA를 추출한 다음, *B. subtilis* AC819 균주에 상동성 유전자재조합을 이용하여 competent cell 형질전환을 시켰다. 얻어진 형질전환체를 *B. subtilis* HL-1이라고 명명하였으며, 그 표현형은 histidine 요구성, streptomycin 내성, tetracyclin 내성을 나타내면서 단백질 분해효소를 생산하지 않았다. 플라스미드 pUB110을 이용한 *B. subtilis* HL-1의 protoplast 형질전환율은 *B. subtilis* MT-2의 형질전환율보다 높았다. 따라서 새로운 *B. subtilis* HL-1균주는 단백질분해효소의 형질전환과 내열성 protease 유전자클로닝에서 숙주로 사용하는데 유용하다.

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References

1. Yang, M.Y., Ferrari, E. and Henner, D.J. (1984) Cloning of the Neutral protease gene of *Bacillus subtilis* and the Use of the Cloned gene to Create an in Vitro-Derived Deletion Mutation. *J. Bacteriol.*, **160**, 15-21
2. Uehara, H., Yamane, K. and Maruo, B. (1979) Thermosensitive, Extracellular Neutral Proteases in *Bacilllus subtilis*: Isolation, Characterization and Genetics. *J. Bacteriol.*, **139**, 583-590
3. Imanaka, T., Fujii, M., Aramori, I. and Aiba, S. (1982) Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. *J. Bacteriol.*, **142**, 824-830
4. Fujii, M., Takagi, M., Imanaka, T. and Aiba, S. (1983) Molecular cloning of a thermostable neutral protease gene from *Bacillus stearothermophilus* in a vector plasmid and its expression in *Bacillus stearothermophilus* and *Bacillus subtilis*. J. Bacteriol., **154**, 831-837
5. Bott, K. and Wilson, G.A. (1967) Development of competence in the *Bacillus subtilis* transformation system. *J. Bacteriol.*, **94**, 562-570
6. Hara, T., Zhang, J.R. and Ueda, S. (1983) Identification of plasmids linked with polyglutamate production in *Bacillus subtilis* (natto). *J. Gen. Appl. Microbiol.*, **29**, 345-354
7. Wyrick, P.B. and Rogers, H.J. (1973) Isolation and characterization of cell wall-defective variants of *Bacillus subtilis*. *J. Bacteriol.*, **116**, 456-465
8. Hara, T., Lee, J.T., Prana, T.K., Akamatsu, T., Fujio, Y. and Ogata, S. (1991) Successive Protoplast Transformation of *Bacillus subtilis* by plasmid DNA under low Concentration of lysozyme. *J. Fac. Agri.*, **36**, 23-28
9. Akamatsu, T. and Sekiguchi, J. (1987) Characterization of chromosome and plasmid transformation in *Bacillus subtilis* using gently lysed protoplasts. *Arch. Microbiol.*, **146**, 353-357
10. Spizizen, J. (1958) Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci.*, **44**, 1072-1078
11. Hagihara, B., Matsubara, H., Nakai, M. and Okunuki, K. (1958) Crystalline bacterial proteinase 1. Preparation of crystalline proteinase of *B. subtilis*. J. Biochem., **45**, 185-194
12. Saito, H. and Miura, K. (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochem. Biophys. Acta.*, **72**, 619-629
13. Dubnau, D., Smith, I., Morell, P. and Marmur, J. (1965) Gene conservation in Bacillus species, conseved genetic and nucleic acid base sequence homologies. *Proc. Natl. Acad. Sci.*, **54**, 491-498
14. Seki, T., Tsunekawa, H., Nakamura, K., Yoshimura, K. and Oshima, Y. (1979) Conserved genes in *Bacillus subtilis* and related species. *J. Ferment. Technol.*, **57**, 488-504

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