Bacillus subtilis (natto) Plasmid Responsible for Polyglutamate Production Encoding r-Glutamyltranspeptidase

Hara, Toshio
Laboratory of Microbial Technology, Department of Food Science & Technology, Faculty of Agriculture, Kyushu University

Fujio, Yusaku
Laboratory of Microbial Technology, Department of Food Science & Technology, Faculty of Agriculture, Kyushu University

Ueda, Seinosuke
Laboratory of Microbial Technology, Department of Food Science & Technology, Faculty of Agriculture, Kyushu University

https://doi.org/10.5109/23815

出版情報：九州大学大学院農学研究院紀要. 30 (2/3), pp.95-105, 1985-12. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 30 (2 • 3), 95-105 (1985)

Bacillus subtilis (*natto*) Plasmid Responsible for Polyglutamate Production Encoding r-Glutamyltranspeptidase

Toshio Hara, Yusaku Fujio and Seinosuke Ueda

Laboratory of Microbial Technology, Department of Food Science & Technology, Faculty of Agriculture, Kyushu University 46-09, Fukuoka 812

(Received July 8, 1985)

Gamma-glutamyltranspeptidase (γ-GTP) activity of PGA-producing strains derived by DNA-mediated transformation was significantly increased compared with that of protease, α-amylase, and alkaline phosphatase in the PGA-nonproducing recipient strain, ***Bacillus subtilis*** Marburg 168. Its enzyme activity was deficient in the stringy-negative mutants cured with acridine orange. It is possible to deduce that γ-GTP activity responsible for PGA production may be under the control of a specific regulatory gene(s) as extrachromosomal element. Parental strains were found to possess a plasmid, but PGA-nonproducing derivatives were missing the plasmid. The molecular weight of the plasmid ***were*** estimated to be 5.7 kilobase (kb) and restriction endonuclease cleavage site map was constructed. Therefore, we conclude that the function of the 5.7-kb plasmid detected in ***B. subtilis*** (*natto*) is concerned with PGA production. This observation suggests that a broader range of functions are associated with plasmid in ***Bacillus*** species.

INTRODUCTION

Japanese "natto", produced by steamed soybeans overgrown ***Bacillus subtilis*** (*natto*), *is* one of the most traditional fermented foods, which probably would not be acceptable to many Occidental. Even some Japanese do not find it palatable. However, "natto" is a low-cost and highly nutritious food in Japan. It consists of polysaccharide (levan-form fructan) and polyglutamate (PGA). The composition of viscous material is mainly γ-PGA containing D- and L-glutamate in varying proportions (Fujii, 1963).

In our previous papers (Aumayr *et al.*, 1981; Hara, *et al.*, 1982), we reported that the genetic trait of high PGA productivity of ***B. subtilis*** (*natto*) Asahikawa was transferred by DNA-mediated transformation to ***B. subtilis*** Marburg 168 which cannot produce PGA and that the activity of γ-glutamyltranspeptidase (γ-GTP) of the representative transformants acquired a high productivity of stringy substance were 20 times higher than that of the original ***B. subtilis*** Marburg 168, although other enzyme activities such as alanine racemase or transaminase among the parental strains and its representative transformants were almost the same. These results suggest the presence of a correlation between PGA production and γ-GTP activity, and of a specific regulatory gene(s) that participates in PGA production by ***B. subtilis*** (*natto*). Re-

cently, we reported that the γ-GTP activity of the three representative transformants, 3F1, F1-9, and M5B4, was 124, 233, and 147 mU/ml, whereas that of the donor strains 476, 90, and 157 mU/ml, respectively (Hara and Ueda, 1982). It is necessary, therefore, to consider whether γ-GTP productivity of PGA-producing derivatives by DNA-mediated transformation is under the control of the multiplication of a specific regulatory gene(s) encoding γ-GTP or the secretion of γ-GTP synthesized by such a regulatory gene(s).

To further investigate the regulatory mechanisms of PGA production in *B. subtilis* (*natto*), we attempted to assay several enzyme activities in the parental strains, its representative PGA-producing transformants, and the PGA-nonproducing cured strains and to analyze genetic elements concerned with PGA production. In this communication, we describe the isolation and characterization of a specific extrachromosomal element encoding γ-GTP responsible for PGA production in *B. subtilis* (*natto*).

MATERIALS AND METHODS

Microorganisms

Three strains (Asahikawa, Miura, and Takahashi No. 5) of *Bacillus subtilis* (*natto*) isolated from the commercial product, "natto", were used in this study. *B. subtilis* Marburg 168 (*trp*$^-$) was used as a recipient strain.

Media

The compositions of buillon-yeast extract medium and minimal medium used for preparation of competent cells were the same as described by Yamaguchi *et al.* (1974). The composition of sucrose-glutamate (SG) medium used for isolation of transformants was sucrose 5 %, L-glutamic acid monosodium salt 1.5 %, KH_2PO_4 0.27 %, $Na_2HPO_3 \cdot 12H_2O$ 0.42 %, NaCl 0.05 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, and biotin 0.1 μg/ml, pH 6.4. The medium used for assay of enzyme activities consisted of peptone 1.2 %, citric acid 0.2 %, glycerol 2 %, NH_4Cl 0.7 %, K_2HPO_4 0.05 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, $FeCl_3 \cdot 6H_2O$ 0.004 %, and biotin 0.1 μg/ml.

Procedure of transformation

Transforming DNA was prepared from exponentially growing cells by the method of Saito and Miura (1963), and filtered through a Millipore filter (type HA, pore size 0.3 μm) to remove spores. Transformation experiments were performed by the method of Yoshikawa (1970). As a selective marker, stringiness (Stg) was taken as the length of string made of a toothpick from the surface of the colonies.

Acridine orange treatment

Acridine orange (AO, 2: 8-*bis* Dimethylaminoacridine, Eastman Kodak Co.) treatment was carried out by the method of Hirota (1960). A cell density of about 10^8/ml at logarithmic phase was inoculated to produce a cell density of about 10^4/ml in nutrient medium containing AO (20 μg/ml). The culture was

incubated overnight at 37°C with aeration. Diluted cultures were then plated by spreading on SG agar plates for single colonies which were counted and tested for PGA productivity.

Assay of γ-glutamyltranspeptidase activity

Determination of γ-glutamyltranspeptidase (γ-GTP) activity has been described previously (Aumayr et al., 1981). One unit of γ-GTP activity is defined as the amount of enzyme which liberates 1 μmol of α-naphthylamine per min at 37°C.

Isolation of **plasmid**

Cleared lysate of ***B. subtilis*** (*natto*) was prepared by the sodium dodecyl sulfate (SDS) -NaCl method of Guerry ***et al.*** (1973) with slight modification. Overnight cultures grown in nutrient broth were inoculated into 200ml fresh medium and cultured at 30°C for 4.5 hr with shaking. Cells were harvested and washed with cold buffer consisting of 0.03 M Tris-HCl (pH 8.0), 0.05 M NaCl, and 5 mM EDTA (TES buffer). Cells were suspended in 5 ml of 25 % sucrose in TES buffer. A 0.75 ml portion of EDTA (0.25 M, pH 8.0) and 1.5 ml of lysozyme (Sigma Chemical Co., 5 mg/ml in TES buffer containing 25 % sucrose) were added, and the mixture was incubated for 30 min at 37°C. A 0.75 ml amount of Pronase E (5 mg/ml in TES buffer predigested for 1.5 hr at 37°C) was added, and the suspension was incubated for an additional 30 min at 37°C. Lysis was brought about room temperature by the addition of 0.75 ml of 10 % SDS in TES buffer. NaCl (0.75 ml, 5 M) was added, and the whole mixture was left overnight at 4°C. The mixture was centrifuged for 30 min at 17,000 rpm and the supernatant fluid was made up to a final volume of 6.0 ml with 50mM EDTA (pH 8.0). Five g of solid CsCl and 0.1 ml of ethidium bromide (EtBr) solution (30 mg/ml in dimethyl sulfoxide) were added, and density was adjusted to 1.575 g/ml. The mixture was centrifuged at 38,000 rpm for 40 hr at 4°C in a Hitachi 65 Ti rotor. The presence of CCC DNA was determined by visualization under ultraviolet illumination. When satelite bands were detected, they were collected and recentrifuged in CsCl-EtBr gradients.

Plasmid DNA was collected from the gradient, extracted with n-buthanol three times and dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and 1 mM EDTA (TE buffer). The purified samples were used for analyses with restriction endonucleases.

Agarose gel electrophoresis

Plasmid DNA samples were analyzed by using 1.0 % agarose (Nakarai Chemicals, Ltd., Kyoto) slab gel according to the method of Sharp ***et al.*** (1973). Electrophoresis was carried out at room temperature with 40 mM Tris-HCl (pH 8.1), 20 mM acetic acid, and 2 mM EDTA. All plasmid DNA bands were identified by the fluorescence from integrated EtBr under ultraviolet irradiation. Plasmid DNA fragments molecular weights were estimated using λ-*Hin*dIII fragments as standards (Robinson and Landy, 1977).

Digestion of plasmid DNA with restriction endonucleases

Restriction endonucleases *Bam*HI, *Hin*cII, and *Hin*dIII were purchased from Takara Brewery Co. Ltd., Kyoto, Japan. The conditions for the reactions for the cleavage of plasmid by the various enzymes were the same as those recommended by Bethesda Research Laboratory.

RESULTS AND DISCUSSION

Transformation of stringiness and tryptophan requirement from *B. subtilis* (*natto*) to *B. subtilis*

Transfer of phenotypic tryptophan nonrequirement (*trp*$^+$) from B. *subtilis* (*natto*) to *B. subtilis* Marburg 168 by DNA-mediated transformation was carried out. The cell-DNA mixture was spread on agar plates containing 1.5 % L-glutamate and 0.1 μg/ml of biotin. When 0.1 μg per ml of the transforming DNA of *B. subtilis* (*natto*) was employed, the frequency of *trp*$^+$ in DNA-mediated transformation was 10^{-5} and that of *stg*$^+$ was 10^{-1} (Table 1). It was thought that the double transformation of nutrient requirement and stringiness might be the result of two independent events. The frequency of the double transformation of *stg*$^+$*trp*$^+$ to the transformation of *trp*$^+$ were 2 to 7 %.

Table 1. Comparison of transformation frequencies in various bacilli".

Source of DNA[b]	*stg*$^+$	*trp*$^+$	*stg*$^+$ *trp*$^+$
Asahikawa	1.1×10^{-1}	7.5×10^{-5}	5.3×10^{-6}
Miura	1.0×10^{-1}	8.5×10^{-5}	5.9×10^{-6}
Takahashi No. 5	1.2×10^{-1}	2.7×10^{-4}	5.9×10^{-6}

[a] These experiments were carried out as described in MATERIALS AND METHODS. After DNA was added, pancreatic DNase (Boehringer Mannheim GmbH) (10 μg/ml) was added and the mixture incubated for 60 min at 37°C and then spread on agar plates containing L-glutamate and biotin.

[b] DNA concentration was 0.1 μg/ml.

Transfer of high γ-GTP activity from *B. subtilis* (*natto*) to *B. subtilis*

The productivity of extracellular enzymes such as protease, α-amylase, alkaline phosphatase, and γ-GTP from the parental strains and its representative transformants obtained PGA productivity are summarized in Table 2. A significant difference in the productivities of protease and a-amylase could not be detected in transformants, but the productivity of alkaline phosphatase in the transformants was four times higher than that of the recipient strain, *B. subtilis* Marburg 168. However, γ-GTP productivity in PGA-producing transformants increased to about fifty times higher compared with that in the recipient strain. Related to the production of α-amylase and protease, genes such as *amy*R, *tmr*, *npr*R, pap, *amy*B, *hpr*, and *sac*U have been revealed by various investigators. Among them, *amy*R and *tmr* regulate only α-amylase production and *npr*R only neutral protease, while *pap*, *amy*B and, *sac*U af-

Table 2. Protease, α-amylase, alkaline phosphatase, and γ-GPT activity in the culture fluids of the parental strains and the representative transformants[a].

Strain	Protease activity (mU/ml)	α-Amylase activity (mU/ml)	Alkaline phosphatase activity (mU/ml)	γ-GTP activity (mU/ml)
Asahikawa	346	559	408	476
3F1	126	35	271	149
Miura	331	417	828	157
M5B4	168	28	229	142
Takahashi No. 5	343	468	1422	90
F1-9	217	120	367	233
168	149	44	62	6

[a] Each strain was cultured in the medium described in MATERIALS AND METHODS at 37°C for 3 days. Cells were removed by centrifugation, and then enzyme activities were determined.

fect the production of α-amylase, protease, levan-sucrase, and flagella (Yoneda and Maruo, 1975; Ayusawa *et al.*, 1975; Steinmetz *et al.*, 1976). The production of extracellular soluble alkaline phosphatase should be independently controlled from the regulation system of extracellular α-amylase and protease (Yamane *et al.*, 1976; Hitotsuyanagi *et al.*, 1978). However, the productivity of γ-GTP in the transformants obtained PGA productivity was increased to almost equal levels as well as the productivity of protease, α-amylase, and alkaline phosphatase was almost similar in the PGA-producing transformants. Therefore, these results suggest that the synthesis of r-GTP may have relation to the multiplication of a specific regulatory gene(s) and that γ-GTP productivity into the culture fluid is dependent on the secreting ability of the recipient strain, *B. subtilis* Marburg 168.

Biochemical function of plasmid with PGA production

The frequency of PGA-producing transformants was exceptionally high compared with that observed for a chromosomal marker (*trp*) used as a control, suggesting a plasmid-related control of PGA synthesis. To investigate the participation of plasmid in PGA production, overnight cultures of strains Asahikawa, Miura, and Takahashi No. 5 were treated with acridine orange (AO). After treatment AO, PGA-nonproducing colonies appeared with frequencies of 64.2 % in Asahikawa, 9.2 % in Miura, and 22.4 % in Takahashi No. 5 (Table 3). Untreated colonies showed high PGA productivity. As shown in Table 4, γ-GTP activity of cured strains was decreased. Cleared lysates of *B. subtilis* (*natto*) and cured strains were prepared to clarify the presence of plasmid-linked PGA production. *B. subtilis* (*natto*) harbored a single plasmid species (Fig. 1). However, no plasmid DNA was detected in the stringiness-negative mutants from each strain of *B. subtilis* (*natto*) with AO as shown in Fig. 1.

Table 3. Effect of AO on PGA production.

Strain	AO concn (fig/ml)	No. of colonies tested	Elimination rate (%)[a]
Asahikawa	0	120	0
	20	120	64. 2
Miura	0	120	0
	20	120	9. 2
Takahashi No. 5	0	120	0
	20	116	22. 4

[a] Colonies deficient in the property of PGA productivity. Overnight cultures were inoculated into nutrient medium (pH 7.6) with or without AO (20 μg/ml) and incubated overnight at 37°C. All samples were plated on SG agar plates, and colonies were tested for their PGA productivity with a toothpick.

Table 4. Comparison between stringiness and γ-GTP activity of the parental strains and the cured strains.

Strain	Stringiness (cm)[a]	γ-GTP activity (mU/ml)[b]
Parental		
Asahikawa	600	434
Cured		
A- 1	0	2. 7
A- 2	0	2. 8
A- 3	0.5	7: 6
A- 4	0	4.6
A- 5	0	4. 0
A- 6	0	3. 6
A- 7		3. 8
A- 8	2	28. 9
A- 9	0	4. 8
A-10	0	3. 0
A-11		3. 6
A-12	0	2. 3
A-13	0	
A-14	0	3.8...
A-15	0	3.93.9
A-16	0	4. 5
Parental		
Miura	100	116
Cured		
M- 1	1	14. 4
Parental		
Takahashi No.'5	450	78. 9
Cured		
T- 1	20	3. 0
T- 2	0	3. 3
T- 3	0	2. 8
T- 4	40	57. 8
T- 5	0	2. 7
T- 6	0	2. 1

[a] Stringiness on SG agar plates was measured with a toothpick.
[b] Cells were cultured in the medium as described in MATERIALS AND METHODS. After cultivation at 37°C for 3 days, cells were removed centrifugation and then enzyme activities in the culture fluids were determined.

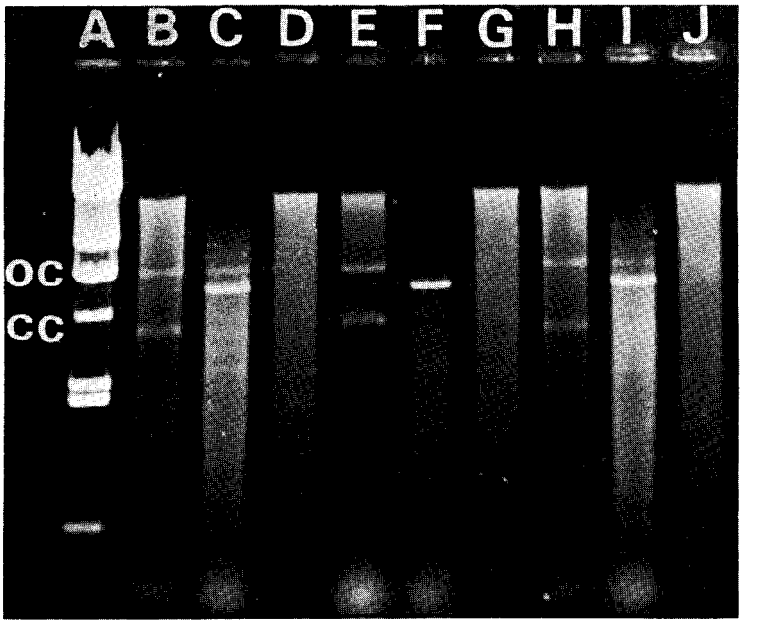

Fig. 1. Agarose gel electrophoresis of cleared lysate of *B. subtilis* (*natto*) and cured strains.
Samples (20 μl of each DNA preparation) were layered on a 0.7 % agarose slab gel. The electrophoresis was carried out at 100 mA for 2 hr. Samples were as follows: (A) lambda phage DNA cleaved with *Hin*dIII; (B) uncleaved lysate of Asahikawa; (C) lysate of Asahikawa cleaved with *Hin*cII; (D) uncleaved lysate of an Asahikawa cured strain, A-l; (E) uncleaved lysate of Miura; (F) lysate of Miura cleaved with *Hin*cII; (G) uncleaved lysate of a Miura cured derivative, M-l ; (H) uncleaved lysate of Takahashi No. 5; (I) lysate of Takahashi No. 5 cleaved with *Hin*cII; (J) uncleaved lysate of a Takahashi No. 5 derivative, T-6.

Isolation and characterization of plasmid responsible for PGA production

The *B. subtilis* (*natto*) strains and its representative transformants were analyzed by preparing cleared lysates that were subsequently centrifuged in CsCl-EtBr gradients. Plasmid preparations obtained thus were digested with three restriction endonucleases *Bam*HI, *Hin*cII, and *Hin*dIII, and subjected to electrophoresis in agarose gels. As shown in Fig. 2, the restriction patterns of the fragments generated by three enzymes were completely similar. Furthermore, it is possible to note that the plasmid was found to stable in the recipient strain, *B. subtilis* Marburg 168. However, γ-GTP productivity of strain Takahashi No. 5 was very low compared with others, although another activity of extracellular enzymes such as protease, a-amylase, and alkaline phosphatase was very high (Table 2). These facts suggest that another factor(s) might be concerned with the secretion of γ-GTP synthesized by regulatory gene(s) located on the 5.7-kb plasmid. The restriction fragment

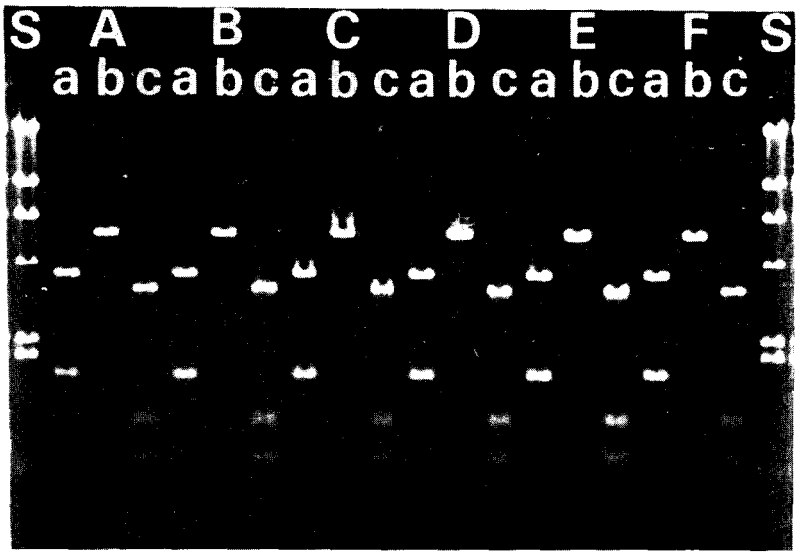

Fig. 2. Agarose gel electrophoresis of plasmid digested by *Bam*HI (a), *Hin*cII (b), or Hind111 (c).
Plasmid DNA from each strain was prepared by CsCl-EtBr gradients. A, B. *subtilis* (*natto*) Asahikawa ; B, transformant 3F1 ; C, *B. subtilis* (*natto*) Miura; D. transformant M5B4; E, *B. subtilis* (*natto*) Takahashi No. 5; F, transformant Fl-9; S, phage λ*Hin*dIII fragments as molecular size markers.

Table 5. Fragments produced on restriction endonuclease digestion of pUHl.

Enzyme	Fragment size (kb)[a]			
	A	B	C	D
*Hin*cII	5.7			
PVUII	5.7			
*Bam*HI	4.2	1.5		
*Eco*RI	5.6	0.1		
*Hae*III	3.6	2.1		
*Pst*I	2.5	2.0	1.2	
Hind111	3.5	1.1	0.7	0.4

[a] Molecular weights were estimated by agarose gel electrophoresis, using Hind111 fragments of phage λDNA as the standards.

catalog of the plasmid, pUHl, is given in Table 5. The restriction endonuclease cleavage map shown in Fig. 3 is based on the sizes of the restriction fragments obtained from single and double digests.

In attempts to ascertain the distribution of extrachromosomal DNA elements in bacilli, most of the plasmids described in spore-forming bacteria such as *B. pumilus* (Lovett, 1973; Lovett and Bramucci, 1975; Lovett and Burdick, 1975), *B. subtilis* (Lovett and Bramucci, 1975; Hegarat and Anagnostopoulos, 1977; Tanaka *et al.*, 1977; Tanaka and Koshikawa, 1977; Bernhard *et al.*, 1978;

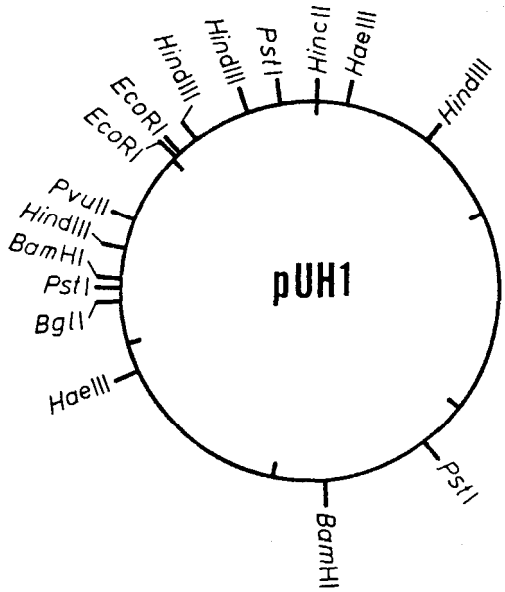

Fig. 3. Restriction endonuclease cleavage site map of pUH1.
The relative distances shown are from average fragment sizes determined on agarose gels.

Gryczan ***et al.,*** 1978 ; Uozumi ***et al.,*** 1980 ; Hara ***et al.,*** 1983 ; Yoshimura ***et al.,*** 1983), and B. ***megaterium*** (Lovett and Burdick, 1975) are cryptic. A few exceptions exist, such as pPL 10 and pPL 7065, which determine bacteriocin production in *B. pumilus* (Lovett et al., 1976, 1977), and pBC7 and pBC16, which determine bacteriocin production and tetracycline resistance, respectively, in ***B. cereus*** (Gryczan ***et al.,*** 1978). However, the very limited information on plasmids in ***Bacillus*** species is due, in part, to the fact that none of the plasmids isolated from strains of ***B. subtilis*** determines resistance to the more common antibiotics. Therefore, other than autonomous replication, the precise functions of the plasmids in ***Bacillus*** strains might remain unknown. Therefore, the observations described here strongly suggest that a broader range of functions are associated with plasmids in ***Bacillus*** species.

REFERENCES

Aumayr, A., T. Hara and S. Ueda 1981 Transformation of ***Bacillus subtilis*** in polyglutamate production by deoxyribonucleic acid from ***B. natto. J. Gen. Appl. Microbiol.,*** **27: 115-123**

Ayusawa, D., Y. Yoneda, K. Yamane and B. Maruo 1975 Pleiotropic phenomena in autolytic enzyme(s) content, flagellation, and simultaneous hyperproduction of extracellular α-amylase and protease in a ***Bacillus subtilis*** mutant. ***J.*** *Bacteriol.,* ***124: 459-469***

Bernhard, K., H. Schrempf and W. Goebel 1978 Bacteriocin and antibiotic resistance plasmids in ***Bacillus*** cereus and ***Bacillus subtilis. J.*** *Bacteriol.,* ***133: 897-903***

Fujii, H. 1963 On the formation of mucilage by ***Bacillus natto***. Part III. Chemical constituents of mucilage in natto. (I). *J. Agric. Chem. Soc. Jpn.*, 37: 407-411

Gryczan, T. J., S. Contente and D. Dubnau 1978 Characterization of ***Staphylococcus aureus*** plasmids introduced by transformation into ***Bacillus subtilis***. *J. Bacteriol.*, ***134: 318-329***

Guerry, P., D. J. LeBlanc and S. Falkow 1973 General method for the isolation of plasmid deoxyriboncleic acid. *J. Bacteriol.*, ***116: 1064-1066***

Hara, T., Y. Fujio and S. Ueda 1982 Polyglutamate production by ***Bacillus subtilis*** (*natto*). *J. Appl.* ***Biochem., 4: 112-120***

Hara, T. and S. Ueda 1982 Regulation of polyglutamate production in ***Bacillus subtilis*** (*natto*): transformation of high PGA productivity. ***Agric.*** *Biol. Chem.*, ***46: 2275-2281***

Hara, T., J. R. Zhang and S. Ueda 1983 Identification of plasmids linked with polyglutamate production in Bacillus ***subtilis*** (*natto*). ***J. Gen.*** *Appl. Microbiol.*, ***29: 345-354***

Hegarat, J. -C. L. and C. Anagnostopoulos 1977 Detection and characterization of naturally occurring plasmids in ***Bacillus subtilis. Mol. Gen. Genet., 157: 167-174***

Hirota, Y. 1960 The effect of acridine dyes on mating type factors in ***Escherichia*** *coli*. *Proc.* ***Natl.*** *Acad.* ***Sci. USA, 46: 57-64***

Hitotsuyanagi, K., K. Yamane and B. Maruo 1978 Independent control of the production and localization of alkaline phosphatase from the production of a-amylase and protease in Bacillus ***subtilis. Agric.*** *Biol.* ***Chem., 42: 585-592***

Lovett, P. S. 1973 Plasmid in ***Bacillus pumilus*** and the enhanced sporulation of plasmid-negative variants. ***J.*** *Bacteriol.*, ***115: 291-298***

Lovett, P. S. and M. G. Bramuci 1975 Plasmid deoxyribonucleic acid in ***Bacillus subtilis*** and ***Bacillus pumilus. J.*** *Bacteriol.*, ***124***: 484-490

Lovett, P. S. and B. D. Burdick 1975 Cryptic plasmid in ***Bacillus pumilus*** ATCC 7065. *Biochem.* ***Biophys. Res.*** *Commun.*, ***54: 365-370***

Lovett, P. S., E. J. Duvall, M. G. Bramuci and R. Taylor 1977 Host function specified by ***Bacillus*** pumilus plasmid pPL 7065. ***Antimicrob. Agents Chemother., 12: 435-437***

Lovett, P. S., E. J. Duvall and K. M. Keggins 1976 ***Bacillus*** *pumilus* plasmid pPL 10: properties and insertion into ***Bacillus subtilis 168*** by transformation. ***J.*** *Bacteriol.*, ***127: 817-828***

Robinson, L. H. and A. Landy 1977 *Hin*dII, *Hin*dIII, and *Hpa*I restriction fragment maps of bacteriophage ADNA. Gene, 2: 1–31

Saito, H. and K. Miura 1963 Preparation of transforming deoxyribonucleic acid by phenol treatment. ***Biochim. Biophys.*** *Acta*, ***72: 619-629***

Sharp, P. A., B. Sugden and J. Sambrook 1973 Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. ***Biochemistry, 12: 3055-3063***

Steinmetz, M., F. Kunst and R. Dedonder 1976 Mapping of mutations affecting synthesis of extracellular enzymes in Bacillus *subtilis*. Identity of the *sac*U, *amy*B and *pap* mutations. ***Mol. Gen. Genet.,*** 148: 281-285

Tanaka, T. and T. Koshikawa 1977 Isolation and characterization of four types of plasmids from ***Bacillus subtilis*** (*natto*). ***J.*** *Bacteriol.*, ***131*** : 699-701

Tanaka, T., M. Kuroda and K. Sakaguchi 1977 Isolation and characterization of four plasmids from ***Bacillus subtilis. J.*** *Bacteriol.*, ***129:*** 1487-1494

Uozumi, T., A. Ozaki, T. Beppu and K. Arima 1980 New cryptic plasmid of ***Bacillus subtilis*** and restriction analysis of other p'asmids found by general screening. ***J.*** *Bacteriol.*, ***142: 315-318***

Yamane, K., T. Miki, H. Saito, Y. Ikeda and B. Maruo 1976 Isolation of mutant secreting extracellular soluble alkaline phosphatase in ***Bacillus subtilis. Agric. Biol.*** *Chem.*, ***40: 2181-2185***

Yoneda, Y. and B. Maruo 1975 Mutation of ***Bacillus*** *subtilis* causing hyperproduction of α–

amylase and protease, and its synergistic effect. *J. Bacteriol., 124: 48-54*

Yoshikawa, H. 1970 Temperature-sensitive mutants of ***Bacillus subtilis.*** I. Multiformed replication and sequential transfer of DNA by a temperature-sensitive mutant. *Proc. Natl. Acad. Sci. USA, 65: 206-213*

Yoshimura, K., 0. Yamamoto, T. Seki and Y. Oshima 1983 Distribution of heterogeneous and homologous plasmids in Bacillus spp. *Appl. Environ. Microbiol., 46: 1268-1275*