

Transformational Construction of a Protein-producing Strain of *Bacillus mesentericus niger*

Hara, Toshio

Laboratory of Microbial Technology, Faculty of Agriculture, Kyushu University

Fujio, Yusaku

Laboratory of Microbial Technology, Faculty of Agriculture, Kyushu University

Ueda, Seinosuke

Laboratory of Microbial Technology, Faculty of Agriculture, Kyushu University

<https://doi.org/10.5109/23750>

出版情報：九州大学大学院農学研究院紀要. 26 (4), pp.205-211, 1982-07. Kyushu University
バージョン：
権利関係：



Transformational Construction of a Protein-producing Strain of *Bacillus mesentericus niger*

Toshio Hara, Yusaku Fujio and Seinosuke Ueda

Laboratory of Microbial Technology, Faculty of Agriculture,
Kyushu University 46-09, Fukuoka 812

(Received February 5, 1982)

A transformant (HS-29) which accumulated a considerable amount of extracellular protein was isolated from *Bacillus mesentericus niger* no. 6021 which produced RNA extracellularly. Strain HS-29 produced 7.2 mg of protein per ml of maltose medium for 5 days in liquid shake culture. This strain excreted various kinds of proteins and the amount of exoprotein increased. The amino acid composition of intra- and extracellular protein was different, and extracellular protein contained relatively less amounts of alanine and glutamic acid compared with intracellular protein.

INTRODUCTION

It has been known that various microorganisms excrete proteins into the culture media. These proteins are detected as enzymes and toxins (Pollock, 1962; Schaeffer 1969; Heyningen 1970). A great deal of work has also been achieved on the production of enzymes for practical purposes. The mechanism of protein excretion has been a subject of extensive studies because the interest and importance of the problem concerning with individual proteins, knowledge on extracellular informational macromolecules and their formation. But its exact mechanism remained unsolved. Therefore, a systematic study on the excretion of proteins by microbial cells might be valuable in revealing information on extracellular macromolecules.

We were interested in constructing a microorganism capable of excreting and accumulating large amounts of proteins. As a first step in such a study, we tried to construct protein-excreting bacteria by using transformation as genetic technique.

MATERIALS AND METHODS

Strains and isolation of transformant

A character for the production of exoprotein in relaxed mutant, *Bacillus mesentericus niger* no. 6021 (Hara and Ueda, 1981a, 1982a, b) was transferred into strain no. 5603 (*thr*⁻) obtained from strain no. 6021 with N-methyl-N'-nitro-N-nitrosoguanidine by the method of Adelberg *et al.* (1965). *Pseudomonas aeruginosa* KYU-1, a DNA-excreting bacteria (Ueda and Hara, 1981;

Hara and Ueda, 1981b, c, 1982c; Hara et al., 1981), is used as the donor strain.

Transforming DNA was prepared from exponentially growing cells by the method of Saito and Miura (1963), and filtrated through Millipore filter (type HA, pore size $0.3\mu\text{m}$) to remove spores. Transformation experiments were carried out by the method of Yoshikawa (1970).

Medium and culture conditions

(1) Bacterial growth was determined by measuring optical density (OD) at 660nm of appropriately diluted culture broth with water.

(2) RNA samples of precipitates obtained by adding three volumes of 95 % ethanol to the supernatant solution of culture broth were subjected to RNA assay by the reaction of orcinol (Mejbaum, 1939) with yeast RNA as standard.

(3) Extracellular protein was estimated as follows: the culture broth was centrifuged to remove cells and an equal volume of 10 % trichloroacetic acid (TCA) was added to the supernatant solution. After allowing to stand for more than 30min at room temperature, the precipitate was washed with 5 % TCA two times, collected by centrifugation, dissolved precipitate was analyzed for protein by the method of Lowry et al. (1951) with bovin serum albumin as standard.

(4) Sugars were estimated by the method of Dubois et al. (1956).

(5) Amino acid composition of proteins was determined as follows. The extracellular protein was precipitated by adding equal volume of 10 % TCA to culture supernatant. The precipitate was dissolved in 0.5 M Tris-HCl buffer (pH 8.0) and dialyzed in a cellophan tube against 5 liters of 0.01 M Tris-HCl buffer (pH 8.0) for 24 hr. A small quantity of precipitate formed during the dialysis was removed by centrifugation and a crude powder of proteins was obtained by lyophilization of the dialysate. To assay intracellular protein, the cells were washed with 0.05 M phosphate buffer (pH 7.2) and then lyophilized. The protein samples were hydrolyzed in 6N HCl at 124°C in sealed tubes for 24 hr and quantitative analysis of amino acid was carried out by using JEOL JLC6AH automatic amino acid analyzer.

(6) Polyacrylamide gel electrophoresis with SDS was performed according to the method of Ames (1974).

RESULTS

Construction of strain producing extracellular protein

Transfer of phenotypic threonine nonrequirement(*thr*⁺) from *B. mesentericus niger* no. 6021 to strain no. 5603 by the DNA-mediated transformation was carried out as shown in Fig. 1. When competent cells of *B. mesentericus niger* no. 5603 were treated with DNA from RNA-producing bacteria, *thr*⁺ transformants appeared on selective plates (Table 1). The number of transformants was proportional to the amount of DNA added. However, the transformation frequencies of *thr*⁺ with DNA from *P. aeruginosa* KYU-1 were about 1/100 of those with a homologous DNA from *B. mesentericus niger* no. 6021.

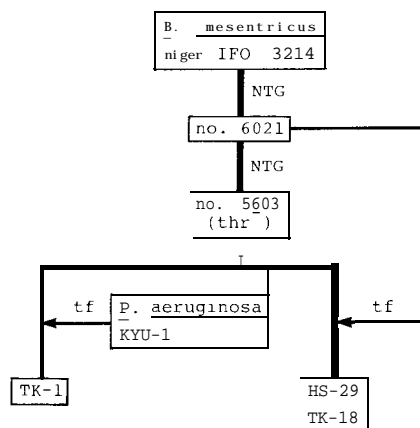


Fig. 1. Construction process of protein-producing bacteria.

Table 1. Comparison of transformation frequency. DNA concentration used was $0.1 \mu\text{g/ml}$. These experiments were carried out as described in Materials and Methods. After addition of DNA, pancreatic DNase (Boehringer Mannheim GmbH) ($10 \mu\text{g/ml}$) was added, followed by incubation for 60 min at 37°C and spreading onto minimal agar plates.

Donor strain	Recipient strain	Transformation frequency
<i>B. mesentericus niger</i> no.6021	<i>B. mesentericus niger</i> no.5603	6.3×10^{-4}
<i>P. aeruginosa</i> KYU-1	(<i>thr</i> ⁻)	1.0×10^{-6}

To isolate protein-producing transformants by the DNA of strain no. 6021, three *thr*⁺ transformants were selected. One hundred and five colonies of the transformants were screened for their ability to produce extracellular protein. One of the transformants (HS-29) was selected and used throughout this work (Table 2).

Table 2. Productivity of RNA and protein in the parental strains and representative transformants. Cultivation was carried out under the conditions as described in Materials and Methods.

Strain		RNA (mg/ml)	Protein (mg/ml)
Donor	no. 6021	4.8	3.4
	KYU-1	0.8	0.6
Recipient	no. 5603	2.7	5.1
	(<i>thr</i> ⁻)	3.2	
Transformant	TK-1	5.2	3.8
	TK-18		5.2
	HS-29	2.4	7.2

Time course of protein production

The time course of the production of extracellular protein by the transformant (HS-29) was examined. Figure 2 shows the time courses of growth and

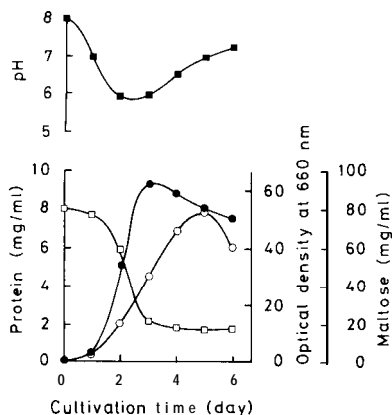


Fig. 2. Time course of extracellular protein production by strain HS-29. Cultivation was carried out under the same conditions as described in Materials and Methods. ○, extracellular protein; ●, growth; □, residual sugars; ■, pH.

protein productivity of strain HS-29. After lag period of about 1 day, bacterial growth became visible and continued until 3 days. Lysis was noted as a decrease in absorbance at 660nm. Sugar utilization was also rapid during the phase of growth and ceased at 3 days. Extracellular protein production proceeded with the growth of cell closely and the amount of extracellular protein amounted to 7.2 mg per ml of the culture broth.

Some characteristics of exoprotein produced by strain HS-29

The SDS polyacrylamide gel electrophoretic patterns of proteins obtained by protein-excretor is shown in Fig. 3. From many bands seen in the gels, the bacteria excreted various kinds of proteins and the amount of each pro-

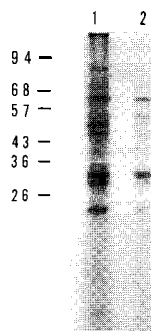


Fig. 3. Electrophoresis of proteins obtained from strain HS-29. Electrophoresis on 10 % polyacrylamide gel was performed according to the method of Ames. Migration was towards the bottom. Numbers on the left of the figure show the band position of marker proteins of various molecular weight ($\times 10^{-3}$). 1, product of strain no.6021; 2, HS-29.

tein varied.

The amino acid composition of intra- and extracellular protein obtained from protein-excreting bacteria, HS-29, was investigated. As shown in Table 3, the amino acid composition of these proteins was different, and exoprotein contained relatively less amounts of alanine and glutamic acid compared with intracellular protein.

Table 3. Amino acid composition of strain HS-29. Intracellular protein was extracted from cell grown for 3 days, and extracellular protein was obtained from culture supernatant as described in Materials and Methods. Each amino acid content was indicated as a basis on leucine content.

Amino acid	Intracellular	Extracellular
Phe	22	8
Try	9	8
Leu	100	100
Ileu	33	14
Met	37	5
Val	48	42
Ala	84	21
Gly	60	46
Glu	114	50
Ser	32	15
Thr	36	30
Asp	76	51
Arg	15	5
His	12	4
Lys	40	16

DISCUSSION

Many reports have appeared on protein excretion. But such proteins described in the literatures were exoenzymes and toxins. Except enzymes and toxins, other functions have also been known and some of them are antibiotics such as various bacteriocins (Nomura, 1967) or neocarzinostatin (Ishida *et al.*, 1965) competence factor for bacterial transformation (Perry and Slade, 1966), cell aggregating substance (Nakamura *et al.*, 1975) and others (Goldfarb *et al.*, 1973; Drucker, 1975).

It has been known that the rate of protein synthesis in growing bacteria is limited by the number of ribosomal RNA per cell (Stent and Brenner, 1961), and in normal cells, RNA synthesis is controlled by the molecular economy of cells. In relaxed mutants, however, the synthesis of ribosomal RNA continues even though the RNA is synthesized at the membrane, it might fail to become incorporated into ribosomes and is excreted.

In the case of the parental strain no. 6021, the productivity of extracellular RNA was depressed by the addition of amino acid (Hara and Ueda, 1982b). This phenomenon suggested that strain no. 6021 may be relaxed mutant. Therefore, the rate of protein synthesis in strain no. 6021 and transformant HS-29 was found to be fast. In fact, dissolved oxygen consumption by these strains was remarkably great (Hara and Ueda, 1982a) and a large amount of

cell mass from maltose resulted.

Recent experiments show that a large amount of protein can be produced in a simple medium containing a sufficient amount of carbon and nitrogen by adding glycine. In *Escherichia coli*, existence of signal peptides at the N-terminal protein of the precursors is one of the common characteristics of the extracytoplasmic proteins including the outer membrane proteins, the periplasmic proteins and the inner membrane proteins.

If the roles of excreted proteins in the microbial world would be clarified, the knowledge on excretion of macromolecules such as exoproteins and DNA could be obtained. Furthermore, enzymatic techniques for introducing recombinant DNA molecules into microbial cells as autonomous replicons would have been established for mass production of biological active substances such as hormone. The possibility for mass production of drug or food protein by fermentation process which is entirely new and may be superior to processes already known.

REFERENCES

- Adelberg, E. A., M. Mandel and G. C. C. Chen 1965 Optimal conditions for mutagenesis by N-methyl-N'-nitroso-N-nitrosoguanidine in *Escherichia coli*. *Biochem. Biophys. Res. Comm.*, 18: 788-795
- Ames, G. F-L. 1974 Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. *J. Biol. Chem.*, 249: 634-644
- Drucker, H. 1975 Regulation of extracellular proteins in *Neurospora crassa*: metabolic requirements of the process. *J. Bacteriol.*, 122: 1117-1125
- Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Robers and F. Smith 1956 Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, 28: 350-356
- Goldfarb, D. M., G. I. Goldberg, L. S. Chernin, L. A. Gukova, I. D. Avdienko, B. N. Kuznetsova and I. Ch. Kushner 1973 A protein produced by male strains of *Escherichia coli* K-12 which increases the yield of recombinants in conjugation: its nature and mode of action. *Molec. Gen. Genet.*, 120: 211-226
- Hara, T., A. Aumayr and S. Ueda 1981 Genetic transformation of *Pseudomonas aeruginosa* with extracellular DNA. *J. Gen. Appl. Microbiol.*, 27: 109-114
- Hara, T. and S. Ueda 1981a RNA production by *Bacillus mesentericus niger*. *J. Ferment. Technol.*, 59: 341-346
- Hara, T. and S. Ueda 1981b Studies on nucleic acid production and application II. A further study of extracellular DNA production by *Pseudomonas aeruginosa* KYU-1. *J. Appl. Microbiol.*, 3: 11-18
- Hara, T. and S. Ueda 1981c A study on the mechanism of DNA excretion from *P. aeruginosa* KYU-1. Effect of Mitomycin C on extracellular DNA production. *Agric. Biol. Chem.*, 45: 2457-2461
- Hara, T. and S. Ueda 1982a Effect of agitation on RNA production by *Bacillus mesentericus niger*. *J. Fac. Agr., Kyushu Univ.*, 26: 79-86
- Hara, T. and S. Ueda 1982b Effect of glycine on RNA production by *Bacillus mesentericus niger*. *J. Ferment. Technol.*, 60: 87-88
- Hara, T. and S. Ueda 1982c Transformation of *Pseudomonas aeruginosa* with chromosomal DNA into *Bacillus subtilis*. *J. Fac. Agr., Kyushu Univ.*, 26: 151-157
- Heyning, W. E. 1970 General characteristics. In "Microbial Toxins," Vol. I. ed. by S. J.

- Ajil, S. Kadis and T. C. Montie, Academic Press, New York, pp. 1-28
- Ishida, N., K. Miyazaki. K. Kumagai and M. Rikimaru 1965 Neocarzinostatin, antitumor antibiotic of high molecular weight. *J. Antibiot., Ser. A*, **18**: 68-76
- Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**: 265-275
- Mejbaum, W. 1939 Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. *Z. Physiol. Chem.*, **258**: 117-120
- Nakamura, J., S. Miyashiro and Y. Hirose 1975 Abstract of papers. The Annual Meeting of Agricultural Chemical Society of Japan, Sapporo. July, 1975, pp. 248 (in Japanese)
- Nomura, M. 1967 Colicins and related bacteriocins. *Ann. Rev. Microbiol.*, **21**: 257-284
- Perry, D. and H. D. Slade 1966 Effect of filtrates from transformable and nontransformable *Streptococci* on the transformation of *Streptococci*. *J. Bacteriol.*, **91**: 2216-2222
- Pollock, M. P. 1962 Exoenzymes. In "The Bacteria," Vol. IV, ed. by I. C. Gunsalus and R. Y. Stainer, Academic Press, New York, pp. 121-178
- Saito, H. and K. Miura 1963 Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta*, **72**: 619-629
- Schaeffer, P. 1969 Sporulation and the production of antibiotics, exoenzymes. and exotoxins. *Bacteriol. Rev.*, **33**: 48-71
- Stent, G. S. and S. Brenner 1961 A genetic locus for the regulation of ribonucleic acid synthesis. *Proc. Natl. Acad. Sci.*, **47**: 2005-2014
- Ueda, S. and T. Hara 1981 Studies on nucleic acid production and application I. Production of extracellular DNA by *Pseudomonas* sp. KYU-1. *J. Appl. Biochem.*, **3**: 1-10
- Yoshikawa, H. 1970 Temperature-sensitive mutants of *Bacillus subtilis* I. Multiforked replication and sequential transfer of DNA by a temperature-sensitive mutant. *Proc. Natl. Acad. Sci.*, **65**: 206-213