微生物における遺伝子工学と食品産業への応用

大阪大学工学部醗酵工学科　今中忠行

①遺伝子工学とその応用
②遺伝子組換え体によるトリプトファン生産
③タンパク質工学とその応用
④プロテアーゼの耐熱化とアスパルテームの生産
⑤各種糖質関連酵素とその改変
⑥機能性食品開発へ向けて

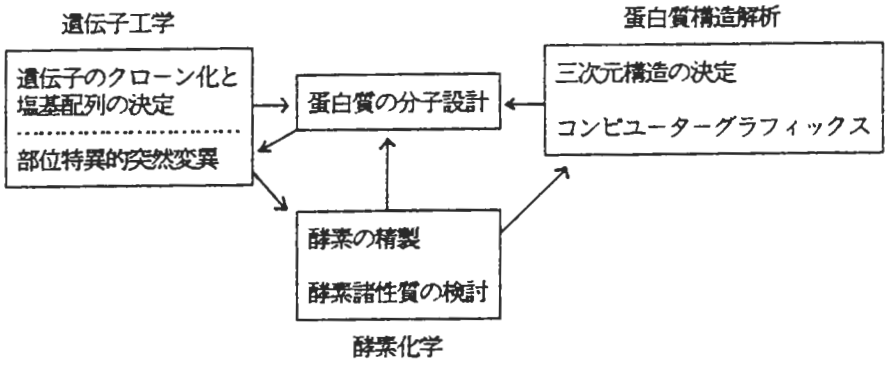


図　　蛋白質工学の基本概念

表　食品の機能

1次機能	栄養素（炭水化物、タンパク質、脂肪、ビタミン、ミネラルなど）の供給による健康の維持
2次機能	食品に含まれる感覚に関係する成分の働き、おもに味、色、匂い、テクスチャーの4つの感覚に作用
3次機能	食品に含まれる成分、またはそれらが消化・吸収の際に生ずる生成物の生理機能への作用

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1982, p. 289–297
0099-2240/82/020289-09$02.00/0

Vol. 43, No. 2

New Approach to Tryptophan Production by *Escherichia coli*: Genetic Manipulation of Composite Plasmids In Vitro

SHUICHI AIBA,* HIROSHI TSUNEKAWA, AND TADAYUKI IMANAKA

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamada-oka, Suita-shi, Osaka, Japan 565

Received 10 August 1981/Accepted 20 October 1981

For the purpose of studying the production of L-tryptophan by *Escherichia coli*, the deletion mutants of the *trp* operon (*trpAE1*) were transformed with mutant plasmids carrying the *trp* operon whose anthranilate synthase and phosphoribosyl anthranilate transferase (anthranilate aggregate), respectively, had been desensitized to tryptophan inhibition. In addition to release of the anthranilate aggregate from the feedback inhibition required for plasmids such as pSC101 *trp*·I15, the properties of *trp* repression (*trpR*) and tryptophanase deficiency (*tnaA*) were both indispensable for host strains such as strain Tna (*trpAE1 trpR tnaA*). The gene dosage effects on tryptophan synthase activities and on production of tryptophan were assessed. A moderate plasmid copy number, approximately five per chromosome, was optimal for tryptophan production. Similarly, an appropriate release of the anthranilate aggregate from feedback inhibition was also a necessary step to ward off the metabolic anomaly. If the mutant plasmid pSC101 *trp*·I15 was further mutagenized (pSC101 *trp*·I15·14) and then transferred to Tna cells, an effective enhancement of tryptophan production was achieved. Although further improvement of the host-plasmid system is needed before commercial production of tryptophan can be realized by this means, a promising step toward this goal has been established.

During the past 2 decades, a number of workers have been engaged in the study of L-tryptophan production by fermentation, using *Escherichia coli* (2, 16, 26), *Bacillus subtilis* (21), *Corynebacterium glutamicum* (10), or other organisms (6). However, the production of L-tryptophan in these studies has not necessarily been satisfactory to meet the economic feasibility of producing the aromatic amino acid by fermentation on an industrial scale.

With the present dissemination of information on genetic manipulation, much attention has been paid to the possibility of enhancing the production of L-tryptophan by improving strains that overproduce amino acids. Tribe and Pittard (26) were harbingers of this direction. The essential feature of their work on *E. coli* could be pinpointed as having: (i) 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthase, anthranilate synthase (ASase), and phosphoribosyl anthranilate transferase (PRTase) desensitized to end product inhibition, (ii) double auxotrophs of phenylalanine and tyrosine secured (Fig. 1), and (iii) *trp* operon enzymes derepressed in addition to the removal of attenuation. They also used ColV *trp* or F'*trp*, which was resistant to 5-methyl-DL-tryptophan, both ASase and PRTase having been desensitized to tryptophan inhibition. Apparently, these plasmids were used to study the gene dosage effect.

Tribe and Pittard dealt mainly with the genetic improvement of *E. coli* rather than with ColV *trp* and F'*trp* (copy number per chromosome, ~one [11] and one to three [7], respectively) for the production of tryptophan. In spite of their endeavors, the production of tryptophan still remains to be improved (26). In contrast, emphasis was placed here on the use of various mutant plasmids. In other words, the various mutants of pSC101 *trp* (copy number, ~five [4]) which became insensitive to feedback inhibition by tryptophan were transferred to *E. coli* W3110 *trpAE1 trpR tnaA* (Table 1) for further improvement in the production of tryptophan by *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains (derivatives of *E. coli* K-12) used are listed in Table 1. *E. coli* W3110 *trpAE1* is a deletion mutant of *trpA-E* and is designated AE1. *E. coli* W3110 *trpAE1 trpR27*(Am) is a *trp* repressor amber (nonsense) mutant and is designated Ram, and *E. coli* W3110 *trpAE1 trpR tnaA* is a *trp* repressor (missense mutant) and tryptophanase-deficient mutant and is designated Tna (14). pSC101 *trp* is a composite plasmid wherein the whole tryptophan operon of *E. coli* is cloned (18). The

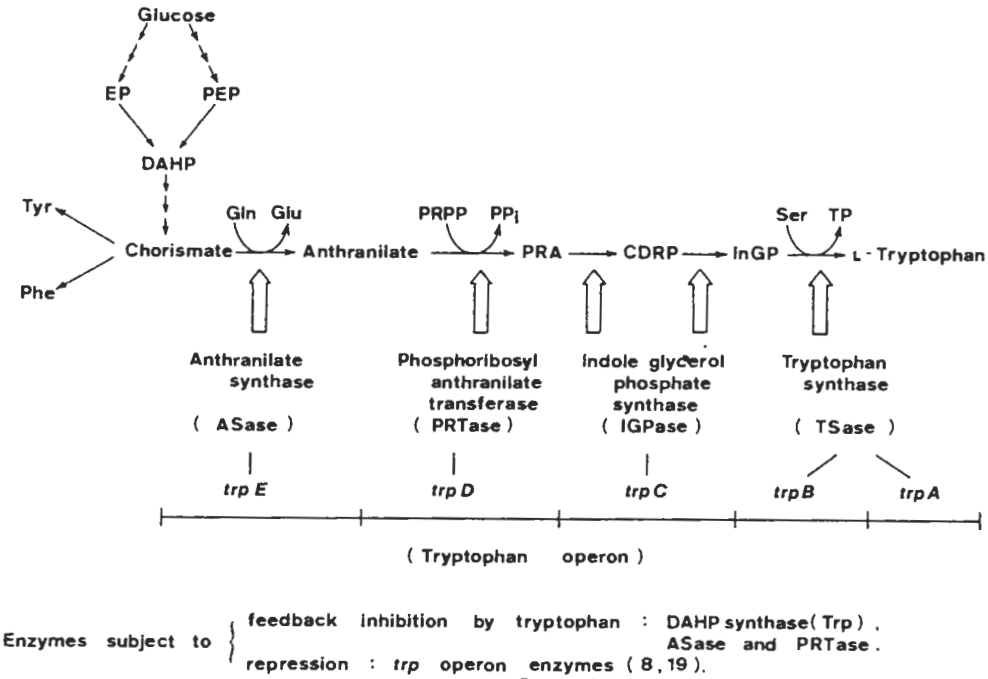


FIG. 1. Main pathways of L-tryptophan biosynthesis. Abbreviations: EP, D-erythrose 4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; Tyr, tyrosine; Phe, phenylalanine; Gln, glutamine; Glu, glutamate; PRPP, 5-phosphoribosyl-1-pyrophosphate; PP_i, pyrophosphate; PRA, 5-phosphoribosyl anthranilate; CDRP, 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate; InGP, indole-3-glycerol phosphate; Ser, serine; TP, triose phosphate.

remaining plasmids in Table 1 will be referred to below.

Media. Several kinds of culture media were used, and glucose was usually added to these media, except for L broth. Glucose and the remaining components in these media were sterilized separately at 121°C for 10 min. The compositions of L broth, L agar (13), minimal medium (MM), and MM agar (14) have been described previously.

MM medium was the basis of the following media designated MTI to MTIII for the production of tryptophan and contained, per liter: KH_2PO_4, 3 g; K_2HPO_4,

TABLE 1. Bacterial strains and composite plasmids

Strain/plasmid	Relevant properties/phenotype	Source/reference
E. coli		
W3110	*trpAE1*[a]	F. Imamoto (14)
W3110	*trpAE1 trpR27*(Am)[b]	F. Imamoto (14)
W3110	*trpAE1 trpR tnaA*[c]	F. Imamoto (14)
Plasmid		
pSC101 *trp*	Tc^r Trp^+	K. Sakaguchi (18)
pSC101 *trp*·I15	Tc^r Trp^+ I^-[d]	This work
pSC101 *trp*·I15·14	Tc^r Trp^+ I^-	
pSC101 *trp*·MI33	Tc^r Trp^+ I^-	
pSC101 *trp*·MI47	Tc^r Trp^+ I^-	
pSC101 *trp*·MI51	Tc^r Trp^+ I^-	
RP4 *trp*·I15	Ap^r Km^r Tc^r Trp^+ I^-	
RSF1010 *trp*·I15	Sm^r Trp^+ I^-	
pBR322 *trp*·I15	Ap^r Tc^r Trp^+ I^-	

[a] Deletion mutant of *trpA-E*, designated AE1.
[b] *trp* repressor amber mutant, designated Ram.
[c] Tryptophanase-deficient mutant, designated Tna.
[d] I^-, Insensitivity to feedback inhibition by tryptophan.

7 g; NH_4Cl, 3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 10 mg. When $FeSO_4 \cdot 7H_2O$ was deleted from MT medium (16) and the medium was supplemented with glucose (30 g) and Casamino Acids (1 g/liter), it was called MTI medium; MTII medium contained, per liter: glucose, 30 g; Casamino Acids, 3 g; anthranilic acid, 0.8 g; and an antibiotic (tetracycline or streptomycin), 10 mg, in addition to the components of MT medium. For MTIII medium, glucose, Casamino Acids, anthranilic acid, and tetracycline were added to MT medium at 50, 10, and 0.5 g/liter and 10 mg/liter, respectively. The initial pH of each medium was adjusted to 7.0 with 2 N NaOH.

Materials. Ampicillin (Ap), tetracycline (Tc), chorismic acid, lysozyme, 5-methyl-DL-tryptophan (5-MT), and 6-fluoro-DL-tryptophan were all purchased from Sigma Chemical Co., St. Louis, Mo. Streptomycin sulfate (Sm) and kanamycin sulfate (Km) were from Meiji Seika Co. Ltd., Tokyo, Japan.

5-Phosphoribosyl-1-pyrophosphate was from P-L Biochemicals, Inc., Milwaukee, Wis. Restriction endonucleases *Eco*RI and *Hin*dIII were purchased from Takara Shuzo Co. Ltd., Kyoto, Japan. Vector plasmid pBR322, T4 ligase, and bovine serum albumin were from Bethesda Research Laboratories Inc., Rockville, Md., and λDNA was from Miles Laboratories, Inc., Elkhart, Ind.

Tryptone, yeast extract, and Casamino Acids were from Difco Laboratories, Detroit, Mich. All other reagents were from Wako Chemical Industries, Osaka, Japan, unless otherwise noted.

Mutagenesis of composite plasmids. Composite plasmids, which encode anthranilate aggregate desensitized to tryptophan inhibition, were obtained originally from 5-MT-resistant mutants of AE1 that harbored pSC101 *trp*. pSC101 *trp* (moderate copy number; see above) was used exclusively, because selection of stable transformants, when needed, was more easy than for RSF1010 *trp* or pBR322 *trp* (larger copy numbers; see below): transformants with the latter plasmids would be potentially unstable due to the larger copy numbers. Both Ram(pSC101 *trp*) and Tna(pSC101 *trp*) exhibited high specific activities of the *trp* operon enzymes ASase and tryptophan synthase (TSase) (14). Consequently, these strains were rarely sensitive to 5-MT and were apparently unsuited to acquiring mutants resistant to 5-MT. On the other hand, AE1(pSC101 *trp*) was suitable, because the specific activities of both ASase and TSase were considerably lower than those in Ram(pSC101 *trp*) and Tna(pSC101 *trp*) (14).

AE1(pSC101 *trp*) was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (200 μg/ml) (1), and mutants were selected on MM agar containing 5-MT, 200 or 2,000 μg/ml. Each mutant was cultivated in MM in test tubes for 16 h at 37°C, and ASase activities of intact cells were then measured. pSC101 *trp*·I15 was extracted from the mutants (resistant to 5-MT at 200 μg/ml), whose ASase activities were not inhibited by tryptophan (0.2 mM). By the same procedure, pSC101 *trp*·MI33, pSC101 *trp*·MI47, and pSC101 *trp*·MI51 were also extracted from other mutants (resistant to 5-MT at 2,000 μg/ml) whose ASase activities were rarely or not inhibited by tryptophan (15 mM).

It was confirmed from transformation of AE1 with each composite plasmid, i.e., pSC101 *trp*·I15 to pSC101 *trp*·MI51, that the insensitivity to feedback inhibition was attributable to a mutation of the *trp* operon, because the plasmid transfer into AE1 was accompanied by the appearance of insensitivity to feedback inhibition by tryptophan (0.2 to 15 mM). Also, pSC101 *trp*·I15·14 was extracted from 5-MT (6,000 μg/ml)-resistant mutants of AE1(pSC101 *trp*·I15), whose ASase activities were rarely inhibited by tryptophan (15 mM).

Transformation, digestion of plasmid DNA with restriction endonuclease, and ligation. The procedures for transformation of host bacteria, digestion of plasmid DNA with *Eco*RI, and ligation with T4 ligase are described elsewhere (13).

Gel electrophoresis. Electrophoresis was carried out in agarose dissolved in Tris-borate buffer as previously described (14). λDNA digested with *Hin*dIII was used as an internal reference (13).

Preparation of plasmid DNA and construction of composite plasmids. Plasmid DNA was extracted by the rapid alkaline extraction procedure of Birnboim and Doly (3). Covalently closed circular DNA was purified by CsCl-ethidium bromide density gradient centrifugation (24) and dialyzed against Tris-sodium EDTA buffer after removal of the dye (13).

Vector plasmids, RSF1010 and RP4, were prepared from RSF1010 *trp* and RP4 *trp* DNA by digestion with *Eco*RI and subsequent ligation with T4 ligase. Both RSF1010 *trp* and RP4 *trp* were obtained from K. Sakaguchi (18). Sm^r Trp^- and Tc^r Trp^- transformants of AE1 were selected for RSF1010 and RP4, respectively. For construction of RSF1010 *trp*·I15, RSF1010 and pSC101 *trp*·I15 DNA were digested with *Eco*RI, followed by ligation with T4 ligase, and Sm^r Trp^+ transformants of AE1 were selected. RP4 *trp*·I15 was constructed likewise by using RP4 and pSC101 *trp*·I15, and Ap^r Km^r Trp^+ transformants of AE1 were selected. Finally, pBR322 *trp*·I15 was constructed from pBR322 and pSC101 *trp*·I15. The construction of these composite plasmids was confirmed by agarose gel electrophoresis.

Enzyme assays. Bacteria were grown until the late logarithmic phase at 37°C in 100 ml of MM. Details of harvesting, washing, and disruption of the cells to obtain crude extract are described elsewhere (14). ASase (EC 4.1.3.27) in the crude extract was assayed by the method of Hiraga (12). However, for screening of the strain that harbors the feedback inhibition-resistant mutant of the *trp* operon plasmid, ASase activity was measured with intact cells for convenience of handling many samples.

PRTase (EC 2.4.2.18) was assayed by the method of Ito and Yanofsky (15), whereas the method proposed by Smith and Yanofsky (23) was used to assay TSase (EC 4.2.1.20).

Protein assay. Protein concentration was measured by the method of Lowry et al. (17), with bovine serum albumin as a standard.

Cultivation. (i) Shake flasks. Bacteria precultured at 37°C overnight in test tubes (L broth, 5 ml, plus Tc or Sm, 10 μg/ml) were inoculated (0.5 ml) into MTI (50 ml) or MTII (50 ml) medium in 500-ml shake flasks. Cultivation continued at 37°C for 36 to 48 h, and the pH of the medium was adjusted to 7.0 with 2 N NaOH every 3 to 5 h.

(ii) Fermentors. A bench-scale minifermentor (capacity, 2 liters; type MD-250, Marubishi Co., Tokyo, Japan) was used. The cells newly transformed with the

plasmid (e.g., pSC101 *trp*·I15) were precultured at 37°C for 16 h in shake flasks (100 ml) containing L broth (20 ml) plus Tc (10 μg/ml). A 2-ml portion of the precultured broth was transferred into another flask (500 ml) that contained L broth (100 ml) plus Tc (10 μg/ml) for more shaking (5 h) at 37°C. The cells were totally transferred into MTIII medium (1.5 liters) in the minifermentor to carry out the run at 37°C under pH control of the medium. The pH was controlled automatically at 7.0 with NH_4OH (12 to 14%, wt/vol).

When anthranilic acid in the medium was reduced to about 0.3 g/liter, the feeding of anthranilic acid was started at a rate of 50 mg/liter per h with a peristaltic pump (type MP, Tokyo Scientific Instruments Co., Tokyo, Japan). An antifoam agent, AF-emulsion (Nakarai Chemical Co., Kyoto, Japan) was used if needed. Air flow rate and agitation speed of an impeller in the fermentor were 1.0 volume of air/volume of liquid per min and 500 rpm, respectively. Culture broth (about 10 ml) was sampled at intervals of 3 h, and immediately after the sampling, the broth was centrifuged (10,000 × *g*, 5 min) at 4°C to obtain supernatant liquid for assays of glucose, anthranilic acid, and tryptophan.

Analyses. Optical density was measured at 660 nm with a spectrophotometer (model 100-20, Hitachi Works Ltd., Tokyo, Japan) for a quick check of the cell density in the sampled broth. Dry cell weight was determined by filtering the broth on a membrane filter (pore size, 0.4 μm; Millipore Corp., Bedford, Mass.) and drying the cells at 100°C for 2 h. One optical density unit at 660 nm corresponded to 0.53 g of dry cells per liter. Glucose was determined by Glucostat reagent (Worthington Diagnostics, Freehold, N.J.) (27). The method presented by Smith and Yanofsky (22) for anthranilic acid was simplified as follows: samples were diluted with 0.5 M citrate buffer solution (pH 4.5), and 1 ml of the solution was mixed with 4 ml of ethyl acetate. Anthranilic acid extracted into ethyl acetate was measured photometrically at 336 nm with a spectrophotometer (model 100-20, Hitachi Works Ltd.). The method described by Dickman and Crockett (5) was used to analyze tryptophan.

Stability of composite plasmids. Phenotypic stability of composite plasmids was assessed by the procedure described elsewhere (14). The stability in this category was defined by the fraction of host cells (harboring composite plasmids) that expressed the phenotype, such as Trp^+ Tc^r, after 10 to 20 generations. Another stability studied was defined by the relative magnitude of specific activities of TSase in the host cells that might change during storage. Initial TSase activity was used as a reference. Storage conditions for media of the host cells were as follows: (i) L agar plus Tc (20 μg/ml), room temperatures or 4°C; (ii) MM agar, room temperatures or 4°C; (iii) L broth plus Tc (10 μg/ml), −80°C; and (iv) MM, −80°C.

RESULTS

Tryptophan production in shake flasks. (i) Minimal requirements for host strains and composite plasmids. AE1, Ram, and Tna were transformed with pSC101 *trp*·I15 to examine the production of tryptophan in shake flasks. Transformants with pSC101 *trp* were used as a control.

Tryptophan in MTI medium (without anthranilic acid) observed in the shaking culture at 37°C for 36 h is shown in Table 2. It is clear that both repression (*trpR*) and inhibition (I^-; feedback resistant) were minimal requirements for a marked production of tryptophan. When both *trpR* and I^- were provided, the accumulation of tryptophan was almost sevenfold higher than when only I^- was given. The phenotypic expression of *trpR* was of importance from the viewpoint of tryptophan production, regardless of a nonsense (in Ram) or missense (in Tna) mutation. In addition to the minimal requirements, the deficiency of tryptophanase activity in the host bacteria was also effective for tryptophan production (Table 2).

According to Kida and Matsushiro (16), who developed a double mutant (*trpR*, I^-) of *E. coli* K-12, the accumulation of tryptophan in MTI medium (shaking culture at 37°C for 42 h) was ~180 μg/ml. Although the difference between the production (180 μg/ml) they found and that in Table 2 (360 μg/ml) could not be attributed to the previous use of the *trp* operon on the chromosome versus the composite and multicopy (ca. five) plasmid here, it was postulated that multicopy plasmids would be useful for the production of a specific metabolite (tryptophan in this study).

(ii) Effect of copy number of composite plasmids. The earlier observation that tryptophan accumulation was enhanced (Table 2) when repression was removed may be supported by the enhanced activities of enzymes relevant to the *trp* operon. Hence, it was considered worthwhile to study the production of tryptophan with

TABLE 2. Shake flask cultures of AE1, Ram, and Tna strains[a]

Strain	Plasmid	Repression	Inhibition	Tryptophanase	Tryptophan (mg/liter)	Plasmid stability (%)
AE1	pSC101 *trp*	+	+	+	7	~100
AE1	pSC101 *trp*·I15	+	−	+	11	~100
Ram	pSC101 *trp*	−	+	+	7	~95
Ram	pSC101 *trp*·I15	−	−	+	70	~95
Tna	pSC101 *trp*	−	+	−	8	~85
Tna	pSC101 *trp*·I15	−	−	−	360	~85

[a] Cells were grown in MTI medium (without anthranilic acid) at 37°C for 36 h.

TABLE 3. TSase activities of AE1 and Tna strains[a]

Strain	TSase (U/mg of protein)	Plasmid		Plasmid stability (%)
		Copy no./ chromosome	Reference	
AE1(RP4 *trp*·I15)	4	1–3	7	~100
AE1(pSC101 *trp*·I15)	25	~5	4	~100
AE1(RSF1010 *trp*·I15)	47	10–50	9	~100
AE1(pBR322 *trp*·I15)	114	60–80	25, 28	~80
Tna(RP4 *trp*·I15)	36	1–3	7	~100
Tna(pSC101 *trp*·I15)	107	~5	4	~85
Tna(RSF1010 *trp*·I15)	215	10–50	9	~35
Tna(pBR322 *trp*·I15)	—[b]	60–80	25, 28	—

[a] Cells were grown in MM at 37°C until the late log phase.
[b] —, Stable transformants difficult to obtain.

various composite and feedback-resistant plasmids transferred to AE1 or Tna. Thus, the gene dosage effect on activities of specific enzymes of the *trp* operon could be assessed if composite plasmids with various copy numbers were used.

Assuming that TSase activity represents those of the *trp* operon enzymes, TSase activities measured with transformants of AE1 or Tna are summarized in Table 3. The copy numbers of RP4, pSC101, RSF1010, and pBR322 are taken as 1 to 3 (7), ~5 (see above), 10 to 50 (9), and 60 to 80 (25, 28), respectively. The copy number of the vector plasmid in the references was assumed to represent, on a relative basis, that of the composite plasmid. The gene dosage effect was then manifested in AE1 (Table 3), because the increase of TSase activity in AE1 cells almost corresponded to the copy number of the plasmids in the host cells.

Although the gene dosage effect could also be noted in Tna, the fact that the activity of TSase in Tna(pBR322 *trp*·I15) was beyond assessment does not necessarily negate the gene dosage effect but was apparently due to the difficulty in obtaining stable transformants. The anomaly of metabolism that might have been triggered by the large copy number of pBR322 *trp* and the absence of the *trp* repressor in Tna cells might have made it difficult to acquire the transformant. In fact, about 4 days were required to obtain the transformant of Tna with pBR322 *trp*·I15 on MM agar (37°C), the period being two- to threefold longer than that needed for acquiring all other transformants listed in Table 3. In addition, colonies of this specific transformant [Tna(pBR322 *trp*·I15)] on MM agar were small in size, suggesting that the growth rate of the transformant was low.

The phenotypic stability of AE1 shown in Table 3 (lines 1 to 3) was nearly 100%, whereas that of AE1(pBR322 *trp*·I15) was about 80%. In other words, the stability deteriorated when TSase activity increased (14). Since TSase activity in Tna was larger than in AE1, phenotypic stability in Tna would not always be warranted. Actually, the stabilities for Tna carrying RP4 *trp*·I15, pSC101 *trp*·I15, and RSF1010 *trp*·I15 were of the order of 100, 85, and 35%, respec-

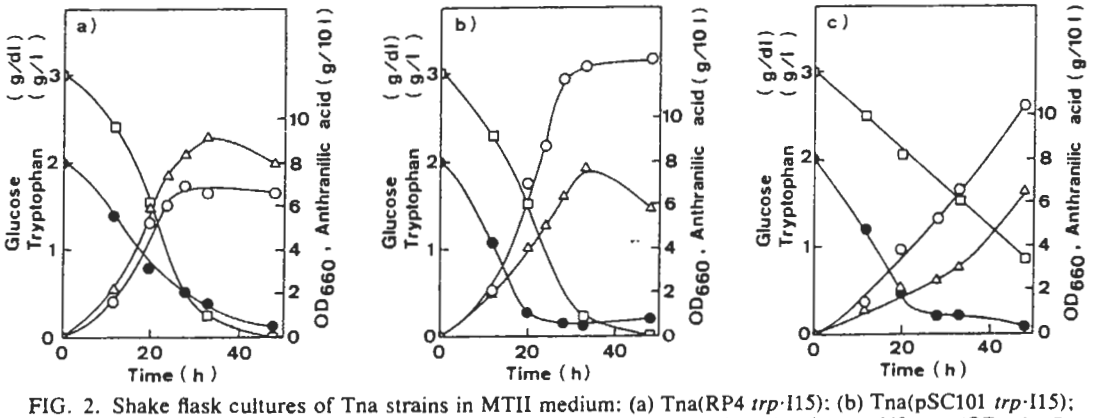


FIG. 2. Shake flask cultures of Tna strains in MTII medium: (a) Tna(RP4 *trp*·I15); (b) Tna(pSC101 *trp*·I15); (c) Tna(RSF1010 *trp*·I15). Symbols: □, glucose; ○, tryptophan; △, optical density at 660 nm (OD_{660}); ●, anthranilic acid.

tively (Table 3), whereas the stability for Ram carrying pSC101 *trp*·I15 was ~95% (Table 2).

We examined the relationship between the copy number of the plasmids and the production of tryptophan rather than the activity of TSase. In this context, shake flask cultures of Tna carrying RP4 *trp*·I15, pSC101 *trp*·I15, or RSF1010 *trp*·I15 in MTII media are shown in Fig. 2a, b, and c, respectively. For RP4 *trp*·I15 and pSC101 *trp*·I15, the maximum concentration of tryptophan (1.7 and 3.1 g/liter, respectively) was nearly linear with the copy number of these plasmids; i.e., the larger the copy number, the higher the product (tryptophan) concentration.

However, the close correlation among copy number, TSase activity, and accumulation of tryptophan in culture medium ceased to exist when the copy number exceeded a certain level. For Tna(RSF1010 *trp*·I15) (Fig. 2c), the maximum tryptophan concentration (2.6 g/liter) apparently deviated from the relationship between the copy number and tryptophan produced, although the TSase activity was still proportional to the copy number.

The gene dosage effect observed here and the de facto limitation most probably suggest the optimum plasmid copy number for tryptophan production. The use of pSC101 *trp*·I15 or its derivative hereafter in this paper originated from the above inference.

It is clear (Fig. 2a, b, and c) that the bacterial growth rate deteriorated conversely with the enhancement of TSase activity. The decrease in the accumulation of tryptophan in Fig. 2c was perhaps due to the poor growth of this strain, Tna(RSF1010 *trp*·I15), caused by the higher TSase activity. This interpretation agrees with the previous observation on the growth of Tna(pBR322 *trp*·I15) in Table 3. Lastly, the difference in the order of magnitude for tryptophan concentration in Table 2 and Fig. 2 must be mentioned. MTI medium (without anthranilic acid) was used in Table 2 primarily for ease of comparison with the earlier work of Kida and Matsushiro (16), whereas in Fig. 2 MTII medium containing anthranilic acid was used, and a higher accumulation of tryptophan in the medium was obtained in shake flasks. The use or feeding of anthranilic acid in this paper was considered.

Tryptophan production in fermentors. (i) Release from feedback inhibition. The degree of release from feedback inhibition was defined by a tryptophan concentration (millimolar) that inhibits ASase activity by one-half of that assayed in the absence of tryptophan. The higher the concentration, the higher the degree of release from feedback inhibition, implying that ASase activity becomes more insensitive to tryptophan and vice versa.

Tryptophan accumulation in MTIII medium in fermentors at 37°C for 30 h was studied with various mutants of pSC101 *trp* or a derivative of pSC101 *trp*·I15 that were transferred to Tna. The concentration of tryptophan observed at the end of each run was plotted against the degree of

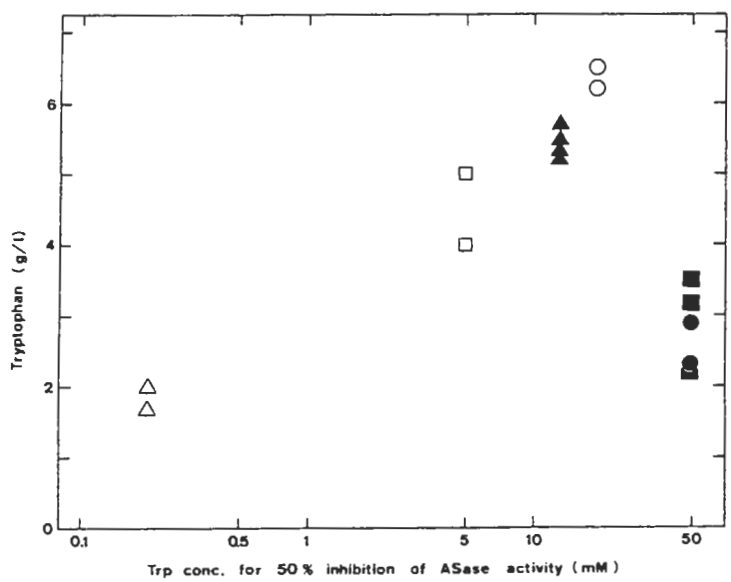


FIG. 3. Production of tryptophan by Tna strains carrying various mutants of pSC101 *trp* or a derivative of pSC101 *trp*·I15 versus degree of release for ASase from feedback inhibition. Cells were grown in minifermentors (MTIII medium) at 37°C and pH 7.0 for about 30 h. Symbols: △, Tna(pSC101 *trp*); ▲, Tna(pSC101 *trp*·I15); ○, Tna(pSC101 *trp*·I15·14); ●, Tna(pSC101 *trp*·MI33); □, Tna(pSC101 *trp*·MI47); ■, Tna(pSC101 *trp*·MI51).

release (Fig. 3). It is interesting that tryptophan production was enhanced in proportion to the release as long as the abscissa reading was less than 20 mM.

When the release became more advanced, tryptophan production was adversely affected (Fig. 3). Supposing that the release was in the range given in Fig. 3, specific activities of ASase, TSase, and PRTase ranged from 16 to 17, 105 to 116, and 6.1 to 7.0 U/mg of protein, respectively. Regarding both Tna(pSC101 *trp*·MI33) and Tna(pSC101 *trp*·MI51), whose release of ASase from feedback inhibition was more complete, the activities were 10.8 to 11.0 U/mg of protein for ASase, 90 to 94 U/mg of protein for TSase, and 3.4 to 3.9 U/mg of protein for PRTase.

A marked decrease in the activities of ASase and PRTase (anthranilate aggregate) in these latter plasmids may be partly responsible for the decrease of tryptophan production when the degree of release was 50 mM (Fig. 3). However, the feedback inhibition of PRTase by tryptophan must be addressed. Different from the previous picture for the ASase, the remaining fraction of PRTase assayed in the presence of tryptophan for all of the composite plasmids (Fig. 3) leveled off when the tryptophan concentration was >1 mM (15). Evidently, a particular feature of PRTase did not permit the convenient use of a 50% inhibition level of tryptophan as for the ASase. Notwithstanding this fact, it was confirmed that the leveling-off fraction of PRTase for a given plasmid (Fig. 3) corresponded to the degree of release for ASase of the same plasmid; i.e., when the release of the ASase increased, the residual fraction of the PRTase (indicative of the release for PRTase) was also found to increase (data not shown). Accordingly, the release from feedback inhibition for ASase activities might be assumed to represent the overall picture for both enzymes.

(ii) Batch culture of Tna(pSC101 *trp*·I15·14). An example of batch cultures of Tna(pSC101 *trp*·I15·14) is shown in Fig. 4. Although the data points (optical density at 660 nm) for cell growth in MTIII medium at 37°C, pH 7.0, exhibited logarithmic growth up to about 9 h after the start of this run, the production of tryptophan did not necessarily correspond to cell growth.

The accumulation of tryptophan terminated when glucose was exhausted. Correspondingly, the concentration of anthranilic acid that had been fed continuously into the medium began to increase due to the cessation of anthranilic acid uptake by the cells.

The concentration of tryptophan at 27 h in this example was 6.2 g/liter. The total amount of anthranilic acid charged into the medium was: 0.5 g (initial) + 50 mg/liter per h × 20 h (feeding

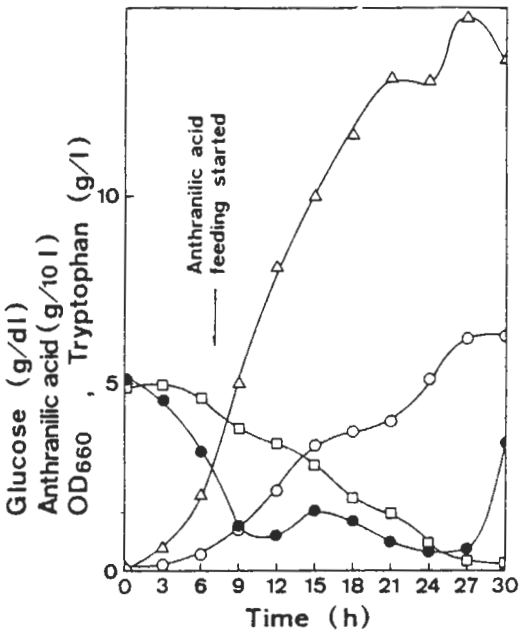


FIG. 4. Batch culture of Tna(pSC101 *trp*·I15·14) at 37°C and pH 7.0 in minifermentors (MTIII medium). Symbols: □, glucose; ○, tryptophan; △, optical density at 660 nm (OD_{660}); ●, anthranilic acid.

period) = 1.5 g. A stoichiometric estimate of tryptophan to be synthesized from this total amount of anthranilic acid was equivalent to 2.25 g (= 1.5 × 1.5). Consequently, it is inferred that nearly 4 g (= 6.2 − 2.25) of tryptophan must have been yielded by using the anthrailic acid that resulted from the initial glucose added (Fig. 1).

DISCUSSION

The advantages of this new approach to the production of tryptophan by *E. coli* were that (i) host strains and composite plasmids could be treated with mutagen independently; (ii) after mutagenesis, mutants of the host strain could be transformed with mutant plasmids; and (iii) more freedom was available for choice of these mutant strains and plasmids for transformation. It is evident that the high production of tryptophan (Fig. 4) could not be realized unless this approach was utilized. Similarly, Tribe and Pittard reported on the production of tryptophan by *E. coli*, using plasmid ColV *trp* or F'*trp* (copy number, one to three per chromosome) (26). Their data showed that 1 g of tryptophan per liter accumulated after 12.5 h of cultivation, the productivity being 0.08 g of tryptophan/liter per h. Their principal concern was in the use of various mutants of the host strain rather than plasmids.

Other recent work on tryptophan production dealt with either *B. subtilis* or *C. glutamicum*, both of which had been subjected to a series of mutageneses. According to Shiio et al., the tryptophan concentration in the culture medium of *B. subtilis* was 6.15 g/liter in 48 h (21), whereas the production with *C. glutamicum* was 12 g/liter in 96 h (10). For tryptophan yield per hour, it is clear that the data in Fig. 4 surpassed the yields for *B. subtilis* and *C. glutamicum*.

It must be emphasized that the host strains used throughout this study were deletion mutants (*trpAE1*) having either *trpR* or deficiency of tryptophanase in addition to *trpR*. Consequently, there was no need to use *recA* strains; therefore, there was no possibility of recombination of plasmid DNA with chromosomes. If the lower growth rate of *recA* strains is taken into account, the fact that the deletion mutants in this work is also significant from the viewpoint of tryptophan production.

Tryptophan analogs other than the 5-MT, 6-fluoro-DL-tryptophan, 4-methyl-DL-tryptophan, 6-methyl-DL-tryptophan, and tryptophan hydroxamate (all from Sigma Chemical Co.) were examined to select feedback-resistant plasmid mutants. However, since all of the mutant strains tested were insensitive to these analogs except 6-fluoro-DL-tryptophan, the use of these analogs was impossible. It must be mentioned that repeated trials to select feedback-resistant plasmids from 6-fluoro-DL-tryptophan-resistant mutants failed for unknown reasons.

It is interesting to point out the following gene dosage effect: the TSase activities of AE1 carrying the composite plasmids were enhanced nearly in proportion to the copy number (Table 3). The effect was more clearly seen in AE1 than in Tna. In Tna cells, the enhanced expression of TSase activities owing to *trpR* might have disturbed the metabolism of the host strain (20). The metabolic disturbance would be responsible for the apparent deviation from the proportionality in Tna. The gene dosage effect on tryptophan production rather than the activities of enzymes for the biosynthesis of tryptophan was recognized to some extent in Tna (Fig. 2).

An extensive analysis of amino acids in the broth of MTIII (Fig. 4) with an amino acid autoanalyzer (model 835-50, Hitachi Works Ltd.) that assays 17 species of amino acids (not glutamine, asparagine, and tryptophan) revealed that concentrations of glycine, serine, and threonine were not detected during the initial 4 to 8 h. The remaining 14 amino acids decreased rather than increased in concentration as the cultivation proceeded (data not shown). The early disappearance of glycine, serine, and threonine from the broth would support the metabolic pathways shown in Fig. 1 functioning normally in the culture of Tna cells harboring pSC101 *trp*·115·14. In this context, however, it is difficult to rule out the possibility that serine arising from glucose may have limited the biosynthesis of tryptophan.

Consequently, an appropriate addition of serine to the medium during the cultivation would be effective to increase tryptophan production. Indeed, ample room is available for further improvement of tryptophan production by enriching the culture medium, by feeding glucose intermittently (rather than the initial dumped supply) to prolong cellular activities, or by securing a double auxotroph of phenylalanine and tyrosine. However, the commendable production of tryptophan already achieved in a simple medium (MTIII) by a routine culture manipulation is worthy of attention (Fig. 4).

Finally, the plasmid stability (TSase activity) during storage of Tna(pSC101 *trp*·115·14) must be emphasized. When the composite plasmids were at −80°C, the enzyme activities remained almost unchanged during 150 days, whereas at 4°C the activities decreased to 0.8, irrespective of L agar (+ Tc, 20 μg/ml) or MM agar, taking the initial TSase activity as unity. Storage at room temperature was prohibitive because of deformation and desiccation of the agar during the 150-day period.

LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. 18:788–795.
2. **Aiba, S., T. Imanaka, and H. Tsunekawa.** 1980. Enhancement of tryptophan production by *Escherichia coli* as an application of genetic engineering. Biotechnol. Lett. 2:525–530.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513–1523.
4. **Cabello, F., K. Timmis, and S. N. Cohen.** 1976. Replication control in a composite plasmid constructed by *in vitro* linkage of two distinct replicons. Nature (London) 259:285–290.
5. **Dickman, S. R., and A. L. Crockett.** 1956. Reactions of xanthydrol. IV. Determination of tryptophan in blood plasma and in proteins. J. Biol. Chem. 220:957–965.
6. **Ebihara, Y., N. Niitsu, and G. Terui.** 1969. Fermentative production of tryptophan from indole by *Hansenula anomala*. J. Ferment. Technol. 47:733–738.
7. **Falkow, S., P. Guerry, R. W. Hedges, and N. Datta.** 1974. Polynucleotide sequence relationships among plasmids of the I compatibility complex. J. Gen. Microbiol. 85:65–76.
8. **Gibson, F., and J. Pittard.** 1968. Pathways of biosynthesis of aromatic amino acids and vitamins and their control in microorganisms. Bacteriol. Rev. 32:465–492.
9. **Guerry, P., J. V. Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. 117:619–630.
10. **Hagino, H., and K. Nakayama.** 1975. L-tryptophan production by analog-resistant mutants derived from a phenylalanine and tyrosine double auxotroph of *Corynebacterium glutamicum*. Agric. Biol. Chem. 39:343–349.
11. **Hickson, F. T., T. F. Roth, and D. R. Helinski.** 1967. Circular DNA forms of a bacterial sex factor. Proc. Natl. Acad. Sci. U.S.A. 58:1731–1738.

12. **Hiraga, S.** 1969. Operator mutants of the tryptophan operon in *Escherichia coli*. J. Mol. Biol. **39**:159–179.
13. **Imanaka, T., T. Tanaka, H. Tsunekawa, and S. Aiba.** 1981. Cloning of the genes for penicillinase, *penP* and *penI*, of *Bacillus licheniformis* in some vector plasmids and their expression in *Escherichia coli*, *Bacillus subtilis*, and *Bacillus licheniformis*. J. Bacteriol. **147**:776–786.
14. **Imanaka, T., H. Tsunekawa, and S. Aiba.** 1980. Phenotypic stability of *trp* operon recombinant plasmids in *Escherichia coli*. J. Gen. Microbiol. **118**:253–261.
15. **Ito, J., and C. Yanofsky.** 1969. Anthranilate synthetase, an enzyme specified by the tryptophan operon of *Escherichia coli*: comparative studies on the complex and the subunits. J. Bacteriol. **97**:734–742.
16. **Kida, S., and A. Matsushiro.** 1965. The application of cellular regulatory theories to the tryptophan accumulation by *Escherichia coli*. (I) Tryptophan accumulation by *Escherichia coli*. J. Ferment. Technol. **43**:302–306.
17. **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.
18. **Nagahari, K., T. Tanaka, F. Hishinuma, M. Kuroda, and K. Sakaguchi.** 1977. Control of tryptophan synthetase amplified by varying the numbers of composite plasmids in *Escherichia coli* cells. Gene **1**:141–152.
19. **Pabst, M. J., J. C. Kuhn, and R. L. Somerville.** 1973. Feedback regulation in the anthranilate aggregate from wild type and mutant strains of *Escherichia coli*. J. Biol. Chem. **248**:901–914.
20. **Rood, J. I., M. K. Sneddon, and J. F. Morrison.** 1980. Instability in *tyrR* strains of plasmids carrying the tyrosine operon: isolation and characterization of plasmid derivatives with insertions or deletions. J. Bacteriol. **144**:552–559.
21. **Shiio, I., K. Ishii, and K. Yokozeki.** 1973. Production of L-tryptophan by 5-fluorotryptophan resistant mutants of *Bacillus subtilis*. Agric. Biol. Chem. **37**:1991–2000.
22. **Smith, O. H., and C. Yanofsky.** 1960. 1-(o-Carboxyphenylamino)-1-deoxyribulose 5-phosphate, a new intermediate in the biosynthesis of tryptophan. J. Biol. Chem. **235**:2051–2057.
23. **Smith, O. H., and C. Yanofsky.** 1962. Enzymes involved in the biosynthesis of tryptophan. Methods Enzymol. **5**:794–806.
24. **Tanaka, T., and B. Weisblum.** 1975. Construcion of a colicin E1-R factor composite plasmid in vitro: means of amplification of deoxyribonucleic acid. J. Bacteriol. **121**:345–362.
25. **Timmis, K. N., S. N. Cohen, and F. C. Cabello.** 1978. DNA cloning and the analysis of plasmid structure and function, p. 1–58. *In* F. E. Hahn, H. Kersten, W. Kersten, and W. Szybalski (ed.), Progress in molecular and subcellular biology, vol. 6. Springer-Verlag, New York.
26. **Tribe, D. E., and J. Pittard.** 1979. Hyperproduction of tryptophan by *Escherichia coli*: genetic manipulation of the pathways leading to tryptophan formation. Appl. Environ. Microbiol. **38**:181–190.
27. **Washko, M. E., and E. W. Rice.** 1961. Determination of glucose by an improved enzymatic procedure. Clin. Chem. **7**:542–545.
28. **Widera, G., F. Gautier, W. Lindenmaier, and J. Collins.** 1978. The expression of tetracycline resistance after insertion of foreign DNA fragments between the *Eco*RI and *Hin*dIII sites of the plasmid cloning vector pBR322. Mol. Gen. Genet. **163**:301–305.

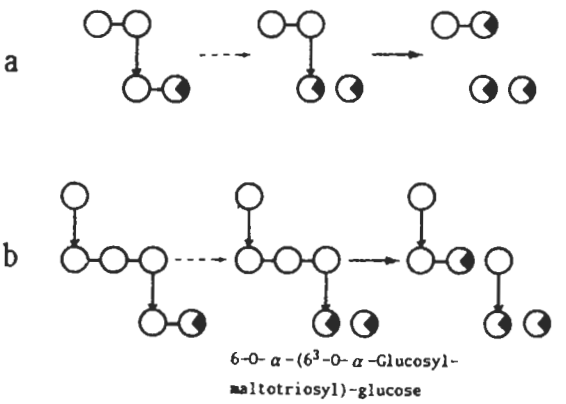


FIG. 5. Schematic representation of a side reaction of neopullulanase on 6^2-*O*-α-maltosyl-maltose (a) and 6^2-*O*-α-(6^3-*O*-α-glucosyl-maltotriosyl)-maltose (b). Broken arrows indicate the very weak side reaction of neopullulanase. Symbols are as in Fig. 1.

(Fig. 3) must be reliable enough to explain the action of neopullulanase on pullulan.

In addition to these main reactions, a slight side reaction of neopullulanase was found. Neopullulanase attacked mainly the α-(1→6) linkage of 6^2-*O*-α-maltosyl-maltose and produced maltose. However, a very small amount of glucose was found (Fig. 2c). Therefore, it was most likely that neopullulanase hydrolyzed the α-(1→4) linkage of 6^2-*O*-α-maltosyl-maltose very slightly and produced glucose and maltose (Fig. 5a). A similar reaction might occur with other substrates. Taking 6^2-*O*-α-(6^3-*O*-α-glucosyl-maltotriosyl)-maltose as an example of an intermediate product, a very small amount of isomaltose might be produced as one of the final products from pullulan after neopullulanase treatment (Fig. 5b). In fact, a faint spot corresponding to isomaltose was found on the paper chromatogram in Fig. 4, spot a. Spot b in Fig. 4 was thought to be the intermediate product 6-*O*-α-(6^3-*O*-α-glucosyl-maltotriosyl)-glucose (Fig. 5b).

Recently, some enzymes that demonstrate dual specificity towards α-(1→4)- and α-(1→6)-glucosidic linkages have been reported (3, 10, 12). *Thermoanaerobium* sp. strain Tok6-B1 pullulanase (10) and *Thermoanaerobium brockii* debranching enzyme (3) can cleave the α-(1→6) linkages [and none of the α-(1→4) bonds] of pullulan and the α-(1→4) linkages of starch. On the other hand, *Thermoactinomyces vulgaris* α-amylase attacks some of the α-(1→6) linkages in partial hydrolysates of pullulan as well as α-(1→4) linkages in starch and pullulan (12). From the viewpoint of the pattern of action on pullulan and partial hydrolysates of pullulan, *B. stearothermophilus* neopullulanase has something in common with *T. vulgaris* α-amylase. When compared on the same basis of a final hydrolysis percentage [(reducing sugar/total carbohydrate) × 100], *T. vulgaris* α-amylase was shown to hydrolyze pullulan and starch 35 and 50%, respectively (14), whereas *B. stearothermophilus* neopullulanase hydrolyzed pullulan efficiently (45%) but hydrolyzed starch less efficiently (13%) (8). Hence, *B. stearothermophilus* neopullulanase should be clearly distinguished from α-amylase.

Further investigations are now in progress to better understand the substrate specificity of the enzyme by using several polysaccharides, such as amylose, amylopectin, glycogen, and β-limit dextrin.

LITERATURE CITED

1. **Abdullah, M., and D. French.** 1970. Substrate specificity of pullulanase. Arch. Biochem. Biophys. **137**:483–493.
2. **Bender, H., and K. Wallenfels.** 1961. Untersuchungen an Pullulan. II. Spezifisher Abbau durch ein Bacterielles Enzyme. Biochem. Z. **334**:79–95.
3. **Coleman, R. D., S. S. Yang, and M. P. McAlister.** 1987. Cloning of the debranching-enzyme gene from *Thermoanaerobium brockii* into *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **169**:4302–4307.
4. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
5. **Fukumoto, J., and S. Okada.** 1963. Studies on bacterial amylase. Amylase types of *Bacillus subtilis* species. J. Ferment. Technol. **41**:427–434.
6. **Kainuma, K., and D. French.** 1969. Action of pancreatic amylase on starch oligosaccharides containing single glucose side chains. FEBS Lett. **5**:257–261.
7. **Kainuma, K., S. Kobayashi, and T. Harada.** 1978. Action of *Pseudomonas* isoamylase on various branched oligo- and polysaccharides. Carbohydr. Res. **61**:345–357.
8. **Kuriki, T., S. Okada, and T. Imanaka.** 1988. New type of pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. J. Bacteriol. **170**:1554–1559.
9. **Pazur, J. H., and T. Ando.** 1960. The hydrolysis of glucosyl oligosaccharides with α-D-(1→4) and α-D-(1→6) bonds by fungal amyloglucosidase. J. Biol. Chem. **235**:297–302.
10. **Plant, A. R., R. M. Clemens, H. W. Morgan, and R. M. Daniel.** 1987. Active-site- and substrate-specificity of *Thermoanaerobium* Tok6-B1 pullulanase. Biochem. J. **246**:537–541.
11. **Robyt, J., and D. French.** 1963. Action pattern and specificity of an amylase from *Bacillus subtilis*. Arch. Biochem. Biophys. **100**:451–467.
12. **Sakano, Y., S. Hiraiwa, J. Fukushima, and T. Kobayashi.** 1982. Enzymatic properties and action patterns of *Thermoactinomyces vulgaris* α-amylase. Agric. Biol. Chem. **46**:1121–1129.
13. **Sakano, Y., N. Masuda, and T. Kobayashi.** 1971. Hydrolysis of pullulan by a novel enzyme from *Aspergillus niger*. Agric. Biol. Chem. **35**:971–973.
14. **Shimizu, M., M. Kanno, M. Tamura, and M. Suekane.** 1978. Purification and some properties of a novel α-amylase produced by a strain of *Thermoactinomyces vulgaris*. Agric. Biol. Chem. **42**:1681–1688.
15. **Ueda, S., K. Fujita, K. Komatsu, and N. Nakashima.** 1963. Polysaccharide produced by the genus *Pullularia*. 1. Production of polysaccharide by growing cells. Appl. Microbiol. **11**:211–215.
16. **Umeki, K., and T. Yamamoto.** 1972. Enzymatic determination of structure of singly branched hexaose dextrins formed by liquefying α-amylase of *Bacillus subtilis*. J. Biochem. (Tokyo) **72**:101–109.

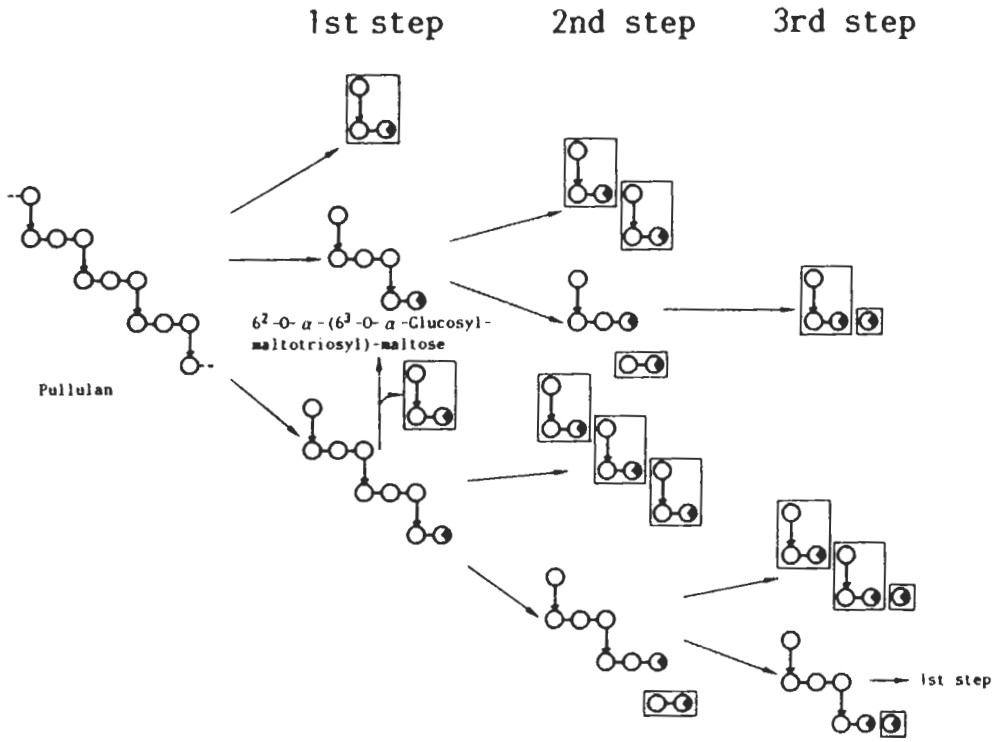


FIG. 3. Model of pullulan hydrolysis by neopullulanase. Symbols are as in Fig. 1. The oligosaccharides enclosed by rectangles are the final products.

Confirmation of the model of action. To examine whether the action model is correct, we analyzed intermediate products produced from pullulan by neopullulanase. A 10% pullulan solution (4.5 ml) and 5 U of neopullulanase were incubated at 50°C for 4 h, and the reaction was stopped by heat treatment at 100°C for 5 min. The reaction mixture was analyzed by preparative-scale paper chromatography, and a part of the reaction mixture was visualized by paper chromatography after treatment by the glucoamylase dip method (6) to convert weakly reducing oligosaccharides into glucose (Fig. 4). Spots A, B, and C corresponded to glucose, maltotriose, and panose, respectively, as final products. Spot D seemed to be a branched DP 4 oligosaccharide, and the fraction was extracted from the paper chromatogram. The sample was hydrolyzed by neopullulanase, and glucose and a branched DP 3 oligosaccharide were produced as final products. The extracted fraction was hydrolyzed by glucoamylase, and only glucose was produced as the final product. These results indicated that spot D was 6^3-*O*-α-glucosylmaltotriose. Spot E corresponded to a branched DP 6 oligosaccharide, and the fraction was extracted. The sample was hydrolyzed by pullulanase to produce maltose and a branched DP 4 oligosaccharide and was also hydrolyzed by glucoamylase to produce glucose and panose. These results suggested that spot E was 6^2-*O*-α-(6^3-*O*-α-glucosyl-maltotriosyl)-maltose. In this context, the extracted sample from the spot E fraction was hydrolyzed by neopullulanase, and panose, maltose, and glucose were produced. From the data mentioned above, the model of action of neopullulanase

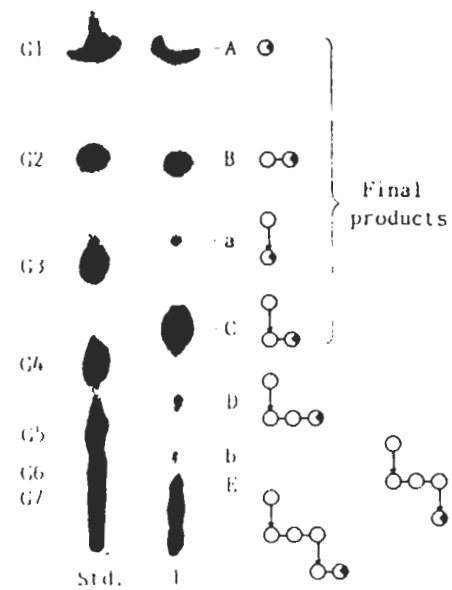


FIG. 4. Paper chromatography of the partial hydrolysis products produced from pullulan by neopullulanase. Lane 1 contains the sample. Maltohexaose and maltoheptaose are indicated by G6 and G7, respectively. Other abbreviations and symbols are as in Fig. 1.

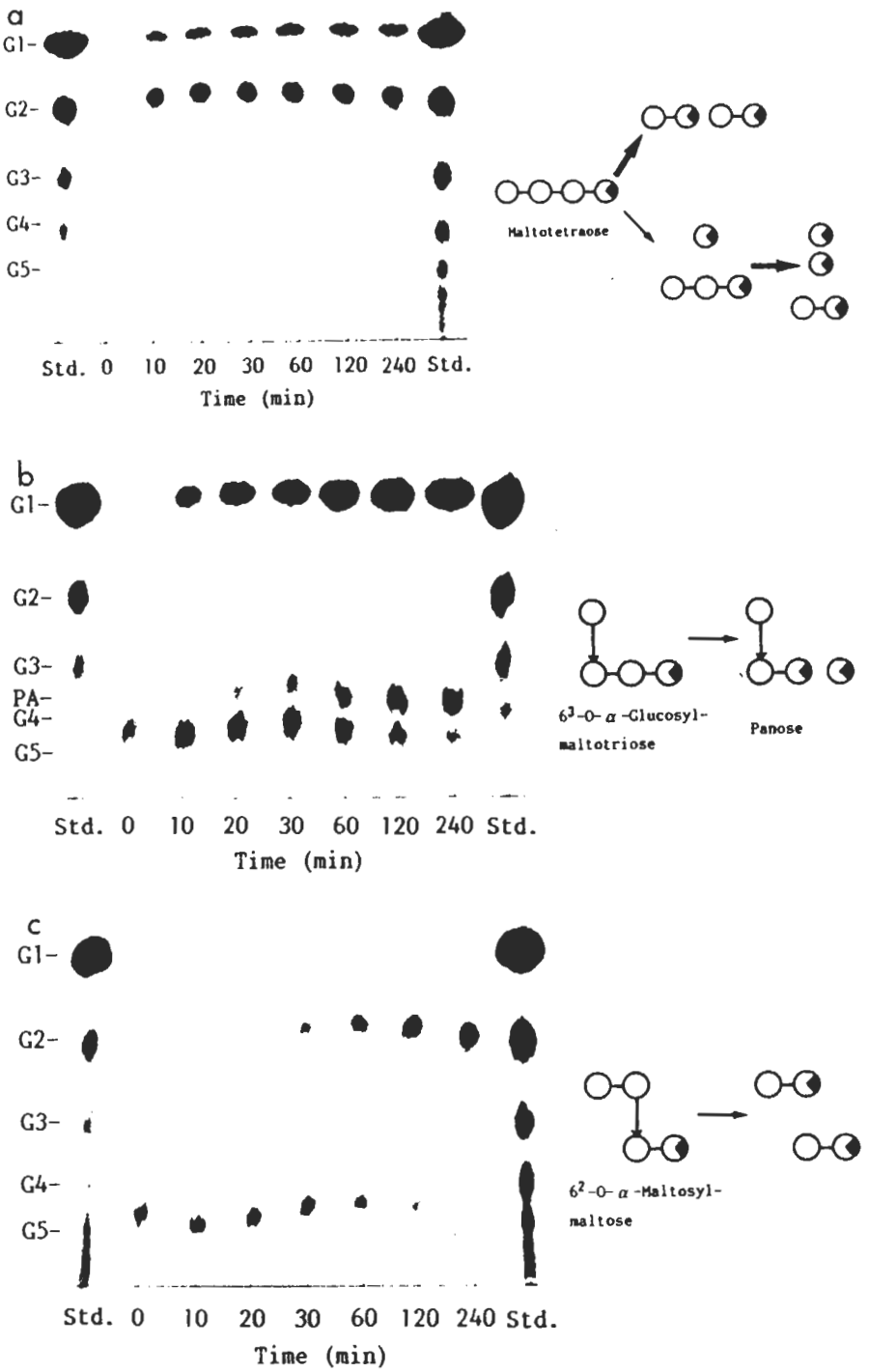


FIG. 2. Action of neopullulanase on DP 4 oligosaccharides. PA, Panose. Other abbreviations and symbols are as in Fig. 1.

were developed seven and four times, respectively. Sugars on the paper were detected by the silver nitrate dip method (11). High-performance liquid chromatography (LC-6A; Shimazu, Kyoto, Japan; ZORBAX-NH_2 column; Du Pont Co., Wilmington, Del.) was also used for the analysis of reaction products. Paper chromatography gave superior discrimination between α-(1→4)- and α-(1→6)-linked oligosaccharides, as compared with high-performance liquid chromatography or thin-layer chromatography. The retardation of an α-(1→6) linkage was about one-half to two-thirds of that of an additional α-(1→4)-linked D-glucose unit (1). Quantitative analysis of reducing sugars in the hydrolysate was performed as described above for the neopullulanase assay. Total carbohydrate was measured by the phenol-sulfuric acid method (4).

Extraction of oligosaccharides from paper chromatograms. After the preparative-scale paper chromatograms were developed and dried, the oligosaccharide fraction was cut out and dipped into boiling water (100 ml) for 5 min. The eluted oligosaccharide solution was collected by filtration (Whatman no. 1 filter paper). These elution steps were repeated twice, and the solution was concentrated by lyophilization. The yield of oligosaccharide was about 100%, with no contamination by other oligosaccharides.

RESULTS AND DISCUSSION

Preparation of branched oligosaccharides. Isomaltose was incubated with starch and cyclodextrin glucanotransferase to generate various branched oligosaccharides terminated by isomaltose at the reducing end (7). The oligosaccharides in the mixture obtained by the coupling reaction with cyclodextrin glucanotransferase were fractionated by preparative-scale paper chromatography. As a branched fraction with a degree of polymerization (DP) of 3, isopanose and 6^1-*O*-α-glucosyl-maltose were produced simultaneously from isomaltose, so it was not feasible to isolate them as individual compounds (1). In the same manner, panose was incubated with starch and cyclodextrin glucanotransferase to generate various branched oligosaccharides terminated by panose at the reducing end, and these oligosaccharides were fractionated. As a branched DP 4 fraction, 6^2-*O*-α-maltosyl-maltose and 6^2-*O*-α-glucosyl-maltotriose were produced (7) and could be separated by paper chromatography. The former migrated more slowly than did the latter (16).

The structures of these branched oligosaccharides were confirmed by pullulanase and glucoamylase analyses. The mixture of isopanose and 6^1-*O*-α-glucosyl-maltose was not hydrolyzed by pullulanase (1) but was hydrolyzed to produce glucose and isomaltose by glucoamylase (9). 6^2-*O*-α-Maltosyl-maltose was hydrolyzed by pullulanase, and maltose was produced (1). It was also hydrolyzed by glucoamylase to produce glucose and to accumulate panose as an intermediate product (9).

On the other hand, waxy maize amylopectin was digested completely by saccharifying α-amylase. The products were analyzed by preparative-scale paper chromatography, and the smallest limit dextrin was extracted as 6^3-*O*-α-glucosyl-maltotriose (16).

Action of neopullulanase on DP 2 and DP 3 oligosaccharides. Maltose, isomaltose, panose, maltotriose, maltotriitol, and the mixture of isopanose and 6^1-*O*-α-glucosyl-maltose were digested with neopullulanase, and the time course of the products was analyzed by paper chromatography. Each reaction mixture (500 μl) consisted of 0.5% substrate in 50 mM acetate buffer (pH 6.0) and 0.05 U of neopullulanase. After an appropriate incubation period at 50°C, 50-μl samples were collected, and the reaction was stopped by heat treatment at 100°C for 5 min. A 10-μl amount of each sample was applied to chromatography paper. Neopullulanase did not hydrolyze maltose, isomaltose, and panose (data not shown). Maltotriose and maltotriitol could be hydrolyzed, and both maltose and glucose were produced, indicating that neopullulanase hydrolyzed α-(1→4) linkages next to both nonreducing and reducing ends (Fig. 1a and b). It must be noted that maltotriitol does not produce a spot on a paper chromatogram with silver nitrate reagent because of the reduction of a reducing end. The mixture of isopanose and 6^1-*O*-α-glucosyl-maltose could be also hydrolyzed, and both maltose and glucose were produced (Fig. 1c). These facts indicated that neopullulanase hydrolyzed not only α-(1→4) linkages but also α-(1→6) linkages.

Action of neopullulanase on DP 4 oligosaccharides. Using the same procedures as those described above, we analyzed the patterns of action of neopullulanase on maltotetraose, 6^3-*O*-α-glucosyl-maltotriose, and 6^2-*O*-α-maltosyl-maltose. Neopullulanase hydrolyzed maltotetraose and produced maltose and a smaller amount of glucose (Fig. 2a). These results showed that the middle position of the three α-(1→4) linkages was mainly cleaved. 6^3-*O*-α-Glucosyl-maltotriose was hydrolyzed, and panose and glucose were produced (Fig. 2b). On the other hand, neopullulanase attacked the α-(1→6) linkage of 6^2-*O*-α-maltosyl-maltose and produced maltose (Fig. 2c). A very small amount of glucose was visible on the paper chromatogram; the reason is discussed below.

Time course of pullulan hydrolysis by neopullulanase. To investigate the pattern of action of neopullulanase more precisely, we quantitatively analyzed the time course of the sugars making up the pullulan hydrolysate by high-performance liquid chromatography (data not shown). Only panose was detected in the early stage of the reaction (0 to 30 min); maltose production followed after 30 min. Glucose could not be detected in the early stage at all but could be detected after 60 min. The final molar ratio of panose, maltase, and glucose (after 360 min) was 3:1:1.

Model of action of pullulan hydrolysis by neopullulanase. According to the results mentioned above, a model of the action of neopullulanase on pullulan is proposed (Fig. 3). In the first step, neopullulanase attacks only α-(1→4)-glucosidic linkages on the nonreducing side of α-(1→6)-glucosidic linkages of pullulan at random and produces panose and several intermediate products composed of some panose units. The endo-cleaving fashion of neopullulanase was confirmed by the fact that the enzyme could hydrolyze β-cyclodextrin (data not shown). In the second step, taking 6^2-*O*-α-(6^3-*O*-α-glucosyl-maltotriosyl)-maltose as an example of one of the intermediate products, neopullulanase attacks either α-(1→4) or α-(1→6) linkages and produces panose or 6^3-*O*-α-glucosyl-maltotriose plus maltose, respectively. In other words, the enzyme can hydrolyze not only α-(1→4) linkages but also α-(1→6) linkages of partial hydrolysates of pullulan (intermediate products), recognizing the structure terminated by 6^2-*O*-α-maltosyl-maltose at the reducing end (Fig. 2c). In the third step, 6^3-*O*-α-glucosyl-maltotriose is hydrolyzed by the enzyme, and glucose is produced together with panose (Fig. 2b). This model is basically consistent with the fact that when pullulan was hydrolyzed by neopullulanase, panose, maltose, and glucose appeared, in that order, with some intervals, that the molar ratio of maltose and glucose was 1:1, and that the amount of panose was greater than those of maltose and glucose.

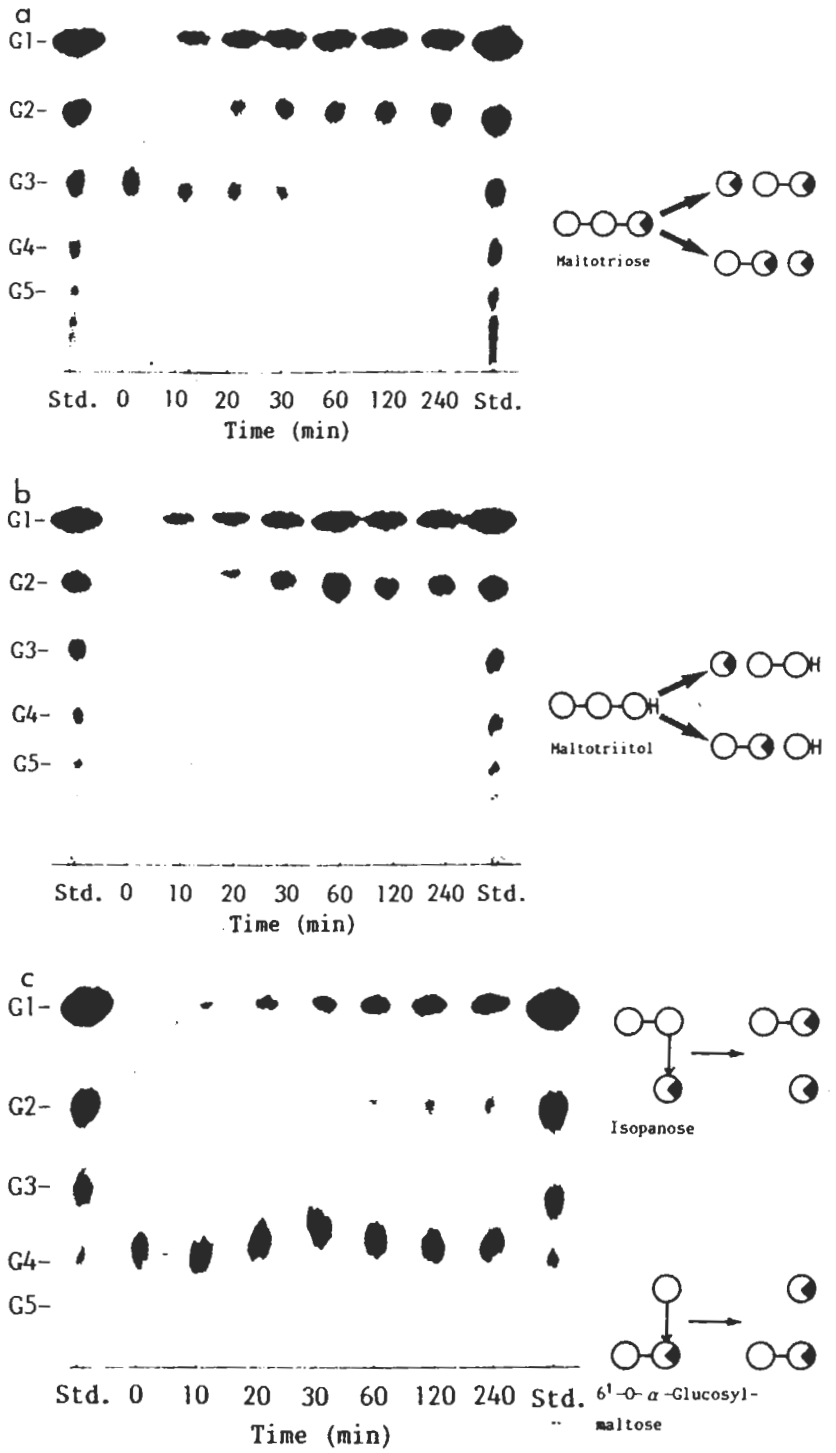


FIG. 1. Action of neopullulanase on DP 3 oligosaccharides. Paper chromatograms and the schematic representation of the results are shown. Glucose, maltose, maltotriose, maltotetraose, and maltopentaose are indicated by G1, G2, G3, G4, and G5, respectively. Std. represents the standard mixture of oligosaccharides. Symbols: ○, glucose; ◔, glucose with a reducing end; OH, sorbitol; —, α-(1→4) linkage; ↓, α-(1→6) linkage. Thick and thin horizontal arrows indicate relatively fast and slow enzyme reactions in the system, respectively.

JOURNAL OF BACTERIOLOGY, Jan. 1989, p. 369–374
0021-9193/89/010369-06$02.00/0

Vol. 171, No. 1

Pattern of Action of *Bacillus stearothermophilus* Neopullulanase on Pullulan

TADAYUKI IMANAKA* AND TAKASHI KURIKI

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamadaoka, Suita-shi, Osaka 565, Japan

Received 18 May 1988/Accepted 18 October 1988

The action of neopullulanase from *Bacillus stearothermophilus* on many oligosaccharides was tested. The enzyme hydrolyzed not only α-(1→4)-glucosidic linkages but also specific α-(1→6)-glucosidic linkages of several branched oligosaccharides. When pullulan was used as a substrate, panose, maltose, and glucose, in that order, were produced as final products at a final molar ratio of 3:1:1. According to these results, we proposed a model for the pattern of action of neopullulanase on pullulan as follows. In the first step, the enzyme hydrolyzes only α-(1→4)-glucosidic linkages on the nonreducing side of α-(1→6) linkages of pullulan and produces panose and several intermediate products composed of some panose units. In the second step, taking 6^2-*O*-α-(6^3-*O*-α-glucosyl-maltotriosyl)-maltose as an example of one of the intermediate products, the enzyme hydrolyzes either α-(1→4) (the same position as that described above) or α-(1→6) linkages and produces panose or 6^3-*O*-α-glucosyl-maltotriose plus maltose, respectively. In the third step, the α-(1→4) linkage of 6^3-*O*-α-glucosyl-maltotriose is hydrolyzed by the enzyme, and glucose and another panose are produced. To confirm the model of the pattern of action, we extracted intermediate products produced from pullulan by neopullulanase and analyzed the structures by glucoamylase, pullulanase, and neopullulanase analyses. The experimental results supported the above-mentioned model of the pattern of action of neopullulanase on pullulan.

Four types of pullulan-hydrolyzing enzymes have been reported: (i) glucoamylase (glucan 1,4-α-glucosidase; EC 3.2.1.3) (15), which hydrolyzes pullulan from nonreducing ends to produce glucose; (ii) pullulanase (α-dextrin endo-1,6-α-glucosidase; EC 3.2.1.41) (2), from *Klebsiella pneumoniae*, which hydrolyzes α-(1→6)-glucosidic linkages of pullulan to produce maltotriose; (iii) isopullulanase (EC 3.2.1.57) (13), from *Aspergillus niger*, which hydrolyzes α-(1→4)-glucosidic linkages of pullulan to produce isopanose (6-*O*-α-maltosyl-glucose); and (iv) neopullulanase, which hydrolyzes α-(1→4)-glucosidic linkages of pullulan to produce panose (6^2-*O*-α-glucosyl-maltose). The last enzyme was reported to be a new type of pullulanase from *Bacillus stearothermophilus* TRS40 in our previous paper (8).

Neopullulanase from *B. stearothermophilus* TRS40 could hydrolyze pullulan efficiently and only hydrolyzed a small amount of starch. When pullulan was used as a substrate, the main product was panose, and small amounts of glucose and maltose were simultaneously produced (8). Therefore, neopullulanase was obviously different from the pullulan-hydrolyzing enzymes previously reported (2, 13–15) and should be considered as a new enzyme (8).

To produce glucose and maltose in addition to panose from pullulan, neopullulanase has to hydrolyze α-(1→6)-glucosidic linkages as well as α-(1→4)-glucosidic linkages. The present investigation was conducted to reveal the attack point of glucosidic linkages of pullulan by neopullulanase.

This paper describes the action of neopullulanase on many oligosaccharides and pullulan. According to these observations, a model of the pattern of action of the enzyme on pullulan is proposed and discussed.

* Corresponding author.

MATERIALS AND METHODS

Substrates and reagents. Maltotriose, maltotriitol, panose, and pullulan were purchased from Hayashibara Co., Ltd., Okayama, Japan. Isomaltose and maltotetraose were obtained from Sigma Chemical Co., St. Louis, Mo. Unless otherwise specified, glucose, maltose, β-cyclodextrin, and all other reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Enzymes. Neopullulanase from *B. stearothermophilus* TRS40 was purified as described previously (8). Saccharifying α-amylase (EC 3.2.1.1) was prepared from *B. subtilis* as described by Fukumoto and Okada (5). Cyclodextrin glucanotransferase (cyclomaltodextrin glucanotransferase; EC 2.4.1.19) from *B. macerans* was purchased from Amano Pharmaceutical Co., Ltd., Nagoya, Japan. Pullulanase from *Klebsiella (Aerobacter) aerogenes* and glucoamylase from *Aspergillus niger* were obtained from Sigma.

Assay of neopullulanase activity. Neopullulanase activity was assayed as described previously (8). The reaction mixture (500 μl) consisted of 1% pullulan in 0.2 M sodium acetate buffer (pH 6.0) and the enzyme. The reaction was stopped after an appropriate incubation period at 60°C by the addition of 3,5-dinitrosalicylic acid reagent (1 ml). The reagent was prepared by mixing 0.5 g of 3,5-dinitrosalicylic acid dissolved in 20 ml of 2 N NaOH and 30 g of sodium potassium tartrate dissolved in 50 ml of deionized water and adjusting the volume to 100 ml with deionized water. One unit of neopullulanase activity was defined as the amount of enzyme which released 1 μmol of reducing sugar as glucose per min under the assay conditions described above.

Analysis of hydrolysis products. Paper chromatography was carried out in the ascending mode on Toyo no. 50 filter-paper with a solvent mixture of *n*-butanol–pyridine–water (6:4:3 or 6:4:4, by volume). Preparative-scale (40- by 40-cm) and analytical-scale (18- by 20-cm) paper chromatograms

higher thermostability in the first and the second halves, respectively. Therefore, the amino acid substitution in thermostable neutral protease was confined to the first half. The substitution Gly to Ala is available for positions 47, 61 and 144. Since Gly 144 is in the α-helix which combines two domains (Fig. 2), the amino acid substitution Gly 144 to Ala 144 (mutation M1) should increase the internal hydrophobicity, stabilize the α-helix and thus improve the thermostability of the protease. Since it requires only the addition of a methyl group this replacement minimizes interruption of function or internal residue packing arrangements. Another mutation, M3 (Thr 66 to Ser 66) should decrease thermostability of the enzyme. Amino acid substitutions were performed by site-directed mutagenesis (Fig. 3).

Thermostability of wild-type (WT) neutral protease of *B. stearothermophilus* and thermolysin was tested at 75 °C (Fig. 4*a*). Thermolysin was more thermostable than the WT protease from *B. stearothermophilus*. When M1 mutation (Gly 144 to Ala 144, GGG to GCG) was introduced, the M1 enzyme was found to be more thermostable than WT enzyme (Fig. 4*a*, *b*). The M3 mutation (Thr 66 to Ser 66, ACC to TCC) was found to produce fairly thermolabile enzyme (Fig. 4*b*). The double mutant M13 was constructed, with both the M1 and M3 mutations. Surprisingly, M13 enzyme had the same thermostability as M3. When a different thermostability increasing mutation, M2 (Gly 61 to Ala 61, GGC to GCC), was added to M3, the double mutant enzyme M23 showed the same stability as M3. But when both mutations M1 and M2 were introduced into M3, the stability of the triple mutant enzyme M123 recovered to some extent compared to that of M3 (Fig. 4*b*). It is interesting that two mutations are required simultaneously to enhance the thermostability of M3 enzyme.

The statistical data in ref. 6 were obtained from many simultaneous replacements of amino acids between thermophilic and mesophilic enzymes. We attempted single amino-acid substitution to test the hypothesis and found that a single substitution does not always enhance thermostability.

The thermostability of an enzyme is influenced by many factors, including amino acid sequence, three-dimensional structure, cofactors and *p*H. The *Bacillus* neutral proteases require Ca ion as the most important enzyme stabilizing factor. Four Ca-binding sites were found in thermolysin (Figs 1 and 2)[14,17]. Some Ca-binding sites, Asp 141, Asp 188, Glu 193 and Asp 194 are conserved for all proteases examined (Fig. 1). However, a Ca-binding site, Glu 180, of thermostable proteases from *B. stearothermophilus* and *B. thermoproteolyticus* is deleted

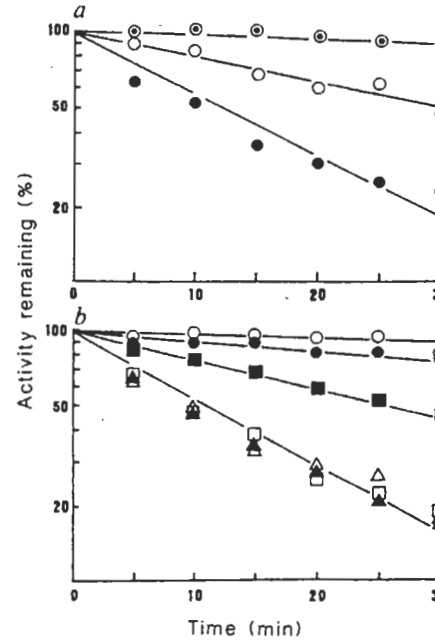


Fig. 4 Thermostability of neutral proteases. Extracellular protease produced by *B. subtilis* carrying *nprT*$^+$ gene was purified by the method described previously[7]. Remaining activity after heating at: *a*, 75 °C; *b*, 65 °C was assayed as described earlier[7] and was expressed as the percent of original activity. ⊙, Thermolysin; ●, wild-type enzyme (WT) from *B. stearothermophilus*; ○, mutant M1; △, mutant M3; ▲, double mutant M13; □, double mutant M23; ■, triple mutant M123.

in the proteases from *B. subtilis* and *B. amyloliquefaciens* (Fig. 1). This might account for the fact that the latter proteases are rather thermolabile. If the amino acid sequence from Thr 177 to Phe 181 were inserted in the *B. subtilis* protease, the enzyme would become more thermostable. Studies on other mutations using this approach are in progress.

We thank S. Aiba for valuable discussion and for comments on the manuscript.

Received 14 July; accepted 8 October 1986.

1. Yutani, K., Ogashara, K., Sugino, Y. & Matsushiro, A. *Nature* **267**, 274–275 (1977).
2. Matsumura, M., Katakura, Y., Imanaka, T. & Aiba, S. *J. Bact.* **160**, 413–420 (1984).
3. Ulmer, K. M. *Science* **219**, 666–671 (1983).
4. Perry, L. J. & Wetzel, R. *Science* **226**, 555–557 (1984).
5. Wells, J. A. & Powers, D. B. *J. biol. Chem.* **261**, 6564–6570 (1986).
6. Argos, P. *et al. Biochemistry* **18**, 5698–5703 (1979).
7. Fujii, M., Takagi, M., Imanaka, T. & Aiba, S. *J. Bact.* **154**, 831–837 (1983).
8. Takagi, M., Imanaka, T. & Aiba, S. *J. Bact.* **163**, 824–831 (1985).
9. Nakajima, R., Imanaka, T. & Aiba, S. *Appl. Microbiol. Biotechnol.* **23**, 355–360 (1986).
10. Titani, K. *et al. Nature new Biol.* **238**, 35–37 (1972).
11. Yang, M. Y., Ferrari, E. & Henner, D. J. *J. Bact.* **160**, 15–21 (1984).
12. Vasantha, N. *et al. J. Bact.* **159**, 811–819 (1984).
13. Kester, W. R. & Matthews, B. W. *J. biol. Chem.* **252**, 7704–7710 (1977).
14. Matthews, B. W. *et al. Nature new Biol.* **238**, 37–41 (1972).
15. Norris, K., Norris, F., Christiansen, L. & Fiil, N. *Nucleic Acids Res.* **11**, 5103–5112 (1983).
16. Carter, P. J., Winter, G., Wilkinson, A. J. & Fersht, A. R. *Cell* **38**, 835–840 (1984).
17. Matthews, B. W. *et al. Nature new Biol.* **238**, 41–43 (1972).

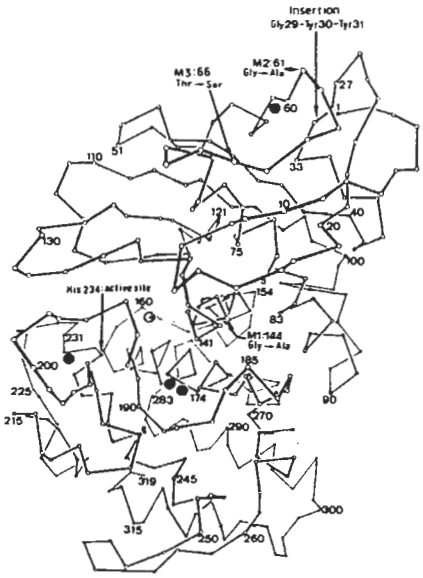


Fig. 2 Three-dimensional structure of thermolysin[14]. Open circles, α-carbon positions. Zinc atom is drawn stippled with its three protein ligands shown diagramatically as broken lines. Four calcium atoms are shown as solid circles. The amino acid number from the NH_2-terminus is for *B. stearothermophilus* neutral protease; originally, Gly-Tyr-Tyr at positions 29-31 are absent in thermolysin.

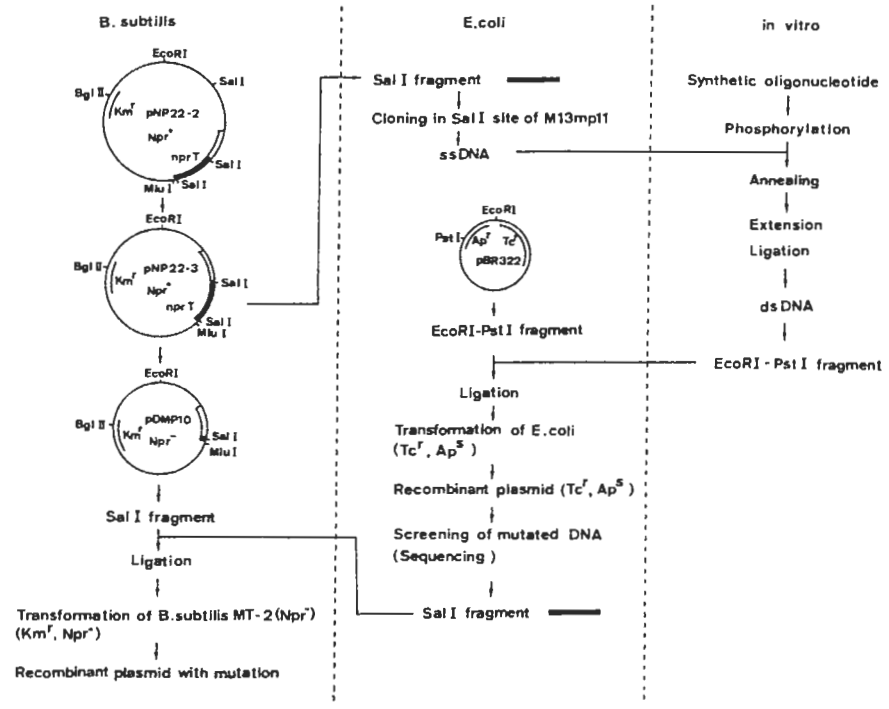


Fig. 3 The scheme of site-directed mutagenesis and DNA construction. The *B. stearothermophilus nprT*$^+$ gene has been cloned in pNP22-2, which is a deletion derivative of pNP22-1 (ref. 8). Since the plasmid contains three *Sal*I sites, one *Sal*I near an *Eco*RI site was eliminated by partial digestion of pNP22-2 with *Sal*I, digestion with Bal31, and ligation. The plasmid thus obtained is designated pNP22-3. The 888 base pair (bp) *Sal*I fragment containing most of the extracellular portion of protease was isolated from pNP22-3 (Npr$^+$), and the resulting plasmid (Npr$^-$) was designated as pDMP10. The 888 bp *Sal*I fragment was cloned as described[15] in M13mp11, and single-stranded (ss) DNA was isolated. The ss DNA was annealed with a synthetic oligonucleotide whose 5′ end had been phosphorylated with polynucleotide kinase. After DNA extension using the primer and ligation, the double-stranded DNA was digested with both *Eco*RI and *Pst*I. The *Eco*RI-*Pst*I fragment was ligated with larged *Eco*RI-*Pst*I fragment of pBR322. The ligation mixture was used to transform *Escherichia coli*. Recombinant plasmids were isolated from the transformants (Tc^r Ap^s). Mutated DNA was screened by either restriction enzyme treatment (mutated primer 5′-CGTCGTGGCGCATGAGT-3′, *Hha*I for mutation M1 and 5′-GACCGATGCCGACAACC-3′, *Sfa*NI for mutation M2; asterisks, indicate mismatches and underlined sequences the new restriction site) or colony hybridization[16] and the mutation was confirmed by DNA sequencing. The 888 bp *Sal*I fragment with mutation was isolated from the recombinant plasmid, and ligated with *Sal*I treated pDMP10. The ligation mixture was used to transform *B. subtilis* MT-2 (Npr$^-$)[7] to Km^r Npr$^+$. *B. subtilis* MT-2 carrying the mutant plasmid was used to produce extracellular neutral protease.

Reprinted from Nature, Vol. 324, No. 6098, pp. 695-697, 18 December 1986

A new way of enhancing the thermostability of proteases

Tadayuki Imanaka, Mitsuyoshi Shibazaki & Masahiro Takagi

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamada-oka, Suita-shi, Osaka 565, Japan

Thermostability of enzyme can be enhanced by single amino acid substitutions[1,2]. Recent advances in genetic engineering have made it possible to create novel proteins in a predictable manner where structural information for the protein is available. This 'protein engineering'[3] has already been used to enhance enzyme thermostability[4,5], but it is usually not clear which amino acid substitutions should be made. We consider that the following approach should be helpful in engineering proteins with enhanced thermostability: highly conserved residues should be left unchanged; the sequences of known mesophilic and thermophilic proteins should be used to suggest the kinds of changes likely to increase thermostability[6]; and substitutions should be made that increase internal hydrophobicity and that stabilize helices for strong internal packing. We describe here the use of this approach to alter the thermostability of the thermostable neutral protease of *Bacillus stearothermophilus*, the sequence of which is known[7,8]. Surprisingly we find that a single mutation that decreases thermostability can require two mutations that increase stability to compensate for it. The effects on stability are not additive, suggesting cooperativity.

Comparison of primary structures of enzymes of the same function but different origins is useful to determine the essential regions for activity, because active and/or substrate-binding sites are highly conserved in the homologous regions[9]. Amino acid sequences of four neutral proteases from *B. stearothermophilus*[8], *Bacillus thermoproteolyticus*[10], *Bacillus subtilis*[11] and *Bacillus amyloliquefaciens*[12] were therefore aligned and compared (Fig. 1).

The amino acid sequence of thermostable neutral protease from *B. stearothermophilus* was homologous (85%) with that of thermolysin from *B. thermoproteolyticus*. Similarly, the sequences for thermolabile neutral proteases from *B. subtilis* and *B. amyloliquefaciens* were also homologous (89%). In contrast, the homology between the thermostable and thermolabile enzymes was lower (~45%) (Fig. 1). Nine regions were found to be conserved in all four enzymes. These highly homologous regions are likely to be essential for the enzyme activity. In fact, it has been reported that His 234 and Arg 206 are the active and substrate-binding sites of neutral protease (or of thermolysin), respectively[13]. Zn ion is essential for the enzyme activity of thermolysin, and the three protein ligands for Zn (His 145, His 149 and Glu 169)[13] are conserved in all enzymes (Fig. 1). Glu 146, which promotes the attack of a water molecule on the carbonyl carbon of the substrate[13], is also conserved in these enzymes (Fig. 1).

The comparison of *B. stearothermophilus* protease and thermolysin revealed that the amino acid frames were completely matched except for additional three amino acids (Gly-Tyr-Tyr, 29-31) in the enzyme from *B. stearothermophilus* (Fig. 1). Therefore, the three-dimensional structure of *B. stearothermophilus* protease would be basically similar to the known tertiary structure of thermolysin[14] shown in Fig. 2. Although His 234 (active site), Arg 206 (substrate-binding site), Leu 205, Glu 193 and Asn 162 are far apart in the primary structure of the enzyme (Fig. 1), these amino acid residues are near the active site in the three-dimensional structure (Fig. 2) and are completely conserved in the four neutral proteases tested. Therefore, alteration of amino acids in the highly conserved sequence should be avoided.

We decided the position and species of amino acid to be replaced to give enhanced enzyme thermostability according to the procedure outlined. The amino acid substitutions between *B. stearothermophilus* protease and thermolysin were examined in the light of statistical data on amino acid substitutions which increase thermostability[6] (Fig. 1). Thermolysin and *B. stearothermophilus* protease contain some amino acids which promote

Fig. 1 Comparison of amino acid sequences of various extracellular neutral proteases. Amino acid residues are shown by single letters: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. A blank indicates the absence of corresponding amino acid of this position. Enzyme sources are: 1, *Bacillus stearothermophilus*[8]; 2, *Bacillus thermoproteolyticus*[10]; 3, *Bacillus subtilis*[11]; 4, *Bacillus amyloliquefaciens*[12]. Homologous sequence regions are surrounded by rectangles. Active and substrate-binding sites of thermolysin are indicated by ○ and □, respectively. Protein ligands for Zn and Ca ions for thermolysin are indicated above the sequence. Substitutions expected to enhance or reduce the thermostability of *B. stearothermophilus* protease in comparison with that of thermolysin from *B. thermoproteolyticus* are indicated above the sequence by + or −, respectively. Vertical arrows, amino acid substitutions in *B. stearothermophilus* protease.

Journal of General Microbiology (1989), **135**, 1521–1528. *Printed in Great Britain*

Nucleotide Sequence of the Neopullulanase Gene from *Bacillus stearothermophilus*

By TAKASHI KURIKI AND TADAYUKI IMANAKA*

Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamadaoka, Suita-shi, Osaka 565, Japan

(*Received 15 December 1988; revised 1 March 1989; accepted 20 March 1989*)

The gene (*nplT*) for a new type of pullulan-hydrolysing enzyme, neopullulanase, from *Bacillus stearothermophilus* TRS40 was sequenced. The DNA sequence revealed only one large open reading frame, composed of 1764 bases and 588 amino acid residues (M_r 69144). Although the thermostable neopullulanase contained eight cysteine residues, they did not provide conformational stability by disulphide bonds. A comparison was made of the amino acid sequences of α-amylase, neopullulanase, isoamylase, pullulanase and cyclodextrin glucanotransferase. All the enzymes examined contained four highly conserved regions which probably constitute the active centres of the enzymes. The amino acid residues required for the specificity of neopullulanase are compared with those of α-amylase and other amylolytic enzymes.

INTRODUCTION

Four types of pullulan-hydrolysing enzymes have been described (Imanaka & Kuriki, 1989): (i) glucoamylase (glucan 1,4-α-glucosidase; EC 3.2.1.3), which hydrolyses pullulan from nonreducing ends to produce glucose; (ii) pullulanase (α-dextrin endo-1,6-α-glucosidase; EC 3.2.1.41), which hydrolyses α-(1→6)-glucosidic linkages of pullulan to produce maltotriose; (iii) isopullulanase (EC 3.2.1.57), which hydrolyses α-(1→4)-glucosidic linkages of pullulan to produce isopanose (6-*O*-α-maltosylglucose); and (iv) neopullulanase, which hydrolyses α-(1→4)-glucosidic linkages of pullulan to produce panose (6^2-*O*-α-glucosylmaltose). The last enzyme was reported to be a new type of pullulan-hydrolysing enzyme from *Bacillus stearothermophilus* TRS40 (Kuriki *et al.*, 1988*a*). We have cloned the genes for the neopullulanase and pullulanase in *Bacillus subtilis* (Kuriki *et al.*, 1988*a*, *b*, respectively). The neopullulanase could hydrolyse pullulan efficiently and only hydrolysed a small amount of starch. Further studies revealed that neopullulanase hydrolysed not only α-(1→4)-glucosidic linkages but also specific α-(1→6)-glucosidic linkages of several branched oligosaccharides (Imanaka & Kuriki, 1989).

It was previously demonstrated that four highly conserved regions existed in eleven different α-amylases (EC 3.2.1.1) (Nakajima *et al.*, 1986) and the regions were most likely to be the active and/or substrate-binding sites of the enzymes (Matsuura *et al.*, 1984). These homologous regions have also been discussed for other kinds of amylolytic enzymes (Amemura *et al.*, 1988; Binder *et al.*, 1986; Kimura *et al.*, 1987; McPherson & Charalambous, 1988; Sakai *et al.*, 1987).

This paper describes the nucleotide sequence of the cloned neopullulanase gene. To further investigate the unique action pattern of the neopullulanase at the molecular level, the amino acid sequence of the neopullulanase is compared with those of α-amylase, isoamylase, pullulanase and cyclodextrin glucanotransferase, and the specificity of the neopullulanase is described.

METHODS

Bacterial strains, plasmids and phages. The *Bacillus* strain used was *Bacillus subtilis* NA-1 (*arg-15 hsmM hsrM* Amy$^-$ Npr$^-$) (Kuriki *et al.*, 1988*a*). *Escherichia coli* MV1184 [*ara* Δ(*lac-pro*) *strA thi* (φ80Δ*lacI*ZΔM15) Δ(*srl-recA*)306::Tn*10*(Tet^r); F′:*traD36 proAB lacI*^{q}ZΔM15] was used as a host for plasmids pUC118 and

pUC119 and for phage M13KO7 (Vieira & Messing, 1987). Plasmid pPP10 [Tc^r *nplT*$^+$ (structural gene of the neopullulanase from *B. stearothermophilus* TRS40)] was described previously (Kuriki *et al.*, 1988*a*).

Plasmid isolation, restriction enzyme treatment, and ligation of DNA. Plasmid DNA was prepared by either the rapid alkaline extraction method or CsCl/ethidium bromide equilibrium density gradient centrifugation as described previously (Imanaka *et al.*, 1982). Treatment of DNA with restriction enzymes and ligation of DNA with T4 DNA ligase were done as recommended by the manufacturer.

Transformation. Transformation of *E. coli* with plasmid DNA, and transformation of competent *B. subtilis* cells, were done as described previously (Imanaka *et al.*, 1981).

Gel electrophoresis for DNA analysis and isolation. For the analysis of DNA, gel electrophoresis with agarose or polyacrylamide was done under standard conditions (Maniatis *et al.*, 1982). A GENECLEAN kit (BIO 101, La Jolla, Calif., USA) was used for the recovery of DNA from agarose.

DNA sequencing analysis. DNA sequencing was done by the dideoxy chain-terminating method (Sanger *et al.*, 1977) with a SEQUENASE kit (United State Biochemical Corporation, Cleveland, Oh., USA). For preparing single-stranded DNA, pUC118/119 and helper phage M13KO7 were used (Vieira & Messing, 1987). Some strands were analysed by the dideoxy sequencing method using alkaline denatured plasmid templates (Hattori & Sakaki, 1986). Although some parts of the DNA were analysed from one strand, those sequences could be determined without any ambiguity on the sequencing gels, because the substitution of dGTP by the nucleotide analogue dITP with the SEQUENASE kit was successful in elimination of compressed regions. All restriction sites were overlapped by sequencing across them.

Detection of neopullulanase-producing colonies and assay of neopullulanase activity. Neopullulanase-producing colonies were selected on PLL agar plates and neopullulanase activity was assayed at 50 °C as described previously (Kuriki *et al.*, 1988*a*).

Purification of neopullulanase. B. subtilis NA-1(pPP10) was cultivated in LSII broth (Kuriki *et al.*, 1988*a*) at 37 °C for 16 h. The culture supernatant was used for enzyme purification. Purification procedures for the neopullulanase were described previously (Kuriki *et al.*, 1988*a*).

Amino acid composition and amino-terminal amino acid sequence. Using neopullulanase purified to homogeneity, the amino acid composition was analysed as described previously (Matsumura *et al.*, 1984). The amino-terminal amino acid sequence was analysed by Edman degradation as described elsewhere (Takagi *et al.*, 1985).

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and DNA polymerase I (Klenow fragment) were purchased from Takara Shuzo Co., Kyoto, Japan. Tetracycline was from Sigma. All other chemicals used were from Wako Pure Chemical Industries, Osaka, Japan.

Analysis of amino acid sequence homology. This was done with an NEC PC-9801 computer (Nippon Electric Co.) and the GENETYX system (Software Development Co., Tokyo, Japan).

RESULTS

Nucleotide sequence of the neopullulanase gene

We have previously shown that the structural gene for the neopullulanase (*nplT*) from *B. stearothermophilus* TRS40 is cloned in a 3·4 kb (2·2 MDa) *Hin*dIII fragment of plasmid pPP10 (Kuriki *et al.*, 1988*a*). Fig. 1 shows the restriction map of the 3·4 kb *Hin*dIII fragment. Using a frame-shift mutation generated by end-filling with DNA polymerase I, we confirmed that a single *Sal*I site was located in *nplT*. The nucleotide sequence of *nplT* was determined (Fig. 2), according to the strategy shown in Fig. 1.

To correlate the nucleotide sequence data with the structure of the neopullulanase, the amino-terminal amino acid sequence of the enzyme from *B. subtilis* NA-1(pPP10) was determined through five cycles of the Edman degradation procedure (Allen, 1981). The first five amino acids of the enzyme were Met–Arg–Lys–Glu–Ala. This sequence completely matched that deduced from the nucleotide sequence only when ATG was taken as the initiation codon at position +1 (Fig. 2).

Starting from the ATG codon at position +1 and terminating in a TAG nonsense codon at position +1765, the single open reading frame was composed of 1764 nucleotides (Fig. 2). The maximum length of other open reading frames was 474 nucleotides in the two other reading frames of the same strand and the three reading frames of the complementary strand.

At 6 bases upstream from the ATG codon, there was a 10-base sequence, AAGGAGGAGA (−16 to −7), which exhibited complementarity with the 3′ end of the 16S rRNA from *B. stearothermophilus* (Kozak, 1983) and *B. subtilis* (Moran *et al.*, 1982); hence it is the most probable ribosome-binding site (Shine–Dalgarno sequence) of *nplT*. The free energy of

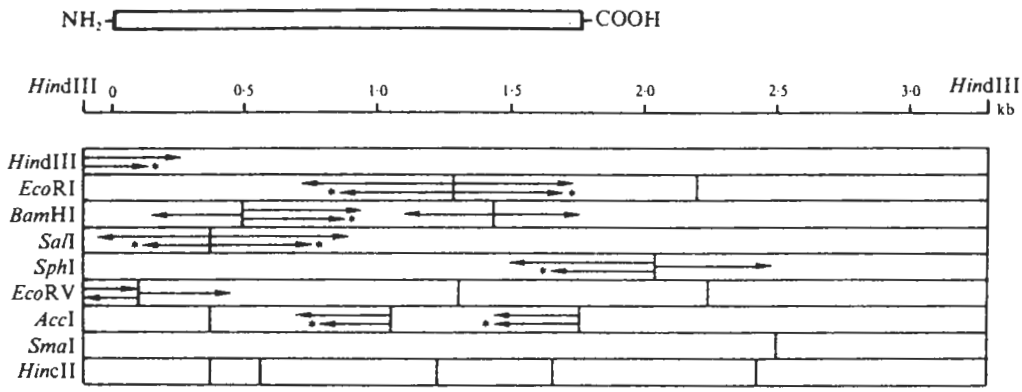


Fig. 1. Restriction map and sequencing strategy of the 3·4 kb *Hin*dIII fragment of pPP10. The location and size of the neopullulanase gene are shown by the open bar. Arrows indicate the direction and length of individual sequence determinations. Asterisks indicate the regions analysed using dITP.

formation of the most stable pairing was calculated as $-18{\cdot}0$ kcal mol^{-1} ($-75{\cdot}3$ kJ mol^{-1}) (Tinoco *et al.*, 1973).

Two putative promoters (-35 and -10 regions) are shown in Fig. 2; one is TTGACTTTTTTTCTCTCTTTTCGTACTCT (-90 to -62) and the other TTTTTTTCTCTCTTTTCGTACTCTTTAAT (-85 to -57). However, these two putative promoters are somewhat different from the consensus sequence for the σ^{43} factor (TTGACA for the -35 region and TATAAT for the -10 region) in *B. subtilis* (Moran *et al.*, 1982). The distance between the -35 region and the -10 region was 17 bp for each promoter, which is the same as the consensus distance in *B. subtilis* (Moran *et al.*, 1982). Furthermore, 39 bases downstream from the termination codon at position $+1765$ was a palindromic sequence (Fig. 2) that might act as a transcription terminator (Rosenberg & Court, 1979).

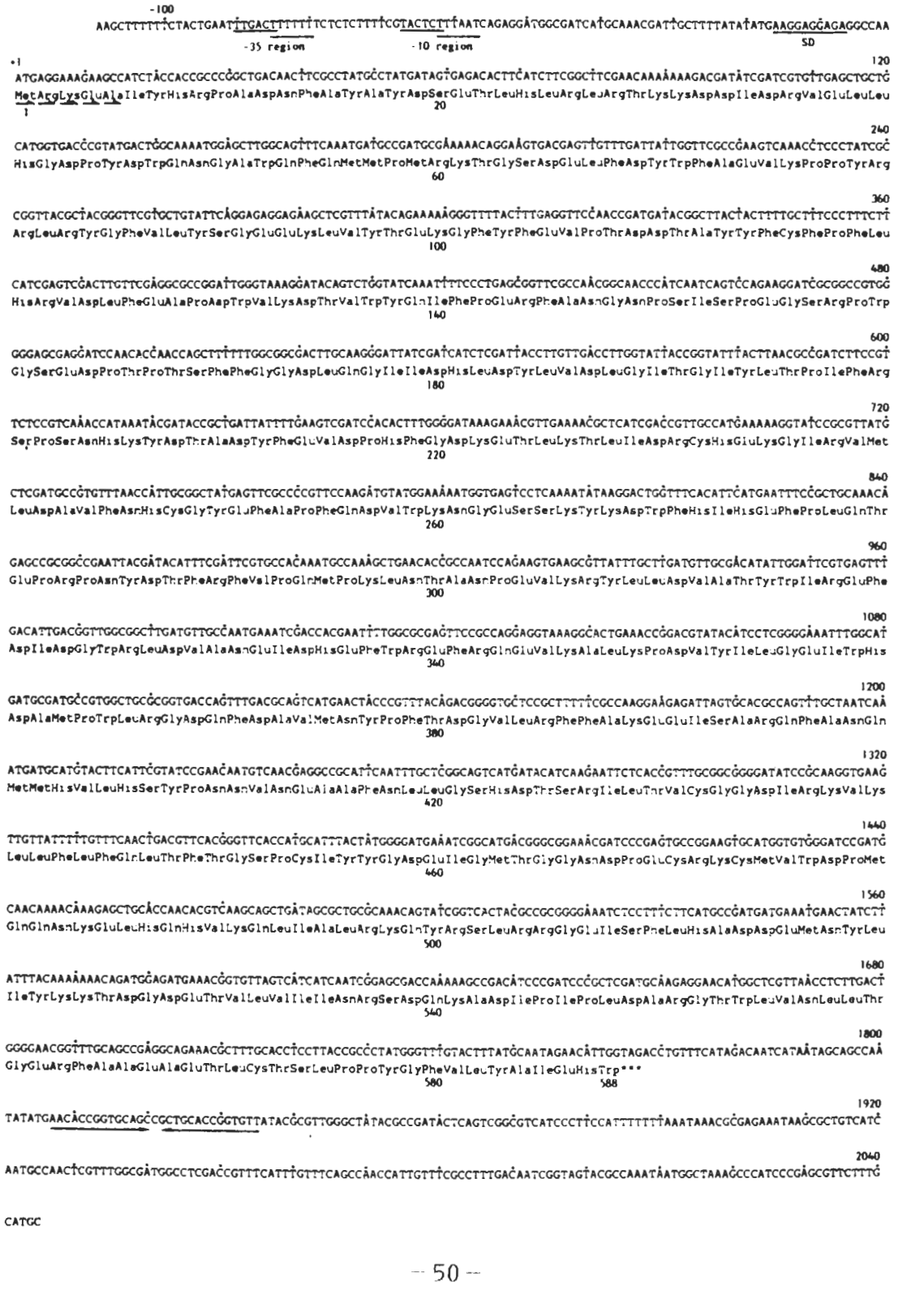
Amino acid analysis

The M_r of the neopullulanase, calculated as 69144 from the amino acid sequence (588 amino acid residues), is in reasonable agreement with the direct assessment (M_r 62000) by SDS polyacrylamide gel electrophoresis (Kuriki *et al.*, 1988*a*). The amino acid composition of the neopullulanase agreed with that deduced from the nucleotide sequence (data not shown). The codon usage was rather randomly distributed, without clear bias for any particular third-position base, and was nearly the same as that of *B. stearothermophilus* α-amylase (Nakajima *et al.*, 1985).

The neopullulanase contained eight cysteine residues (Fig. 2). If some disulphide bonds existed, they might provide conformational stability (Perry & Wetzel, 1984). To examine whether disulphide bonds were present in the neopullulanase, we compared the thermostability of the enzyme in the presence or absence of a reducing agent. 2-Mercaptoethanol (final concentration 10 mM) was added to the enzyme solution and the thermostability at 60, 65 and 70 °C was measured as described previously (Kuriki *et al.*, 1988*a*). The thermostability and specific activity of the reduced neopullulanase were rather higher than those of the native (non-reduced) enzyme, although the reason is not clear yet (data not shown). This result implied that the neopullulanase did not contain any disulphide bonds necessary for enzyme activity or for stability.

Comparison of the amino acid sequences of homologous regions in several amylolytic enzymes

We analysed the homologous amino acid sequences for α-amylases (Nakajima *et al.*, 1986), the neopullulanase, isoamylase (Amemura *et al.*, 1988), pullulanase (Katsuragi *et al.*, 1987) and cyclodextrin glucanotransferases (Binder *et al.*, 1986; Kimura *et al.*, 1987; Sakai *et al.*, 1987). As


-100
-35 region
-10 region
SD
+1
1
20
60
100
140
180
220
260
300
340
380
420
460
500
540
580
588
120
240
360
480
600
720
840
960
1080
1200
1320
1440
1560
1680
1800
1920
2040
MetArgLysGluAlaIleTyrHisArgProAlaAspAsnPheAlaTyrAlaTyrAspSerGluThrLeuHisLeuArgLeuArgThrLysLysAspAspIleAspArgValGluLeuLeu
HisGlyAspProTyrAspTrpGlnAsnGlyAlaTrpGlnPheGlnMetMetProMetArgLysThrGlySerAspGluLeuPheAspTyrTrpPheAlaGluValLysProProTyrArg
ArgLeuArgTyrGlyPheValLeuTyrSerGlyGluGluLysLeuValTyrThrGluLysGlyPheTyrPheGluValProThrAspAspThrAlaTyrTyrPheCysPheProPheLeu
HisArgValAspLeuPheGluAlaProAspTrpValLysAspThrValTrpTyrGlnIlePheProGluArgPheAlaAsnGlyAsnProSerIleSerProGluGlySerArgProTrp
GlySerGluAspProThrProThrSerPhePheGlyGlyAspLeuGlnGlyIleIleAspHisLeuAspTyrLeuValAspLeuGlyIleThrGlyIleTyrLeuThrProIlePheArg
SerProSerAsnHisLysTyrAspThrAlaAspTyrPheGluValAspProHisPheGlyAspLysGluThrLeuLysThrLeuIleAspArgCysHisGluLysGlyIleArgValMet
LeuAspAlaValPheAsnHisCysGlyTyrGluPheAlaProPheGlnAspValTrpLysAsnGlyGluSerSerLysTyrLysAspTrpPheHisIleHisGluPheProLeuGlnThr
GluProArgProAsnTyrAspThrPheArgPheValProGlnMetProLysLeuAsnThrAlaAsnProGluValLysArgTyrLeuLeuAspValAlaThrTyrTrpIleArgGluPhe
AspIleAspGlyTrpArgLeuAspValAlaAsnGluIleAspHisGluPheTrpArgGluPheArgGlnGluValLysAlaLeuLysProAspValTyrIleLeuGlyGluIleTrpHis
AspAlaMetProTrpLeuArgGlyAspGlnPheAspAlaValMetAsnTyrProPheThrAspGlyValLeuArgPhePheAlaLysGluGluIleSerAlaArgGlnPheAlaAsnGln
MetMetHisValLeuHisSerTyrProAsnAsnValAsnGluAlaAlaPheAsnLeuLeuGlySerHisAspThrSerArgIleLeuThrValCysGlyGlyAspIleArgLysValLys
LeuLeuPheLeuPheGlnLeuThrPheThrGlySerProCysIleTyrTyrGlyAspGluIleGlyMetThrGlyGlyAsnAspProGluCysArgLysCysMetValTrpAspProMet
GlnGlnAsnLysGluLeuHisGlnHisValLysGlnLeuIleAlaLeuArgLysGlnTyrArgSerLeuArgArgGlyGluIleSerPheLeuHisAlaAspAspGluMetAsnTyrLeu
IleTyrLysLysThrAspGlyAspGluThrValLeuValIleIleAsnArgSerAspGlnLysAlaAspIleProIleProLeuAspAlaArgGlyThrTrpLeuValAsnLeuLeuThr
GlyGluArgPheAlaAlaGluAlaGluThrLeuCysThrSerLeuProProTyrGlyPheValLeuTyrAlaIleGluHisTrp***
TATATGAACACCGGTGCAGCCGCTGCACCGGTGTTATACGCGTTGGGCTATACGCCGATACTCAGTCGGCGTCATCCCTTCCATTTTTTTAAATAAACGCGAGAAATAAGCGCTGTCATC
AATGCCAACTCGTTTGGCGATGGCCTCGACCGTTTCATTTGTTTCAGCCAACCATTGTTTCGCCTTTGACAATCGGTAGTACGCCAAATAATGGCTAAAGCCCATCCCGAGCGTTCTTTG
CATGC

		Region 1		Region 2		Region 3		Region 4	
AMY	Consensus	D A V I N H		G F R L D A A K H		E V I D		F V D N H D	
AMY	*A. ory.*	117 D V V A N H	202	G L R I D T V K H	230	E V L D	292	F V E N H D	
AMY	*B. stearo.*	101 D V V F D H	230	G F R L D A V K H	264	E Y W S	326	F V D N H D	
AMY	*B. amylo.*	98 D V V L N H	227	G F R I D A A K H	261	E Y W Q	323	F V E N H D	
AMY	*B. sub.*	97 D A V I N H	172	G F R F D A A K H	208	E I L Q	264	W V E S H D	
AMY	Rat	96 D A V I N H	190	G F R L D A A K H	230	E V I D	292	F V D N H D	
AMY	Mouse, s	96 D A V I N H	193	G F R L D A S K H	233	E V I D	295	F V D N H D	
AMY	Mouse, p	96 D A V I N H	190	G F R L D A A K H	230	E V I D	292	F V D N H D	
AMY	Hog	96 D A V I N H	193	G F R I D A S K H	233	E V I D	295	F V D N H D	
AMY	Human, s	99 D A V I N H	196	G F R I D A S K H	236	E V I D	298	F V D N H D	
AMY	Human, p	99 D A V I N H	196	G F R L D A S K H	236	E V I D	298	F V D N H D	
AMY	Barley	101 D I V I N H	127	D G R L D W G P H	218	E V W D	299	F V D N H D	
NPL	*B. stearo.*	242 D A V F N H	324	G W R L D V A N E	357	E I W H	419	L L G S H D	
IAM	*P. amylo.*	291 D V V Y N H	370	G F R F D L A S V	454	E W S V	502	F I D V H D	
PUL	*K. aero.*	600 D V V Y N H	671	G F R F D L M G Y	704	E G W D	827	Y V S K H D	
CGT	*K. pne.*	130 D Y A D N H	219	A I R I D A I K H	257	E W F G	328	F M D N H D	
CGT	*B. mace.*	135 D F A P N H	225	G I R F D A V K H	258	E W F L	324	F I D N H D	
CGT	Alk. *B.*	135 D F A P N H	225	G I R V D A V K H	268	E Y H Q	323	F I D N H D	
CGT	*B. stearo.*	131 D F A P N H	221	G I R M D A V K H	253	E W F L	319	F I D N H D	

Fig. 3. Highly conserved regions among α-amylase, neopullulanase, isoamylase, pullulanase and cyclodextrin glucanotransferase. The amino acid residues identical with the consensus sequence of α-amylase are shaded. Amino acid residues are shown by single letters as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Enzymes are abbreviated as: AMY, α-amylase; NPL, neopullulanase; IAM, isoamylase; PUL, pullulanase; CGT, cyclodextrin glucanotransferase. Enzyme sources are abbreviated as: *A. ory.*, *Aspergillus oryzae*; *B. stearo.*, *Bacillus stearothermophilus*; *B. amylo.*, *Bacillus amyloliquefaciens*; *B. sub.*, *Bacillus subtilis*; *P. amylo.*, *Pseudomonas amyloderamosa*; *K. aero.*, *Klebsiella aerogenes*; *K. pne.*, *Klebsiella pneumoniae*; *B. mace.*, *Bacillus macerans*; Alk. *B.*, alkalophilic *Bacillus*; s, saliva; p, pancreas. For the reference of each amino acid sequence, see the text. Numbering of the amino acid sequences of the enzymes starts at the amino-terminal amino acid of each mature enzyme. Active sites and substrate binding sites proposed by Matsuura *et al.* (1984) for Taka-amylase A from *A. oryzae* are indicated by ○ and □, respectively.

the results demonstrate (Fig. 3), the four highly conserved regions previously demonstrated in α-amylases were also found in the neopullulanase, isoamylase, pullulanase and cyclodextrin glucanotransferases. The arrangement of regions 1, 2, 3 and 4 from the amino-termini among these enzymes were the same. The other parts of the neopullulanase showed almost no similarity to those of α-amylases, isoamylase, pullulanase and cyclodextrin glucanotransferases.

DISCUSSION

We have determined the complete nucleotide sequence of the gene for a new type of pullulan-hydrolysing enzyme, designated neopullulanase, from *B. stearothermophilus* TRS40. An open reading frame, composed of 1764 bases and 588 amino acid residues, was found. The thermostable neopullulanase contained eight cysteine residues. However, they did not provide conformational stability through disulphide bond formation.

Fig. 2. Nucleotide and amino acid sequences of the neopullulanase gene. The nucleotide sequence is presented from the *Hin*dIII site (nucleotide −109) to the *Sph*I site (nucleotide +2045). The nucleotide sequence is counted from the first base of open reading frame. The amino acid sequence is shown beneath the nucleotide sequence. The amino-terminal amino acid sequence of the neopullulanase, determined by the Edman method, is denoted by half-head arrows. The first amino acid of the translation (Met) is counted as 1. A probable Shine–Dalgarno sequence (nucleotide −16 to −7) and putative promoter regions (−35 and −10 regions) are shown by solid lines below the nucleotide sequence. Asterisks indicate a stop codon. The sequence containing inverted repeat structures downstream from the termination codon TAG is designated by →←.

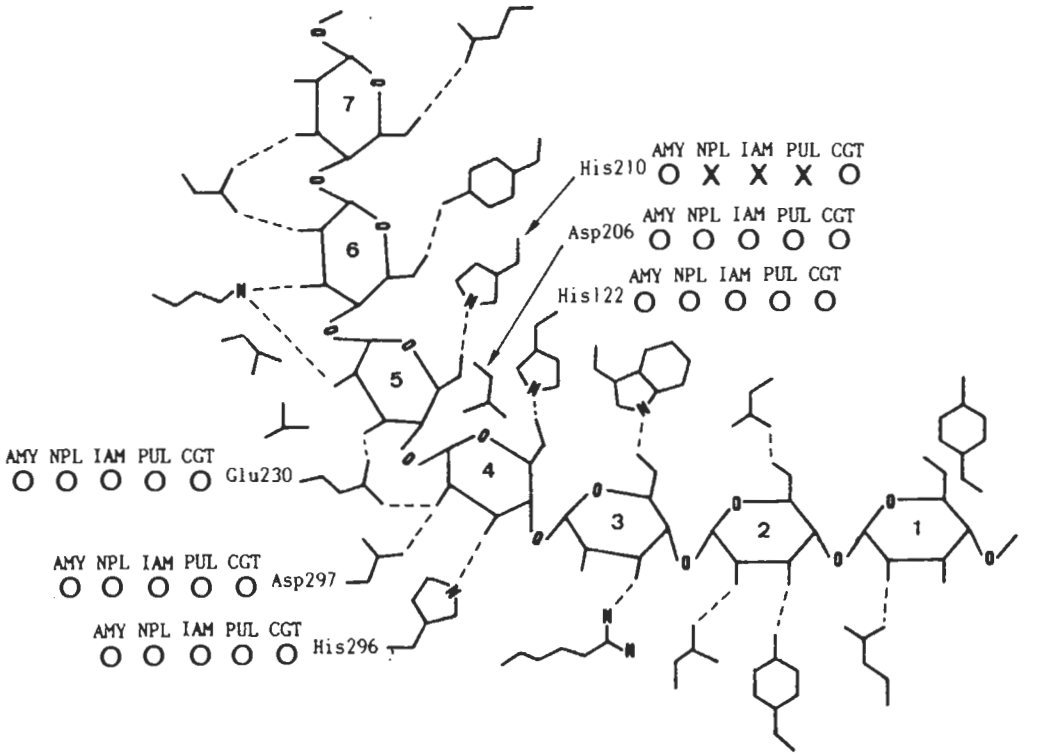


Fig. 4. Proposed substrate-binding model of Taka-amylase A (Matsuura *et al.*, 1984) and conservation of corresponding amino acid residues in other α-amylases, neopullulanase, isoamylase, pullulanase and cyclodextrin glucanotransferases (see Fig. 3). The glucose residues of amylose (as a substrate) are specified by the numbers 1 to 7. Enzymes are abbreviated as in Fig. 3. ○, Conserved residue; ×, non-conserved residue.

Table 1. *Action of various amylolytic enzymes*

Relative hydrolysis and transglucosylation rates are indicated as follows: ++, strong; +, medium; ±, weak; −, none. α-(1→4), α-(1→4)-glucosidic linkage; α-(1→6), α-(1→6)-glucosidic linkage.

	Pullulan		Starch/amylopectin		Glycogen		
Enzyme	α-(1→4)	α-(1→6)	α-(1→4)	α-(1→6)	α-(1→4)	α-(1→6)	Trans-glucosylation
α-Amylase	−	−	++	−			±
Neopullulanase	++	+	±	±			
Isoamylase	−	−	−	++	−	++	
Pullulanase	−	++	−	++	−	±	
Cyclodextrin glucanotransferase	−	−	++	−			++

Although most of the amylolytic enzymes are secretory proteins, no typical signal sequence was found in the amino-terminal region of the neopullulanase. When *B. subtilis* NA-1(pPP10) was cultivated for 13 h (early stationary phase), about 20% of the total neopullulanase activity was detected in the culture supernatant. Since a major portion of the activity (about 80%) was found in the cell extract, which was prepared by sonication (19·5 KHz, 10 min), we purified the intracellular neopullulanase, and the amino-terminal amino acid sequence was compared with that of the enzyme purified from the culture supernatant. The sequences of the first five amino

acids of the enzymes from the two different preparations were identical. Since the neopullulanase was exclusively extracellular in *B. stearothermophilus* TRS40, the different localization of the neopullulanase in *B. subtilis* remains to be investigated.

We found four highly conserved regions in α-amylase, cyclodextrin glucanotransferase, and even in the neopullulanase, isoamylase and pullulanase, whose different patterns of action are summarized in Table 1. This finding might be very interesting to point out the homologies between α-(1→4)-glucanohydrolases (α-amylase and cyclodextrin glucanotransferase) and α-(1→6)-glucanohydrolases (isoamylase and pullulanase). Pullulanase hydrolyses pullulan, whereas isoamylase does not. The neopullulanase hydrolyses not only α-(1→4)- but also α-(1→6)-glucosidic linkages (Imanaka & Kuriki, 1989; Kuriki *et al.*, 1988*a*). The enzyme could hydrolyse pullulan efficiently and only a small amount of starch (Kuriki *et al.*, 1988*a*). Therefore, the neopullulanase is a novel enzyme which might be classified between α-amylase and pullulanase from the viewpoint of its action pattern.

In this context, we analysed the homologies of other glucanohydrolases, such as glucoamylase (EC 3.2.1.3) (Yamashita *et al.*, 1985), β-amylase (EC 3.2.1.2) (Rhodes *et al.*, 1987), cellulase (EC 3.2.1.4) (Béguin *et al.*, 1985; Fukumori *et al.*, 1986), maltase (α-D-glucosidase; EC 3.2.1.20) (Hong & Marmur, 1986) and isomaltase (oligo-1,6-glucosidase; EC 3.2.1.10) (Hunziker *et al.*, 1986). However, no significant homologies were found (data not shown).

A molecular model and catalytic residues of Taka-amylase A from *Aspergillus oryzae* were reported by Matsuura *et al.* (1984). Using the alignment of amino acid residues in homologous regions (Fig. 3), this model was applied for other α-amylases, the neopullulanase, isoamylase, pullulanase and cyclodextrin glucanotransferases (Fig. 4). Glu230 and Asp297 were proposed as active sites of Taka-amylase, and His122, Asp206, His210 and His296 were proposed as substrate-binding sites (Matsuura *et al.*, 1984). These active and substrate-binding sites were surprisingly conserved in other α-amylases, the neopullulanase isoamylase, pullulanase and cyclodextrin glucanotransferases (Figs 3 and 4). However, one substrate-binding-site residue, corresponding to His210 of Taka-amylase, was substituted by Glu, Val or Tyr in the neopullulanase, isoamylase or pullulanase, respectively. It is interesting that only these three enzymes can hydrolyse α-(1→6)-glucosidic linkages (Table 1).

Plant *et al.* (1987) suggested the presence of a carboxyl group with p*Ka* 4·3 at the active site of *Thermoanaerobium* pullulanase. It was considered most likely that the p*Ka* value of 4·3 represented the ionization of a Glu or Asp carboxyl group, and this view was confirmed by chemical modification with a carboxyl-group-specific reagent, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (Plant *et al.*, 1987). This finding is consistent with the hypothesis that Glu and/or Asp might be active-site residues in all the amylolytic enzymes shown in Figs 3 and 4.

To further investigate the role of the amino acid residues for catalytic activity and action pattern of the neopullulanase, and also to improve the characterization of the enzyme at the molecular level, amino acid substitution in the probable active site by site-directed mutagenesis is now in progress.

REFERENCES

ALLEN, G. (1981). Sequencing of proteins and peptides. In *Laboratory Technique in Biochemistry and Molecular Biology*, pp. 161–236. Edited by T. S. Work & R. H. Burdon. Amsterdam: North-Holland Publishing Co.

AMEMURA, A., CHAKRABORTY, R., FUJITA, M., NOUMI, T. & FUTAI, M. (1988). Cloning and nucleotide sequence of the isoamylase gene from *Pseudomonas amyloderamosa* SB-15. *Journal of Biological Chemistry* **263**, 9271–9275.

BÉGUIN, P., CORNET, P., & AUBERT, J.-P. (1985). Sequence of a cellulase gene of the thermophilic bacterium *Clostridium thermocellum*. *Journal of Bacteriology* **162**, 102–105.

BINDER, F., HUBER, O. & BÖCK, A. (1986). Cyclodextrin-glycosyltransferase from *Klebsiella pneumoniae* M5a1: cloning, nucleotide sequence and expression. *Gene* **47**, 269–277.

FUKUMORI, F., SASHIHARA, N., KUDO, T. & HORIKOSHI, K. (1986). Nucleotide sequences of two cellulase genes from alkalophilic *Bacillus* sp. strain N-4 and their strong homology. *Journal of Bacteriology* **168**, 479–485.

HATTORI, M. & SAKAKI, Y. (1986). Dideoxy sequencing method using denatured plasmid templates. *Analytical Biochemistry* **152**, 232–238.

HONG, S. H. & MARMUR, J. (1986). Primary structure of the maltase gene of the *MAL6* locus of *Saccharomyces carlsbergensis*. *Gene* **41**, 75–84.

HUNZIKER, W., SPIESS, M., SEMENZA, G. & LODISH,

H. F. (1986). The sucrase–isomaltase complex: primary structure, membrane-orientation, and evolution of a stalked, intrinsic brush border protein. *Cell* **46**, 227–234.

IMANAKA, T. & KURIKI, T. (1989). Pattern of action of *Bacillus stearothermophilus* neopullanase on pullulan. *Journal of Bacteriology* **171**, 369–374.

IMANAKA, T., TANAKA, T., TSUNEKAWA, H. & AIBA, S. (1981). Cloning of the genes for penicillinase, *penP* and *penI*, of *Bacillus licheniformis* in some vector plasmids and their expression in *Escherichia coli*, *Bacillus subtilis*, and *Bacillus licheniformis*. *Journal of Bacteriology* **147**, 776–786.

IMANAKA, T., FUJII, M., ARAMORI, I. & AIBA, S. (1982). Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. *Journal of Bacteriology* **149**, 824–830.

KATSURAGI, N., TAKIZAWA, N. & MUROOKA, Y. (1987). Entire nucleotide sequence of the pullulanase gene of *Klebsiella aerogenes* W70. *Journal of Bacteriology* **169**, 2301–2306.

KIMURA, K., KATAOKA, S., ISHII, Y., TAKANO, T. & YAMANE, K. (1987). Nucleotide sequence of the β-cyclodextrin glucanotransferase gene of alkalophilic *Bacillus* sp. strain 1011 and similarity of its amino acid sequence to those of α-amylases. *Journal of Bacteriology* **169**, 4399–4402.

KOZAK, M. (1983). Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. *Microbiological Reviews* **47**, 1–45.

KURIKI, T., OKADA, S. & IMANAKA, T. (1988*a*). New type of pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. *Journal of Bacteriology* **170**, 1554–1559.

KURIKI, T., PARK, J.-H., OKADA, S. & IMANAKA, T. (1988*b*). Purification and characterization of thermostable pullulanase from *Bacillus stearothermophilus* and molecular cloning and expression of the gene in *Bacillus subtilis*. *Applied and Environmental Microbiology* **54**, 2881–2883.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (editors) (1982). Gel electrophoresis. In *Molecular Cloning: a Laboratory Manual*, pp. 149–185. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

MATSUMURA, M., KATAKURA, Y., IMANAKA, T. & AIBA, S. (1984). Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic bacilli in comparison with that encoded by plasmid pUB110. *Journal of Bacteriology* **160**, 413–420.

MATSUURA, Y., KUSUNOKI, M., HARADA, W. & KAKUDO, M. (1984). Structure and possible catalytic residues of Taka-amylase A. *Journal of Biochemistry* **95**, 697–702.

MCPHERSON, M. J. & CHARALAMBOUS, B. M. (1988). Functional analysis of the starch debranching enzyme pullulanase. *Biochemical Society Transactions* **16**, 723–724.

MORAN, C. P., JR, LANG, N., LEGRICE, S. F. J., LEE, G., STEPHENS, M., SONENSHEIN, A. L., PERO, J. & LOSICK, R. (1982). Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Molecular and General Genetics* **186**, 339–346.

NAKAJIMA, R., IMANAKA, T. & AIBA, S. (1985). Nucleotide sequence of the *Bacillus stearothermophilus* α-amylase gene. *Journal of Bacteriology* **163**, 401–406.

NAKAJIMA, R., IMANAKA, T. & AIBA, S. (1986). Comparison of amino acid sequences of eleven different α-amylases. *Applied Microbiology and Biotechnology* **23**, 355–360.

PERRY, L. J. & WETZEL, R. (1984). Disulfide bond engineered into T4 lysozyme: stabilization of the protein toward thermal inactivation. *Science* **226**, 555–557.

PLANT, A. R., CLEMENS, R. M., MORGAN, H. W. & DANIEL, R. M. (1987). Active-site and substrate-specificity of *Thermoanaerobium* Tok6-B1 pullulanase. *Biochemical Journal* **246**, 537–541.

RHODES, C., STRASSER, J. & FRIEDBERG, F. (1987). Sequence of an active fragment of *B. polymyxa* beta amylase. *Nucleic Acids Research* **15**, 3934.

ROSENBERG, M. & COURT, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annual Review of Genetics* **13**, 319–353.

SAKAI, S., KUBOTA, M., YAMAMOTO, K., NAKADA, T., TORIGOE, K., ANDO, O. & SUGIMOTO, T. (1987). Cloning of cyclodextrin glucanotransferase genes from *B. stearothermophilus* and *B. macerans*. *Journal of the Japanese Society of Starch Science* **34**, 140–147.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.

TAKAGI, M., IMANAKA, T. & AIBA, S. (1985). Nucleotide sequence and promoter region for the neutral protease gene from *Bacillus stearothermophilus*. *Journal of Bacteriology* **163**, 824–831.

TINOCO, I., JR, BORER, P. N., DENGLER, B., LEVINE, M. D., UHLENBECK, O. C., CROTHERS, D. M. & GRALLA, J. (1973). Improved estimation of secondary structure in ribonucleic acids. *Nature New Biology* **246**, 40–41.

VIEIRA, J. & MESSING, J. (1987). Production of single-stranded plasmid DNA. *Methods in Enzymology* **153**, 3–11.

YAMASHITA, I., SUZUKI, K. & FUKUI, S. (1985). Nucleotide sequence of the extracellular glucoamylase gene *STA1* in the yeast *Saccharomyces diastaticus*. *Journal of Bacteriology* **161**, 567–573.