Cloning and Expression of Thermostable α-Amylase Gene from *Bacillus stearothermophilus* in *Bacillus stearothermophilus* and *Bacillus subtilis*

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The structural gene for a thermostable α-amylase from *Bacillus stearothermophilus* was cloned in plasmids pTB90 and pTB53. It was expressed in both *B. stearothermophilus* and *Bacillus subtilis*. *B. stearothermophilus* carrying the recombinant plasmid produced about fivefold more α-amylase (20.9 U/mg of dry cells) than did the wild-type strain of *B. stearothermophilus*. Some properties of the α-amylases that were purified from the transformants of *B. stearothermophilus* and *B. subtilis* were examined. No significant differences were observed among the enzyme properties despite the difference in host cells. It was found that the α-amylase, with a molecular weight of 53,000, retained about 60% of its activity even after treatment at 80°C for 60 min.

The genus *Bacillus* produces a large variety of extracellular enzymes, some of which are of industrial importance, e.g., neutral protease is used for brewing and α-amylase is used for starch liquefaction and brewing (5). As far as the extracellular and industrially significant enzymes are concerned, it is a matter of course that enzymes which are thermostable rather than thermolabile are more versatile. The structural gene of thermostable neutral protease from *Bacillus stearothermophilus* in both *B. stearothermophilus* and *Bacillus subtilis* has already been cloned (7). The cloning of the thermostable α-amylase gene was attempted in this work, although the α-amylase genes from *Bacillus amyloliquefaciens* and *Bacillus coagulans* have been cloned in vector plasmids previously (4, 14).

The purpose of this paper is to describe the cloning of the α-amylase gene of *B. stearothermophilus* in both *B. stearothermophilus* (thermophile) and *B. subtilis* (mesophile) and to discuss the expression of the gene in each host bacterium.

MATERIALS AND METHODS

Media. L broth and L agar have been described previously (9). LS agar was L agar supplemented with 1.0% (wt/vol) soluble starch.

Bacterial strains and plasmids. Bacterial strains used in this study are listed in Table 1. *B. stearothermophilus* CU21, which produces a thermostable amylase, was used as a donor of the gene. Amylase-negative strains could not be obtained from *B. stearothermophilus* CU21 by a single mutagenic treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Conse-

quently, amylase-negative strain AN174 was obtained from strain CU21 by two successive NTG treatments (7). The intermediate was a poorly producing mutant.

Amylase-negative strain *B. subtilis* UN103 was also obtained from *B. subtilis* MI113 by treatment with NTG. Since the mutant strain *B. subtilis* UN103 exhibited a low growth rate, another strain, *B. subtilis* MI111, was transformed by the competent cell procedure with chromosomal DNA from *B. subtilis* UN103. Amylase-negative transformants were acquired by congression of Leu⁺. An amylase nonproducing strain, *B. subtilis* TN106, thus obtained was used as host cell.

pTB90 (encoding resistance to both kanamycin and tetracycline (Km^r Tc^r) (9) and pTB53 (Km^r Tc^r) (8, 10) were used as vector plasmids for *B. stearothermophilus* and *B. subtilis*, respectively.

Preparation of plasmid and chromosomal DNA. Either the rapid alkaline extraction method or CsCl-ethidium bromide equilibrium density gradient centrifugation as described previously (9) were used to prepare plasmid DNA, whereas chromosomal DNA was prepared as described elsewhere (10).

Transformation. Transformation of *B. stearothermophilus* protoplasts with plasmid DNA was done as described previously (9). For transformation of *B. subtilis* with chromosomal DNA, competent cells were prepared as described previously (10). The protoplast transformation procedure developed by Chang and Cohen (3) was used when *B. subtilis* was transformed with plasmid DNA.

Detection of amylase-producing and -nonproducing colonies on plates. Colonies of *B. stearothermophilus* were transferred by replica plating onto LS agar plates and incubated overnight at 55°C. Likewise, *B. subtilis* colonies were transferred onto LS agar and incubated at 37°C. After pouring the iodine reagent (0.01 M I₂–KI solution) on these plates, colonies with and without clear halos were detected as amylase-producing

TABLE 1. Bacterial strains used

Strain	Characteristics	Origin or reference
B. stearother-mophilus		
CU21	Sm^r Amy$^+$ (high activity)	9
AN17	Sm^r Amy$^+$ (low activity)	CU21, this work
AN174	Sm^r Amy$^-$	AN17, this work
B. subtilis		
MI113	*arg-15 trpC2* r_M^- m_M^- Amy$^+$	8
UN103	*arg-15 trpC2* r_M^- m_M^- Amy$^-$, low growth rate	MI113, this work
MI111	*arg-15 leuA8* r_M^- m_M^- Amy$^+$	8
TN106	*arg-15* r_M^- m_M^- Amy$^-$	MI111, this work

(Amy$^+$) and amylase-nonproducing (Amy$^-$) strains, respectively. The phenotypic ability to form halos was used to quickly identify amylase producers.

Assay of amylase activity. Bacteria were grown to stationary phase in L broth at either 55°C (*B. stearothermophilus*) or 37°C (*B. subtilis*). Samples were taken at intervals. The supernatant of the culture broth after centrifugation (8,000 × *g*, 10 min) was used for the assay of extracellular amylase. Cell concentration was measured as dry weight.

The method for assaying saccharolytic activity was based on the measurement of reducing power increase in a soluble starch solution as described by Bernfeld (2). The reaction mixture contained 1 ml of substrate solution [2% soluble starch in 40 mM potassium phosphate buffer (pH 6.0), 1 mM CaCl$_2$] and 1 ml of the enzyme solution. The reaction at 40°C was stopped after an appropriate incubation period by the addition of 3,5-dinitrosalicylic acid reagent (2 ml). By dissolving 1 g of 3,5-dinitrosalicylic acid in 20 ml of 2 N NaOH and 30 g of potassium sodium tartarate in 50 ml of deionized water, the reagent was prepared by mixing the two solutions, followed by adjustment of the volume to 100 ml with deionized water. For the blank test, the same amount (2 ml) of 3,5-dinitrosalicylic acid reagent was mixed with substrate solution before the addition of enzyme solution.

Brown color was developed by holding all of the assay tubes in a boiling water bath for 5 min. The content of each tube was then diluted with 20 ml of distilled water, and the absorbance at 540 nm was determined for each tube. One saccharolytic unit of amylase activity was defined as the quantity required to produce 1 mg of reducing sugar (as maltose) at 40°C for 3 min. This assay procedure was used throughout unless otherwise stated.

A dextrinogenic assay was also done by the method of Saito and Yamamoto (16), using either amylose or soluble starch as substrate.

Purification of extracellular amylase. *B. stearothermophilus* AN174 that carried a recombinant plasmid was cultivated in 1 liter of L broth containing kanamy-

cin (5 μg/ml) at 55°C overnight. After centrifugation (8,000 × *g*, 10 min) of the culture broth, ammonium sulfate (30% saturation) was added to the supernatant, and the solution was kept at 4°C for 5 h. The precipitate was removed by centrifugation (16,000 × *g*, 20 min). Ammonium sulfate was again added to the supernatant to a final concentration of 45% saturation, and it was maintained at 4°C overnight. After centrifugation (16,000 × *g*, 20 min), the precipitate was dissolved in about 20 ml of 50 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM CaCl$_2$ and dialyzed against the same buffer. The enzyme solution was applied to a column (1.6 by 43 cm) of DEAE-Sephadex A25 that had been equilibrated with the same buffer. Most of the amylase activity was eluted in the void volume. The enzyme fractions, pooled in a dialysis tube, were dehydrated in polyvinylpyrrolidone solution. The concentrated enzyme solution was used for isoelectric focusing.

B. subtilis TN106 carrying a recombinant plasmid was also cultivated in L broth at 37°C for 24 h. The purification of amylase was exactly the same as before.

Isoelectric focusing. Electrofocusing of amylase was done as described elsewhere (9a) except that Ampholine (pH 3.5 to 10.0) was used (2%) and the voltage was at 800 V initially for 24 h and at 1,200 V for another period of 24 h. Right after the isoelectric focusing, the fractionation was done to measure pH, and each fraction (1 ml), after addition of 0.1 ml of 40 mM potassium phosphate buffer (pH 6.0) containing 1 mM CaCl$_2$, was subjected to the determination of amylase activity.

Protein assay. The protein concentration as measured by the Lowry method, using bovine serum albumin as standard (11). After isoelectric focusing, protein was assayed by the method of Bensadoun and Weinstein (1), i.e., 24% trichloroacetic acid solution (1 ml) was added to the sample to precipitate protein. The precipitate obtained by centrifugation (3,300 × *g*, 30 min) was assayed by the Lowry method.

Paper chromatography of starch hydrolysate. Chromatography of the starch hydrolysate was carried out in the ascending mode on Whatman no. 1 filter paper with a solvent mixture of *n*-butanol–pyridine–water (6:4:3, by volume) overnight, and sugars were detected by spraying with silver nitrate solution (17).

Thermostability of enzyme. Amylase (about 20 U/ml) in 40 mM potassium phosphate buffer (pH 6.0)–1 mM CaCl$_2$ was treated at constant temperatures (65, 70, and 80°C). Glass tubes containing samples taken at appropriate time intervals were cooled quickly with ice water, and the remaining amylase activity was assayed at 40°C.

Other procedures. Procedures for mutagenesis, digestion of DNA with restriction endonucleases, ligation of DNA with T4 DNA ligase, agarose gel electrophoresis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis are described elsewhere (7–9). Unless otherwise specified, all chemicals used in this work came from the same sources described in the previous papers (7–9).

RESULTS

Cloning of amylase gene in *B. stearothermophilus*. The chromosomal DNA from *B. stearother-

mophilus CU21 (about 2 μg) was digested with restriction endonuclease *Hin*dIII and ligated with a *Hin*dIII digest (about 1 μg) of pTB90 in total volume of 200 μl. The ligation mixture was used to transform *B. stearothermophilus* AN174. Km^r transformants were transferred by replica plating onto LS agar containing 5 μg of kanamycin per ml for a quick check of amylase-positive colonies. Among about 50,000 colonies tested, two amylase-producing colonies were obtained. The strains were also resistant to tetracycline at 55°C. Plasmid DNAs were prepared from the two specific strains (Km^r Tc^r Amy$^+$) and designated as pKA11 and pKA21, respectively. The two plasmids were indistinguishable in molecular size and *Hin*dIII digestion patterns in preliminary examinations (data not shown). Since pKA11 and pKA21 seemed to be sisters resulting from the same transformed cell, pKA11 was used for further experiments.

The transformation of *B. stearothermophilus* AN174 with pKA11 yielded many Km^r Tc^r Amy$^+$ colonies, indicating that the ability to produce amylase was associated with the recombinant plasmid.

pKA11 DNA was digested with several restriction endonucleases and subjected to agarose gel electrophoresis. *Hin*dIII digestion of pKA11 yielded seven fragments (Fig. 1, lane A; fourth band from the top was doublet). Six DNA fragments might have been cloned incidentally. The sum of *Hin*dIII fragments, estimated as about 30

FIG. 1. Agarose gel electrophoresis of *Hin*dIII digests of plasmids. Lane A, pKA11; lane B, pTB90; lane C, pAT9; lane D, pTB53; lane E, pAT5; lane F, λ cI857S7. Electrophoresis was done in 1.0% agarose gel. Arrow indicates the position of the 4.8-Md *Hin*dIII fragment.

megadaltons (Md), was consistent with that obtained with other fragments of different restriction endonucleases.

To obtain a smaller plasmid that harbors the amylase gene, pKA11 was cleaved with *Hin*dIII, ligated with the *Hin*dIII digest of pTB90, and used to transform *B. stearothermophilus* AN174. A new small recombinant plasmid, pAT9, emerged from a transformant (Km^r Tc^r Amy$^+$). DNA of pAT9 transformed *B. stearothermophilus* AN174 again to Km^r Tc^r Amy$^+$. The plasmid pAT9 was digested with several restriction endonucleases, whose digestion patterns were examined by agarose gel electrophoresis. pAT9 was composed of two *Hin*dIII fragments (6.7 and 4.8 Md; Fig. 1, lane C). This fact indicates that the gene(s) for amylase is coded on the 4.8-Md *Hin*dIII fragment. A restriction map of pAT9 is shown in Fig. 2.

Cloning of the amylase gene in *B. subtilis*. pAT9 DNA prepared by CsCl-ethidium bromide density gradient centrifugation was used to transform *B. subtilis* TN106. Although many Km^r Tc^r Amy$^+$ transformants were obtained, plasmid pAT9 was quite unstable. Consequently, the construction of another recombinant plasmid was needed in *B. subtilis*.

A low-copy-number plasmid, pTB53 (Km^r Tc^r) (10), was used as a vector. *Hin*dIII digests of pAT9 and pTB53 were mixed, ligated, and used for the transformation of *B. subtilis* TN106. Among about 500 Km^r colonies, nine Amy$^+$ transformants were detected. A new recombinant plasmid, pAT5, was obtained from one of these transformants (Km^r Tc^r Amy$^+$). pAT5 transformed *B. subtilis* TN106 to Km^r Tc^r Amy$^+$ and was stably maintained in the host strain.

pAT5 consisted of two *Hin*dIII fragments (11.2 and 4.8 Md; Fig. 1, lane E). A restriction map of pAT5 is shown in Fig. 2. The restriction maps of pTB90 and pTB53 are also shown for clarity.

Expression of the amylase gene in *B. stearothermophilus* and *B. subtilis*. The amylase activities of culture supernatants from both *B. stearothermophilus* and *B. subtilis* with and without recombinant plasmids were examined (Table 2). Parental strain *B. stearothermophilus* CU21 produced 3.9 U/mg of dry cell. Neither host strain AN174 nor the strain carrying vector plasmid pTB90 produced a detectable amount of amylase. In contrast, *B. stearothermophilus* AN174 carrying pAT9 produced five times more amylase (20.9 U/mg of dry cell) than did the original strain, *B. stearothermophilus* CU21.

B. subtilis MI113 exhibited a low amylase activity (0.3 U/mg of dry cell), although the enzyme was substantially different from the thermostable amylase of *B. stearothermophilus*. Host strain *B. subtilis* TN106, with and without
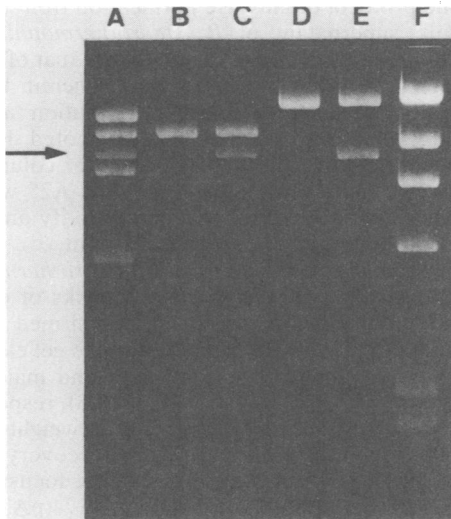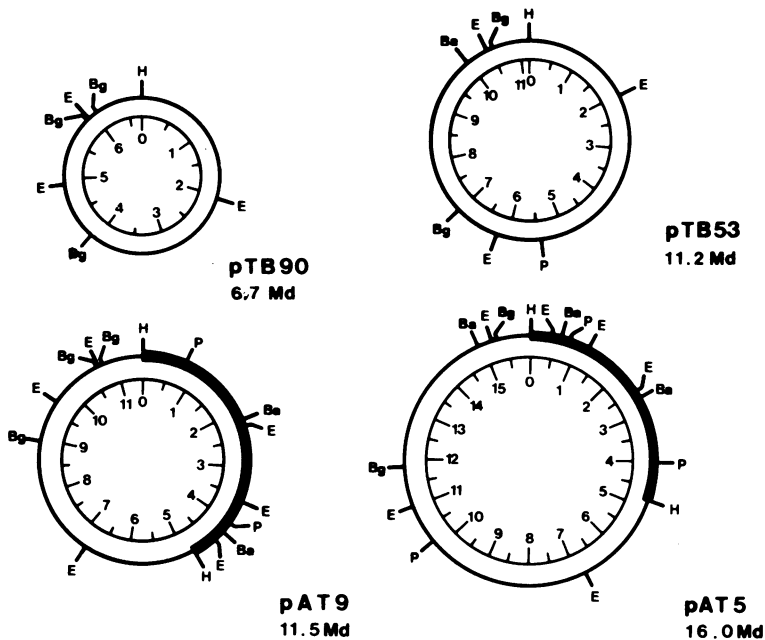
FIG. 2. Cleavage maps with restriction endonucleases for pTB90, pAT9, pTB53, and pAT5. Arabic numbers inside the circles indicate molecular size in Md. Heavy lines indicate the 4.8-Md HindIII fragment. Abbreviations: Ba, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI.

vector plasmid pTB53, did not produce amylase, whereas the strain carrying pAT5 produced amylase (1.1 U/mg of dry cell). These results show that the amylase gene encoded on the 4.8-Md HindIII fragment is expressed also in B. subtilis.

Proof that the structural gene of amylase is coded on the HindIII fragment (4.8 Md). To

TABLE 2. Production of extracellular amylase by B. stearothermophilus and B. subtilis

Strain	Temp (°C)	Amylase concn (U/mg of dry cell)[a] at:		
		Late-log phase	Early-stationary phase	Late-stationary phase
B. stearothermophilus				
CU21	55	1.6	2.4	3.9
AN174	55	ND[b]	ND	ND
AN174(pTB90)	55	ND	ND	ND
AN174(pAT9)	55	2.7	12.4	20.9
B. subtilis				
MI113	37	ND	0.1	0.3
TN106	37	ND	ND	ND
TN106(pTB53)	37	ND	ND	ND
TN106(pAT5)	37	0.1	0.3	1.1

[a] Samples were taken at three growth phases [late logarithmic, early stationary, and late stationary (24 h)] for each culture. Saccharolytic activity was assayed.

[b] ND, Not detectable (<0.01 U/ml).

determine whether the amylase structural gene is coded on the HindIII fragment (4.8 Md), properties of amylase from the recombinant plasmid-carrier strains of B. stearothermophilus and B. subtilis were studied.

The protocol of amylase purification from the culture supernatant of B. stearothermophilus AN174(pAT9) is shown, together with that of B. subtilis TN106(pAT5), in Table 3, wherein the specific activity of the enzyme solution and recovery in the consecutive steps are noted. For both species, the enzyme solution after column chromatography on DEAE-Sephadex A25 was subjected to isoelectric focusing to purify amylase and determine the isoelectric point.

Extracellular amylase from B. stearothermophilus AN174(pAT9) showed two peaks of enzyme activity (Fig. 3A). It was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis that both peaks [minor and major, for fractions 67 (pH 6.9) and 84 (pH 8.8), respectively] corresponded to a molecular weight of 53,000 (Fig. 4, lanes A and B). The recovery of total enzyme activity in the isoelectric focusing step for B. stearothermophilus AN174(pAT9) was 97.4%.

Fig. 3B shows another isoelectric focusing of amylase from B. subtilis, exhibiting again two peaks of enzyme activity [fractions 49 (pH 6.6) and 66 (pH 8.7)]. The recovery of total enzyme activity in this step was 85.7%. Similarly, the

TABLE 3. Summary of purification of thermostable amylase

Species	Fraction	Total enzyme activity (U)	Enzyme activity (U/ml)	Protein concn (mg/ml)	Sp act (U/mg)	Recovery (%)
B. stearothermophilus AN174(pAT9)	Culture supernatant	880	3.9	5.0	0.8	100
	Salting-out by ammonium sulfate	720	140	1.7	84	82
	DEAE-Sephadex A25 chromatography	500	20	0.2	100	57
	Isoelectric focusing					
	Fraction 67	58	58	0.02	2,900	7
	Fraction 84	84	84	0.03	2,800	10
B. subtilis TN106(pAT5)	Culture supernatant	690	1.6	5.3	0.3	100
	Salting-out by ammonium sulfate	150	7.7	1.0	8.0	22
	DEAE-Sephadex A25 chromatography	110	5.4	0.2	27	16
	Isoelectric focusing					
	Fraction 49	6.8	6.8	0.03	230	1
	Fraction 66	25	25	0.03	820	4

enzymes corresponding to the two peak fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As a result, amylases from *B. subtilis* TN106(pAT5) showed the same electrophoretic mobility as amylases from *B. stearothermophilus* (Fig. 4, lanes C and D).

The thermostability of these enzymes is shown in Fig. 5. All of the enzymes showed the same profile for thermostability regardless of differences in isoelectric point and host cell. About 60% of the initial enzyme activity remained intact even after treatment at 80°C for 60 min, showing the thermostability of the amylase. Indeed, only about 20% of the initial activity was detected after treatment of amylase from *B. subtilis* MI113 at 65°C for 10 min (data not shown). In contrast, no loss of amylase activity was observed by treatment of the enzyme of *B. subtilis* TN106(pAT5) at 65°C for 60 min. The fact that the thermostable amylase was pro-
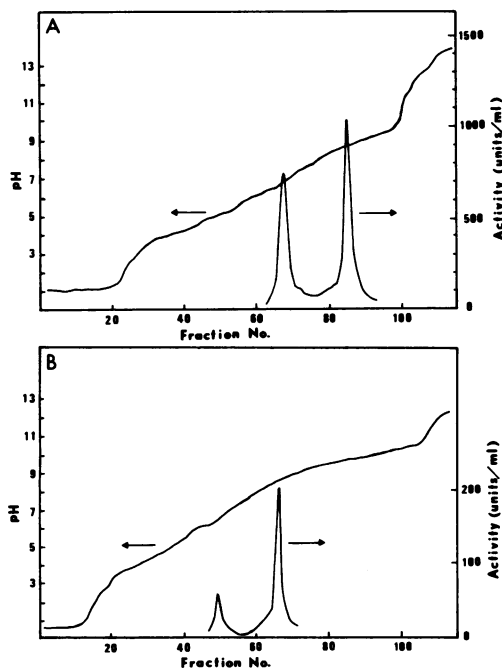

FIG. 3. Isoelectric focusing of amylase. (A) *B. stearothermophilus* AN174(pAT9); (B) *B. subtilis* TN106(pAT5).
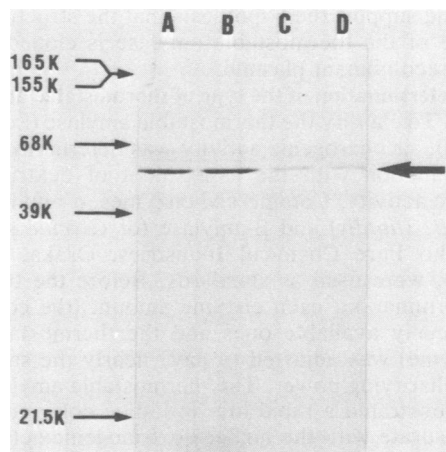

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of amylases. Lane A, amylase (pI = 8.8) from *B. stearothermophilus* AN174(pAT9); lane B, amylase (pI = 6.9) from *B. stearothermophilus* AN174(pAT9); lane C, amylase (pI = 8.7) from *B. subtilis* TN106(pAT5); lane D, amylase (pI = 6.6) from *B. subtilis* TN106(pAT5). Arrow indicates the position of amylase (molecular weight, 53,000).
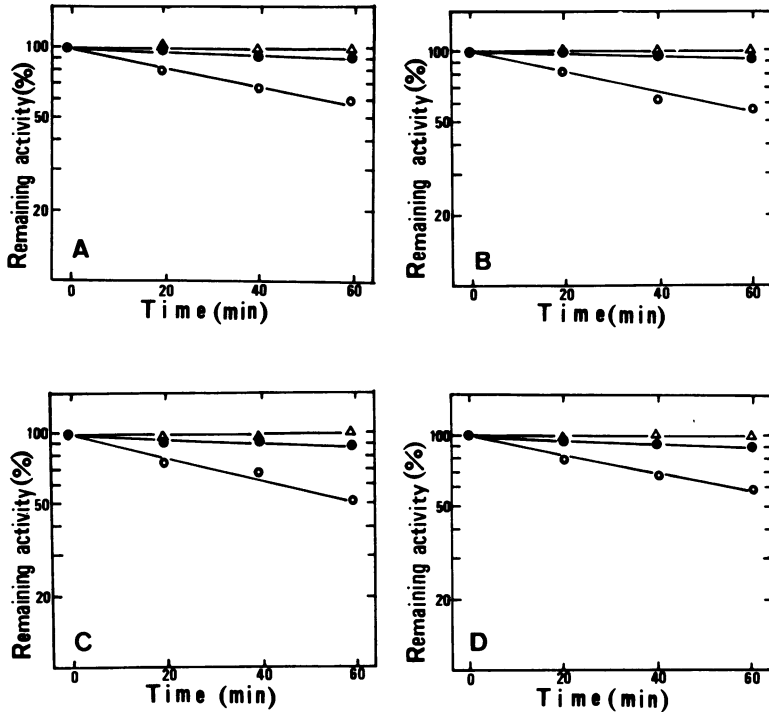
FIG. 5. Heat inactivation of amylase with time. Enzymes were kept at constant temperatures: 65°C ($\triangle$), 70°C ($\bullet$), and 80°C ($\bigcirc$). Portions were sampled at the time indicated, cooled quickly, and assayed for the remaining activity at 40°C. (A) Amylase (pI = 6.9) from *B. stearothermophilus* AN174(pAT9); (B) amylase (pI = 8.8) from *B. stearothermophilus* AN174(pAT9); (C) amylase (pI = 6.6) from *B. subtilis* TN106(pAT5); (D) amylase (pI = 8.7) from *B. subtilis* TN106(pAT5).

duced by the *B. subtilis* transformant with pAT5 would support the hypothesis that the structural gene of the thermostable amylase is cloned in the recombinant plasmid.

Determination of the type of thermostable amylase. To classify the thermostable amylase (i.e, α or β), saccharogenic activity was determined in conjunction with the assessment of dextrinogenic activity. Commercial enzymes, α-amylase (of *B. subtilis*) and β-amylase (of *Glycine* sp.) (Wako Pure Chemical Industries, Osaka, Japan), were used as standards. Before the type determination, each enzyme amount (the commercially available ones and the thermostable enzyme) was adjusted to have nearly the same saccharifying power. The thermostable amylase demonstrated a rapid loss in iodine color commensurate with the higher dextrinogenic activity. The same pattern was observed for α-amylase, but β-amylase exhibited lower dextrinogenic activity (data not shown). In addition, the paper chromatography of starch digests with the thermostable amylase revealed the presence of glucose, maltose, and oligosaccharides containing three, four, five, and more glucose units (data not shown). These observa-

tions identified the thermostable enzyme as α-amylase.

DISCUSSION

We cloned the structural gene for extracellular α-amylase from a thermophile, *B. stearothermophilus*, into vector plasmids pTB90 and pTB53. The gene was expressed in both *B. stearothermophilus* and *B. subtilis*. However, the level of gene expression in *B. subtilis* was lower than that in *B. stearothermophilus*. This phenomenon might be due to the following: (i) low efficiency of the expression of the amylase gene from a thermophile in *B. subtilis*, (ii) low secretion efficiency of thermostable amylase in *B. subtilis*, and (iii) a low copy number of the recombinant plasmid pAT5 in *B. subtilis*. The thermostable α-amylases were produced and secreted by the recombinant plasmid-carrier strains of *B. stearothermophilus* AN174 and *B. subtilis* TN106. It has been reported that penicillinase and thermostable neutral protease are secreted by both *B. stearothermophilus* and *B. subtilis* carrying the specific recombinant plasmids, although the extent of secretion was different (6, 7, 9a, 10). These observations would suggest that some

secretion mechanisms are shared by these meso-philic and thermophilic *Bacillus* species.

The extracellular α-amylase of *B. stearother-mophilus* CU21 is considerably thermostable, but the enzyme was quite unstable in the absence of Ca^{2+}, similar to other α-amylases of *B. stearothermophilus* (12, 13, 15). Actually, when the α-amylase in this work was treated at 65°C for 15 min in the absence of Ca^{2+}, less than 10% of the initial activity was detected. In contrast, the enzyme activity was not inactivated at all in the presence of Ca^{2+}, even after treatment at 65°C for 60 min. For this particular reason, Ca^{2+} was added throughout to the assay solution.

No significant difference was observed between the properties (molecular weight and thermostability) of α-amylases produced by *B. stearothermophilus* and *B. subtilis* despite the difference in host cells. However, isoelectric focusing exhibited two peaks of the enzyme activity (pI, 6.6 to 6.9 and 8.7 to 8.8) for *B. stearothermophilus* AN174(pAT9) and *B. subtilis* TN106(pAT5), respectively. The appearance of these two α-amylase fractions might be ascribed to the following reasons, either separately or collectively: (i) two structural genes of α-amylase were simultaneously cloned in the 4.8-Md *Hind*III fragment, (ii) the α-amylase from a single structural gene was processed in the secretion through cytoplasmic membrane, and (iii) extracellular α-amylase was combined with charged materials such as polyamines. Further work to delineate the intriguing phenomenon of two α-amylases, different in pI values but essentially the same in specific activity, is now in progress.

LITERATURE CITED

1. **Bensadoun, A., and D. Weinstein.** 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. **70**:241–250.
2. **Bernfeld, P.** 1955. Amylases, α and β. Methods Enzymol. **1**:149–158.
3. **Chang, S., and S. N. Cohen.** 1979. High frequency trans-formation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
4. **Cornelis, P., C. Digneffe, and K. Willemot.** 1982. Cloning and expression of a *Bacillus coagulans* amylase gene in *Escherichia coli*. Mol. Gen. Genet. **186**:507–511.
5. **Fogarth, W. M., P. J. Griffin, and A. M. Joyce.** 1974. Enzymes of *Bacillus* species—part 1. Process Biochem. **9**:11–24.
6. **Fujii, M., T. Imanaka, and S. Aiba.** 1982. Molecular cloning and expression of penicillinase genes from *Bacillus licheniformis* in the thermophile *Bacillus stearothermophilus*. J. Gen. Microbiol. **128**:2997–3000.
7. **Fujii, M., M. Takagi, T. Imanaka, and S. Aiba.** 1983. Molecular cloning of a thermostable neutral protease gene from *Bacillus stearothermophilus* in a vector plasmid and its expression in *Bacillus stearothermophilus* and *Bacillus subtilis*. J. Bacteriol. **154**:831–837.
8. **Imanaka, T., M. Fujii, and S. Aiba.** 1981. Isolation and characterization of antibiotic resistance plasmids from thermophilic bacilli and construction of deletion plasmids. J. Bacteriol. **146**:1091–1097.
9. **Imanaka, T., M. Fujii, I. Aramori, and S. Aiba.** 1982. Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. J. Bacteriol. **149**:824–830.
9a.**Imanaka, T., W. Oshihara, T. Himeno, and S. Aiba.** 1983. Comparative studies on extracellular penicillinases of the same structural gene, *penP*, expressed in *Bacillus licheniformis* and *Bacillus subtilis*. J. Gen. Microbiol. **129**:2621–2628.
10. **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.
11. **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.
12. **Manning, G. B., and L. L. Campbell.** 1961. Thermostable α-amylase of *Bacillus stearothermophilus*. I. Crystallization and some general properties. J. Biol. Chem. **236**:2952–2957.
13. **Ogasawara, K., A. Imanishi, and T. Isemura.** 1970. Studies on thermophilic α-amylase from *Bacillus stearothermophilus*. I. Some general and physico-chemical properties of thermophilic α-amylase. J. Biochem. **67**:65–75.
14. **Palva, I.** 1982. Molecular cloning of α-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*. Gene **19**:81–87.
15. **Pfueller, S. L., and W. H. Elliott.** 1969. The extracellular α-amylase of *Bacillus stearothermophilus*. J. Biol. Chem. **244**:48–54.
16. **Saito, N., and K. Yamamoto.** 1975. Regulatory factors affecting α-amylase production in *Bacillus licheniformis*. J. Bacteriol. **121**:848–856.
17. **Trevelyan, W. E., D. P. Procter, and J. G. Harrison.** 1950. Detection of sugars on paper chromatograms. Nature (London) **166**:444–445.