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ISOLATION OF A *Bacillus subtilis* TRANSFORMANT PRODUCING THERMOSTABLE α-AMYLASE BY DNA FROM A THERMOPHILIC BACTERIUM

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Summary: A *Bacillus subtilis* transformant producing thermostable α-amylase was isolated using DNA from a thermophilic bacterium, *Thermophile* V2. The extracellular α-amylase did not crossreact with a rabbit antiserum against *B. subtilis* α-amylase. The structural gene for the thermostable α-amylase was integrated at a different locus from *B. subtilis* α-amylase. It was linked to *pyrA*. The transformant was not thermophilic, and its upper temperature for growth was similar to that of the host bacterium.

INTRODUCTION

α-Amylase is an enzyme distributed from microorganisms to higher plants and animals. We have been studying whether the structural gene(s) for the enzyme in a thermophilic bacterium are able to be introduced into *Bacillus subtilis* Marburg strains by DNA mediated transformation or not. In this study, a *B. subtilis* strain which lacks modification and restriction systems was used as a recipient strain. When a DNA isolated from a thermophilic bacterium, *Thermophile* V2, was used as a donor, one transformant producing thermostable α-amylase was isolated among transformed amino acid prototrophs. The structural gene for α-amylase seemed to be integrated into a different chromosomal locus from that of the *B. subtilis* α-amylase structural gene.

MATERIALS AND METHODS

B. subtilis 207-21 ($r^-_{168}m^-_{168}$, *amyE07, aroI906, lys21, leuA8, metB5*) is an α-amylase negative derivative of *B. subtilis* RM125 isolated by Uozumi *et al.*(1), and *B. subtilis* 61469 ($r^+_{168}m^+_{168}$, *lys21, metB5, trpC2*), a derivative of *B. subtilis* Marburg 168, was kindly supplied by H. Saito. *Thermophile* V2 is a thermophilic bacterium producing thermostable α-amylase (2). DNA from

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Thermophile V2 was extracted from the cells cultured at 60°C by the phenol-pH9 method (3) and purified by 5-20% sucrose gradient centrifugation in the presence of 0.1% sodium dodecyl sulfate. Other DNAs from mesophiles were prepared by the same method. The procedure for DNA mediated transformation was the modified method of Wilson and Bott (4). Transformants for amino acid requirements and α-amylase were selected at 37°C. α-Amylase activity was assayed by the modified method of Fuwa (5). The enzyme produced by a transformant was purified according to a literature (2). *B. subtilis* NA64 and antiserum against α-amylase of *B. subtilis* were described in a previous report (6). Ribonuclease A (Sigma Chemical Co.) and Pronase E (Kaken Kagaku Ltd.) were commercial products.

RESULTS AND DISCUSSION

When competent cells of *B. subtilis* 207-21 were treated with the DNA from *Thermophile* V2 (3 μg/ml, Preparation A in Table 1), a few hundred Lys^+ or Leu^+ transformants appeared on selective plates (Table 1). Treatment of the DNA preparation with ribonuclease A or Pronase E did not affect the transformation activity. The number of transformants was proportional to the amount of the DNA added. However, the transformation frequencies of Lys^+ and Leu^+ with the DNA from *Thermophile* V2 were about $1/10^3$ of those with a homologous DNA from *B. subtilis* var. *amylosaccharitícus* (SAC). Although it was reported that the restriction and modification in *B. subtilis* does not

Table 1. Transformation of *B. subtilis* 207-21 and 61469 by DNAs isolated from *Thermophile* V2, *B. amyloliquefacience* F and *B. subtilis* var. *amylosaccharitícus*.

Preparations of Donor DNA	DNA recipient strains	No. of transformants per 0.1 ml	
		Lys^+	Leu^+
Thermophile V2(A)(3.0 μg/ml)	*B. subtilis* 207-21	366	576
Thermophile V2(B)(3.4 μg/ml)	($r^-_{168}m^-_{168}$)	0	7
Thermophile V2(C)(0.6 μg/ml)		0	11
Thermophile V2(D)(27 μg/ml)		13	4
B. amyloliquefaciens(1.2 μg/ml)		1	1
B. subtilis SAC(0.1 μg/ml)		$>10^5$	$>10^5$
none		0	0
		Lys^+	Met^+ Trp^+
Thermophile V2(A)(3.0 μg/ml)	*B. subtilis* 61469	1	3 0
Thermophile V2(D)(27 μg/ml)	(r^+m^+)	2	0 -
none		0	- 0

DNA preparations A, B, C, and D of *thermophile* V2 were extracted from the cells cultured at 60°C for 4 h, 4 h, 5 h and 3 h, respectively, and purified by sucrose gradient centrifugation.

affect transformation (7), only a few transformants were detected when strain 61469 was used as the recipient cells. The transformation activity of the thermophile DNA differed from preparation to preparation as shown in Table 1. Other DNA preparations than preparation A generated only a small number of Lys^+ or Leu^+ transformants. We could not clarify the reason for the difference in the transforming activity among the DNA preparations.

To isolate Amy^+ transformants by the DNA of *Thermophile* V2, three Lys^+ transformants were selected as intergenotes. They were once more transformed into leucine prototrophy by the treatment with the same DNA preparation. Among about 10,000 colonies of Lys^+ and Leu^+ transformants, three colonies were Amy^+. One of the transformants (207-SV1) was selected for further studies. The transformant 207-SV1 carried the genetic markers of 207-21 except Lys^-, Leu^-, and Amy^-.

The mutation *amyE07* in strain 207-21 produces an immunologically cross-reacting material for antiserum against α-amylase of *B. subtilis*. α-Amylase produced by *B. subtilis* NA64 was almost completely inactivated by heating at 70°C for 10 min while α-amylase activity in 207-SV1 and *Thermophile* V2 retained more than 50% of its activity after treatment at 80°C for 10 min (Fig. 1). The purified α-amylase extracted from 207-SV1 showed similar thermal stability.

The thermostable α-amylase [α-amylase(SV1)] of 207-SV1 did not cross-react with rabbit antiserum against α-amylase of *B. subtilis*. This result indicates that the primary structure of α-amylase(SV1) is different from that of *B. subtilis*. The structural gene of this enzyme was tentatively named $amyV2^+$. The structural gene ($amyE^+$) for α-amylase of *B. subtilis* is located near *aroI* locus (8); the frequency of $amyE^+$ in $aroI^+$ transformants is 30-35 % in DNA mediated transformation. However, $amyV2^+$ did not link to *aroI* but linked to *pyrA* as shown by PBS1 mediated transduction (data not shown).

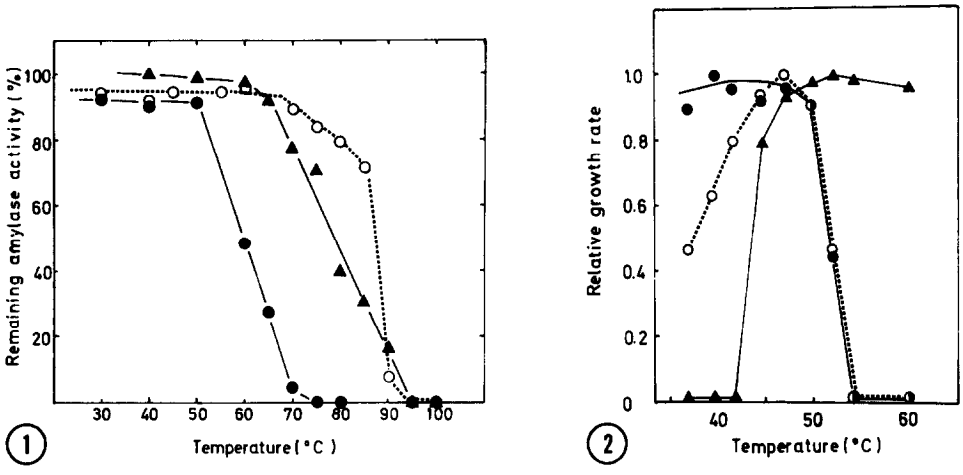

Fig. 1. Effect of heat treatment on α-amylase activity. α-Amylase preparations from *B. subtilis* NA64 (—●—), transformant 207-SV1 (—▲—), and *Thermophile* V2 (--o--) were heated at various temperature for 10 min. The heated solutions were chilled in ice water and the remaining activity was measured.

Fig. 2. Effect of temperature on growth rate of *B. subtilis* 207-21 (—●—), transformant 207-SV1 (--o--), and *Thermophile* V2 (—▲—). The growth at various temperature was followed by absorbance at 660 nm.

Mesophilic bacterium 207-21 shows good growth at 37°C-47°C. On the other hand, maximum growth of *Thermophile* V2 was observed at 55°C-60°C, and its growth rate was poor at 37°C-45°C. Although the growth rate of 207-SV1 was rather poor at 37°C (Fig. 2), the transformant, like the host 207-21, could not grow at 55°C or higher.

Lindsay and Creaser (9), and Mojica *et al.*(10) reported that DNA from thermophilic bacilli could transform a mesophilic *B. subtilis* generating transformants which were able to grow at higher temperatures. Conservation of the DNA sequences near the origin of DNA replication and ribosomal and tRNA functions has been shown by DNA mediated transformation between *Bacillus* spp (11). If these regions of DNA can be replaced by the DNA sequence of thermophilic bacterium, it may cause wide pleiotrophic effects. If so, it might be possible that a mesophilic bacterium will be able to transform to a thermophilic bacterium by introduction of a small number of genes located in the conserved region.

Of the enzymes of 207-SV1 tested, only α-amylase was thermostable; i.e. thermostability of its proteases, alkaline phosphatase and phosphodiesterase was identical with that of the corresponding ones from 207-21. The poor growth of 207-SV1 at 37°C may be due to the introduction of a small number of genes including Lys^+ and Leu^+ from *Thermophile* V2 and no growth at higher temperatures may be due to the fact that almost all the genes of the transformant still remained mesophilic.

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