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Cloning in Bacillus subtilis of an extremely thermostable alpha amylase: Comparison with other cloned heatstable alpha amylases

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A heatstable alpha amylase gene was shotgun cloned from Bacillus licheniformis RP01 into Bacillus subtilis. Restriction endonuclease analysis of the recombinant plasmid revealed a map which was identical to a previously cloned alpha amylase from B. licheniformis FD02 and very similar to the restriction map of a high temperature amylase from Bacillus coagulans. The thermostability and temperature optimum of the cloned alpha amylase was measureably different from those of the previously reported cloned alpha amylases.

The degradation of starch into simpler, more utilisable sugar moieties has become an increasingly important industrial process in recent years (e.g. the glucose syrup industry). There are two stages defined in the degradative pathway (3): First gelatinisation, whereby an aqueous solution of starch is heated above 70°C. The temperature required for complete gelatinisation depends on the source of starch, but generally 110 - 115° C is sufficient. The second stage is the liquefaction or hydrolysis of the gelatinised starch slurry.

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Hydrochloric acid was the traditional agent used in the acid liquefaction process but acid hydrolysis can lead to the formation of undesireable by-products, e.g. 5-hydroxymethyl 2-furfuraldehyde (4). The inclusion of bacterial amylases as sole liquefying agents or in conjunction with acid hydrolysis has eliminated the problem of by-product formation and the thermostability of some of the alpha amylases, particularly those of Bacillus licheniformis (7) has the added advantage of being close to the operational temperatures of the process.

In this report we describe how the gene coding for an extremely heatstable alpha amylase (E. C. 3.2.1.1.) from B. licheniformis was shotgun cloned into Bacillus subtilis using the vector pBD64 and the method of plasmid homology assist (6). Restriction endonuclease analysis of the recombinant plasmid showed it to be identical to a cloned alpha amylase from another strain of B. licheniformis (8) and very similar to a cloned heatstable alpha amylase from Bacillus coagulans (1) However, the thermostability and temperature optimum of our cloned alpha amylase was measureably different from those of the previously cloned alpha amylases.

The bacterial strains and plasmids used in this study are listed in Table 1. EcoRI cut chromosomal DNA (12ug) from B. licheniformis RP01 was ligated to EcoRI cut pBD64 (2.5ug) and transformed into B. subtilis SO103 using the method of plasmid assist (2). DNA was isolated using a rapid miniscreen procedure from one amy^+ transformant and used to transform B. subtilis SO113 to obtain amy^+ transformants without pUB110. These transformants were selected on chloramphenicol (Cm) and screened for the presence or absence of pUB110. After

Table 1 Bacterial Strains and Plasmids

Strain	Relevant Genotype	Source/Reference
B. licheniformis RP01	wild type	this study
B. subtilis JF211	trpC2 amy3 (pRP1)	this study
B. subtilis S0103	his met amy3 (pUB110)	S.A. Ortlepp
B. Subtilis S0113	trpC2 amy3	S.A. Ortlepp

Plasmids	Marker.
pUB110	Kanamycin.
pBD64	Chloramphenicol, Kanamycin
pRP1	Chloranphenicol, Kanamycin, amy^+.

several rounds of retransformation colonies which were amy^+, Cm^r and Kanamycin resistant (Km^r) and which did not contain pUB110 (as determined by visual examination of ethidium bromide stained agarose gels) were isolated. One clone designated JF211 was chosen for further study.

Plasmid DNA was prepared from B. subtilis JF211 by purification on a Caesium chloride gradient (5). When this plasmid, designated pRP1, was retransformed into two amylase negative strains of B. subtilis, S0103 and S0113, 100% of the Cm^r transformants were amy^+. When JF211 was cured of pRP1 by selection of Cm^s colonies following growth in the absence of drug the strain reverted to an amy^- phenotype. Both of these results indicated that pRP1 carried the gene encoding alpha amylase.

Initial restriction endonuclease analysis of pRP1 revealed that a 3,500bp fragment had been cloned into the EcoRI site of pBD64. This fragment was similar in size to the cloned alpha amylase fragment from B. licheniformis PD02 in the recombinant plasmid

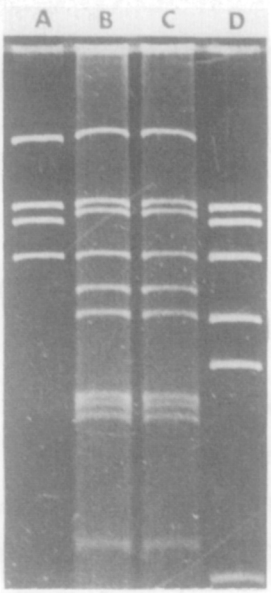

Fig. 1 Restriction endonuclease cleavage analysis of recombinant amylase plasmids. Caesium chloride gradient purified plasmid DNA's were incubated with endonuclease MboI as described previously[8]

The resultant restriction fragments were separated on a 6% Polyacrylamide Gel and visualized by UV photography of the Ethidium Bromide stained gel. Lane A, pBD64; Lane B, pRP1; Lane C, pSA33; Lane D, pUB110.

pSA33 (8) and when we compared pRP1 with pSA33 that had been cleaved with MboI we found no visible difference between the restriction pattern of both recombinant plasmids (Fig. 1). Further endonuclease analysis of the 3,500bp fragment of pRP1 revealed a physical map which was identical to the cloned insert of pSA33 that contains the B. licheniformis FD02 alpha amylase. The sites for the restriction enzymes BclI, ClaI, HindIII and HincII within the 3,500bp fragment of pRP1 mapped to positions close to, if not identical with the corresponding site on the 3,500 bp insert of pSA33 (Fig. 2). Furthermore the physical maps of the cloned inserts of pRP1 and pSA33 are very similar to the restriction map of pAMY2 (1) for the restriction enzymes PstI,

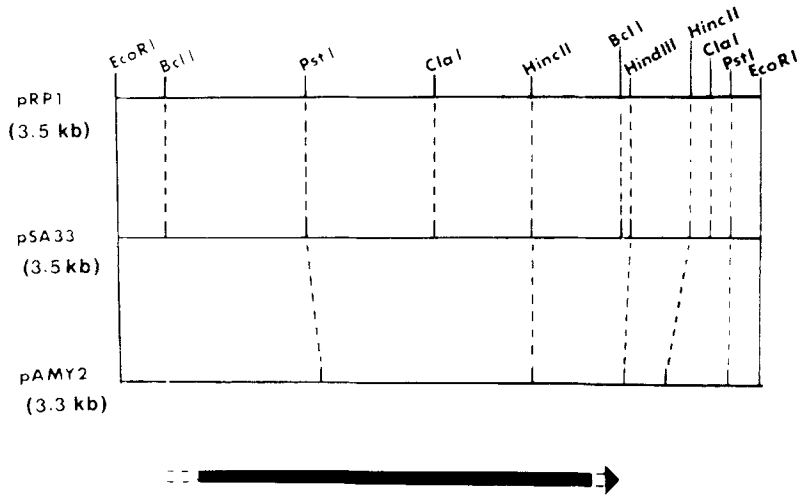

Fig. 2 Restriction endonuclease cleavage maps of the cloned amylase inserts within the plasmids pRP1, pSA33 and pAMY2. The horizontal lines represent the cloned EcoRI fragments within the three recombinant plasmids indicated. The reported molecular weights (in kilobase pairs) of these EcoRI fragments are given in parenthesis. The map position of each of the restriction enzyme sites shown was plotted as a fraction of the total insert size. No restriction endonuclease mapping information for the enzymes Cla I or Bcl I is available for pAMY2.

Shown beneath these physical maps is the approximate location and the direction of transcription of the high temperature amylase gene (8,1 and unpublished observations)

HincII, and HindIII (Fig. 2). This observation indicates a high degree of genetic conservation within the structure of the high temperature alpha amylase gene among different Bacilli.

We have observed however that the amylase proteins encoded by these genes display functional heterogeneity. B. licheniformis RP01 produced an alpha amylase with a temperature

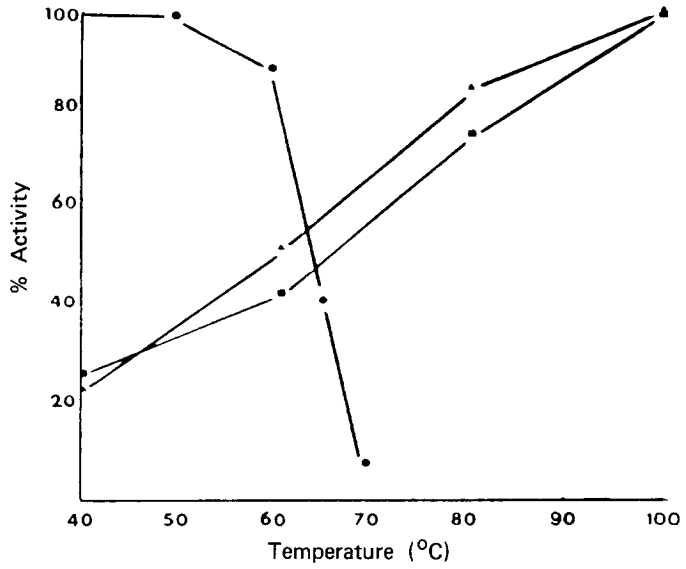

Fig. 3 Temperature profile of pRP1 encoded alpha amylase activity. Bacterial cultures were grown to stationary phase and assayed for amylase activity as described previously (8). B. subtilis 168 (●——●), B. licheniformis RP01 (■——■) and B. subtilis JF211 (▲——▲).

optimum of at least 100°C which contrasts sharply with the alpha amylase of B. subtilis 168 which has a temperature optimum between 40° and 50°C (Fig. 3). When assayed over the temperature range 40°C to 100°C the alpha amylases of B. licheniformis RP01 and B. subtilis JF211 had nearly identical profiles (Fig. 3) which indicated that the plasmid pRP1 coded for the B. licheniformis RP01 alpha amylase. This enzyme had a higher temperature optimum than the amylase encoded by pSA33 which has a temperature optimum of 93°C [8] determined by at least four independant measurements. The thermoresistance of the alpha amylase encoded by pRP1 was measured by assaying the activity at 50°C before and after heating the enzyme at 80°C for 10 minutes. The results of this

Table 2 Thermoresistance of the alpha amylases encoded by the plasmids pRPl, pSA33 and pAMY2[a]

Plasmid	Activity Before Heating units/ml	Activity After Heating units/ml	% Residual Activity
pRPl	142.5	153.5	107.4
pSA33	106	102.5	96.6
pAMY2	35.8	33.4	93.2

Culture supernatants prepared as described (8) were incubated at 80°C for 10 minutes. Amylase activities were determined at 50°C (8) prior to and subsequent to this heat treatment.

a. Taken from Ref. 1.

experiment (Table 2) show that the activity of the cloned alpha amylase is increased by 7% following heat treatment. This result was in contrast to the change in activity displayed by the pSA33 encoded alpha amylase. As shown in Table 2 this enzyme decreases after heating by 3.5%. This decrease in activity is similar to the results obtained for the cloned Bacillus coagulans alpha amylase (1) and included in Table 2 for comparative purposes. Thus the alpha amylases produced by these cloned genes were measureably different in temperature optima and in resistance to incubation at elevated temperature.

Extensive restriction endonuclease analysis of these two clones has shown that the DNA regions encoding the heat stable alpha amylases are highly conserved. This leads us to speculate that a difference of only a few amino acid residues can account for the observed difference in thermoresistance and temperature optima of these two heat stable alpha amylases. Indeed Perutz has shown (9) for the ferredoxins from two mesophile clostridia, that

the replacement of two base pairs can change a heat labile protein to a heat stable one. We are now determining the nucleotide sequence of the amylase coding regions of both pRP1 and pSA33 and this sequence information should allow us to predict the amino acid residues that account for this heterogeneity. It will then be interesting to test these predictions directly by site-specific mutagenesis.

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