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Genetics

Bacillus stearothermophilus contains a plasmid-borne gene for α-amylase

(thermophilic bacteria/recombinant DNA/carbohydrase/industrial enzyme)

JONATHAN R. MIELENZ

Moffett Technical Center, CPC International, Inc., Argo, IL 60501

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ABSTRACT **The gene for thermostable α-amylase from the thermophilic bacterium *Bacillus stearothermophilus* has been cloned and expressed in *Escherichia coli*. Each α-amylase-producing colony contained at least a 9.7-kilobase-pair (kb) chimeric plasmid composed of the vector pBR322 and a common 5.4-kb *Hin*dIII fragment of DNA. *B. stearothermophilus* contains four plasmids with sizes from 12 kb to over 108 kb. Restriction endonuclease analysis of these naturally occurring plasmids showed they also contain a 5.4-kb *Hin*dIII fragment of DNA. Cloning experiments with the four plasmids yielded α-amylase-producing *E. coli* that contained the same 9.7-kb chimeric plasmid. Restriction endonuclease analysis and further recombinant DNA experiments identified a 26-kb plasmid that contains the gene for α-amylase. A spontaneous mutant of *B. stearothermophilus* unable to produce α-amylase was missing the 26-kb plasmid but contained a 20-kb plasmid. A 6-kb deletion within the region of the 5.4-kb *Hin*dIII fragment yielded the 20-kb plasmid unable to code for α-amylase. A nick-translated probe for the α-amylase coding region did not hybridize to either plasmid or total cellular DNA from this mutant strain of *B. stearothermophilus*. These results demonstrate the gene for α-amylase is located exclusively on a 26-kb plasmid in *B. stearothermophilus* with no genetic counterpart present on the chromosome.**

α-Amylase (EC 3.2.1.1) catalyzes the cleavage of the α-1,4-glucosidic linkages between glucose molecules in starch. This hydrolysis of starch is a common first step in conversion of starch into a utilizable substrate for fermentation or for conversion to dextrose and high fructose syrups. α-Amylase producers of commercial importance include *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* (1). *Bacillus stearothermophilus*, a Gram-positive thermophilic bacterium, produces an α-amylase during growth at temperatures of 55–70°C (2, 3). This α-amylase is highly thermostable compared to *B. subtilis* α-amylase (3, 4) and, therefore, it is more desirable for industrial applications of the enzyme. Many genes involved in the synthesis of extracellular α-amylase have been identified in *B. subtilis* (5). The structural gene for the α-amylase enzyme, *amyE*, and its regulatory gene, *amyR*, are closely linked on the genetic map of the *B. subtilis* chromosome (6–8). Furthermore, the gene order surrounding this amylase cistron is similar for three distinct strains of *B. subtilis* (9). The location of the gene for α-amylase from *B. stearothermophilus* has been examined by using recombinant DNA methods. In contrast to the gene for α-amylase from *B. subtilis*, the gene for α-amylase in *B. stearothermophilus* is located on a naturally occurring plasmid.

MATERIALS AND METHODS

Strains and Medium. *B. stearothermophilus* was isolated from a compost pile in Tokyo, Japan. Similar strains can be obtained elsewhere [American Type Culture Collection 31195–31199 (10)]. *Escherichia coli* RR1 and C600 have been described (11). Plasmid pBR322 (12) was obtained from Ray Rodriguez. *B. stearothermophilus* was grown at 56–65°C in a medium containing 0.5% Maltrin 100 (Grain Processing, Muscatine, IA), 0.06% $CaCl_2 \cdot 2H_2O$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.04% KH_2PO_4, 0.025% yeast autolysate Amber BYF (Amber Laboratories, Milwaukee, WI) at pH 7, or tryptose blood agar base (Difco) with 1% Maltrin 100 or 0.25% glucose. Restriction nucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories and used as described by the supplier.

DNA Isolation and Analysis. *B. stearothermophilus* cells were suspended in 12.5% sucrose/25 mM Tris·HCl, pH 8, and incubated with lysozyme (2 mg/ml) for 1 hr on ice. Cells were warmed to room temperature and EDTA was added to 50 mM prior to lysis with 1% $NaDodSO_4$. After 15 min, 1 mg of Pronase (Calbiochem) was added per ml and the mixture was incubated at 37°C for 30 min. The mixture was diluted with 0.25 vol of 10 mM Tris·HCl/1 mM EDTA, pH 7.5, and then extracted twice with phenol. The DNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in 15 mM NaCl/1.5 mM sodium citrate, pH 7.0. The DNA was centrifuged in a 55% (wt/vol) CsCl solution in a Beckman Ti 75 rotor for 24 hr (20°C at 54,000 rpm) followed by dialysis into 10 mM Tris·HCl/1 mM EDTA, pH 7.5.

Plasmid DNA from *B. stearothermophilus* was isolated after treatment with lysozyme, as above, followed by the addition of EDTA (pH 8) to 100 mM, 0.5 vol of 3% Brij 58, 1.2% deoxycholate, and 0.25 vol of 10 mM Tris·HCl/1 mM EDTA, pH 7.5. After complete lysis, plasmid DNA was isolated by CsCl/ethidium bromide centrifugation (13). The fluorescent DNA band was collected and dialyzed into 10 mM Tris·HCl/1 mM EDTA, pH 7.5. *E. coli* plasmid DNA was isolated (14, 15) and transformed into *E. coli* as described (16). Electrophoresis of DNA in gels of agarose has been described (17). The gels were scanned with a Zeineh soft laser scanning densitometer (Biomed). Horizontal gels were run with 2 μg of ethidium bromide per ml in the agarose and buffer. DNA fragments were isolated from agarose with KI gradients as described (18). An [α-^{32}P]CTP-labeled probe (2,000–3,000 Ci/mmol; 1 Ci = 3.7×10^{10} Bq), a gift of R. Coleman, was prepared by nick-translating (19) a portion of the 5.4-kilobase-pair (kb) α-amylase fragment. The probe was hybridized (20) to *Hin*dIII-cleaved DNA fractionated by electrophoresis through a 1% agarose gel and transferred to nitrocellulose (21).

DNA Cloning. *B. stearothermophilus* DNA (3.4 μg) and the vector, pBR322 (0.9 μg), were cleaved with *Hin*dIII, mixed, and ligated. The ligated mixture was transformed into *E. coli* RR1 and transformants were selected on LB plates containing ampicillin (100 μg/ml). The presence of cloned DNA was ver-

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Abbreviation: kb, kilobase pair(s).

ified by replica-plating colonies onto LB plates containing tetracycline (15 μg/ml). Clones expressing α-amylase activity were detected by replica-plating the bacterial colonies onto a plating medium of 0.6% Na_2HPO_4/0.3% KH_2PO_4/0.05% NaCl/0.1% NH_4Cl/0.1% yeast extract (Difco)/1% peptone (Difco)/1.0% Lintner's starch/1.5% agar. Three hours after plating, a 5-ml overlay of plating medium containing about 10^7 bacteriophage T4 or 3 mg of D-cycloserine was added to the plates. After overnight incubation at 37°C, α-amylase zones were detected by addition of a solution of 2.5% Lugol's iodine. Positive colonies were surrounded by a clear zone in the plating medium. The clear zone resulted from α-amylase activity as confirmed by a standard α-amylase assay (22).

RESULTS

Cloning the Gene for α-Amylase. DNA from *B. stearothermophilus* was completely digested with *Hin*dIII and cloned into *E. coli* on plasmid pBR322. About 14,700 ampicillin-resistant colonies of *E. coli* were selected for analysis. Insertion of DNA into the *Hin*dIII site of pBR322 usually inactivates a gene necessary for tetracycline resistance, so the frequency of tetracycline resistance yields an estimate of the number of colonies containing cloned DNA (11). Analysis showed 16% of the ampicillin-resistant transformants were sensitive to tetracycline. Therefore, at least 2,350 of the 14,700 colonies contained *B. stearothermophilus* DNA inserts.

Large-scale screening for α-amylase among the 14,700 colonies relied on detection of a zone of hydrolysis of starch around colonies on appropriate solid medium after iodine staining. This assay was first developed for *Bacillus* that produce extracellular α-amylases. In contrast, *E. coli* K12 produces few extracellular enzymes (23). Therefore, a cloned α-amylase enzyme can only be detected when the *E. coli* cells are lysed. After cell lysis, the released α-amylase causes starch hydrolysis. By using this approach, 18 of the 14,700 colonies were surrounded with large clear zones. These are presumptive α-amylase clones.

Plasmid DNA was isolated from many of the presumptive α-amylase clones to confirm their chimeric plasmids contained the gene for α-amylase. Agarose gel analysis showed most of the colonies examined (10/15) contained a common 9.7-kb plasmid, while five of the strains contained plasmids larger than 9.7 kb (Fig. 1*A*). The size of the DNA insert was determined by digestion with *Hin*dIII restriction endonuclease. All clones contained a common 5.4-kb fragment of DNA, including the five larger plasmids. The genetic nature of the extra fragments of DNA in the five larger plasmids is not known. They may contain genetically linked genes or be the result of ligation of other *Hin*dIII fragments of DNA. Nevertheless, they are not essential for the α-amylase function. To further demonstrate the α-amylase gene was on pBR322, chimeric plasmids from five clones were extracted and transformed into two strains of *E. coli*, RR1 and C600. The DNA was free of viable cells, so any α-amylase activity must have resulted from a transfer of the chimeric plasmid. There was a 100% correlation of growth of the resulting transformants on ampicillin and the production of α-amylase in both strains of *E. coli*. Analysis of plasmid DNA from these transformants confirmed they contained the same size of plasmids as that of the donor strain.

The cloning experiment with *B. stearothermophilus* DNA in *E. coli* produced a larger number of α-amylase-producing clones than expected. From theoretical considerations of the size of the *Hin*dIII fragments of *B. stearothermophilus* DNA relative to the genome size *Bacillus*, only about 0.1% of the *E. coli* transformants should contain the gene for α-amylase if it is present as a single copy on the chromosome. However, 0.8%

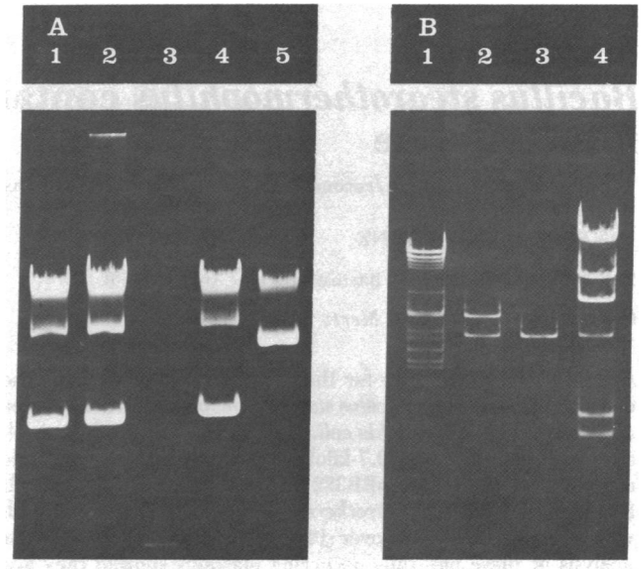

FIG. 1. Analysis of chimeric plasmids containing the gene for α-amylase. (*A*) Plasmids partially purified from four α-amylase-producing colonies of *E. coli* were separated by gel electrophoresis on 1% agarose. Lanes 1, 2, and 4, a 9.7-kb plasmid; lane 5, a larger chimeric plasmid containing the 5.4-kb *Hin*dIII fragment along with other unrelated cloned fragments; lane 3, purified pBR322. (*B*) Chimeric α-amylase plasmid and control DNAs digested by restriction enzyme *Hin*dIII and separated on a 1% agarose gel. Lane 1, plasmid DNA from *B. stearothermophilus*; lane 2, 9.7-kb chimeric α-amylase plasmid; lane 3, pBR322; lane 4, bacteriophage λ DNA.

of the recombinant colonies were shown to express the α-amylase gene. This suggests that the gene coding for α-amylase is present in about eight copies per chromosome equivalent in *B. stearothermophilus*. Cryptic plasmids had been found in *B. stearothermophilus* (S. Mickel, personal communication). These plasmids may be the source of the gene amplification, so the plasmids of *B. stearothermophilus* were examined.

Cleavage of these plasmids with endonuclease *Hin*dIII yielded many fragments, one of which was the same size as the cloned DNA containing the gene for α-amylase (Fig. 1*B*). To test if the plasmids contained the gene for α-amylase, plasmid DNA isolated from *B. stearothermophilus* was cloned into *E. coli* on pBR322 and ampicillin-resistant transformants were analyzed for their resistance to tetracycline. By using the starch-plate screening procedure as described above, 11% of the colonies that contained recombinant DNA had α-amylase activity. Analysis of their recombinant plasmids showed that they were also 9.7 kb in size. Moreover, the observed frequency of positive α-amylase colonies is consistent with the gene for α-amylase being located on one of the readily visible fragments of DNA produced by *Hin*dIII cleavage of the *B. stearothermophilus* plasmids. Taken together, these results verify the plasmid-borne nature of a gene for α-amylase in *B. stearothermophilus*.

Identification of the α-Amylase Plasmid. *B. stearothermophilus* contains multiple plasmids, so further analysis was needed to determine which plasmids contained the gene for α-amylase. Large quantities of plasmid from *B. stearothermophilus* were isolated and analyzed on a 0.5% horizontal agarose gel. Four plasmids were identified and their molecular weights were estimated relative to standards. Plasmids of 12, 15, 26, and >56 kb were present in differing amounts. Each plasmid was isolated by preparative horizontal gels and their purity was verified by analytical gel electrophoresis. These plasmids were digested with *Hin*dIII and compared to the total digest of the mixture of *B. stearothermophilus* plasmids (Fig. 2). The 12-kb

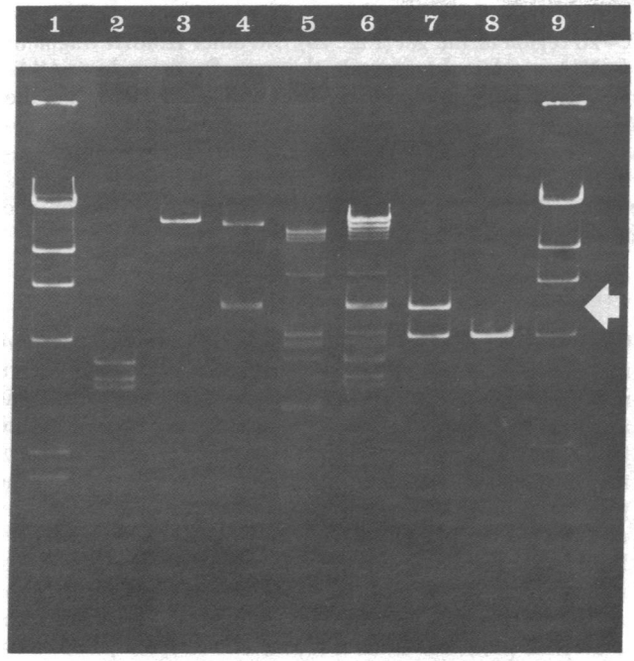

FIG. 2. Restriction enzyme digestion of isolated plasmids from *B. stearothermophilus*. All of the DNAs were digested with enzyme *Hin*dIII and separated on 1% agarose. Lanes 1 and 9, bacteriophage λ DNA; lane 2, 12-kb plasmid; lane 3, 15-kb plasmid; lane 4, 26-kb plasmid; lane 5, 108-kb plasmid; lane 6, all four plasmids; lane 7, chimeric α-amylase plasmid from *E. coli*; lane 8, pBR322. Arrow points to the 5.4-kb fragment of DNA that was cloned in *E. coli*.

plasmid generated five fragments between 3.8 and 0.7 kb, whereas the 15-kb plasmid was cut once (Table 1). The 26-kb plasmid contained four *Hin*dIII fragments, two of which are 5.4 kb. These two bands comigrated with the cloned fragment of DNA that is known to produce α-amylase in *E. coli*. The largest plasmid yielded a large number of fragments after digestion with *Hin*dIII, none of which was 5.4 kb in size. The sum of the sizes of these fragments was about 108 kb. Furthermore, each of the *Hin*dIII-generated fragments found in the composite plasmid preparation can be assigned to each of the individual plasmids (Fig. 2). These results suggest the 26-kb plasmid contains the gene for α-amylase in *B. stearothermophilus*.

The presence of the gene for α-amylase on the 26-kb plasmid

Table 1. Sizes of *Hin*dIII fragments of *B. stearothermophilus* plasmids

Fragment size, kb*			
12-kb plasmid	15-kb plasmid	26-kb plasmid	108-kb plasmid
3.8, 3.4, 3.3,	15.0	14.5	12.5, 12.5†
1.2, 0.7		5.4, 5.4†	11.5, 11.0, 7.0
12.4		0.9	4.4, 4.4†
		26.2	4.1, 4.1†
			3.7, 3.3
			2.8, 2.8, 2.8, 2.8†
			2.5, 2.1, 2.0
			1.8, 1.8, 1.8, 1.8†
			1.6, 1.4, 1.0, 0.9
			108.5

* As determined by agarose gel electrophoresis with fragments of λ DNA used as standards.

† The presence of comigrating fragments was confirmed by densitometer traces.

Table 2. Cloning of individual plasmids from *B. stearothermophilus*

Plasmid	*Hin*dIII fragments, no.	Recombinants, no.	α-Amylase producers, no.
12-kb	5	36	0
15-kb	1	8	0
26-kb	4	40	24
108-kb	26	74	0
Amylase-negative*	35	601	0

The number of *Hin*dIII fragments is from Table 1. A recombinant is a colony of *E. coli* that grew in the presence of ampicillin but failed to grow in the presence of tetracycline. α-Amylase producers are colonies that produce a characteristic clear zone on a starch medium after lysing and staining the colonies (see text). All α-amylase-producing colonies were also sensitive to tetracycline.

* Plasmids isolated from the strain of *B. stearothermophilus* that failed to produce α-amylase.

was confirmed by shotgun cloning each isolated *B. stearothermophilus* plasmid into *E. coli* on pBR322 by using standard methods. Colonies resistant to ampicillin were screened for resistance to tetracycline and α-amylase production (Table 2). The only *B. stearothermophilus* plasmid that generated α-amylase-producing *E. coli* was the 26-kb plasmid. The 26 kb-plasmid yielded 24 α-amylase-producing *E. coli*. Plasmid screens showed only one of the two 5.4-kb fragments contains the gene for α-amylase (data not shown). The 12-, 15-, and about 108-kb plasmids did not supply the gene for α-amylase, even though each *Hin*dIII fragment should have been represented three or more times among the recombinant colonies. Again, the only plasmid that contains the gene for α-amylase among the four identified from *B. stearothermophilus* was the 26-kb plasmid.

Lack of a Chromosomal Gene for α-Amylase. Simultaneous loss of a suspected plasmid function and alteration or elimination of a plasmid supplies strong evidence the gene in question is plasmid-borne. Such a correlation was determined after analysis of the plasmids present in a mutant strain of *B. stearothermophilus* that was unable to produce α-amylase (amylase-negative). The mutant strain was isolated by growing *B. stearothermophilus* on a rich liquid medium containing glucose followed by plating on the same medium. About 1% (1/110) of the colonies did not produce α-amylase. The strain was not defective in α-amylase transport since lysis of the cells failed to liberate intracellular enzyme activity. Furthermore, the mutant strain was confirmed to be *B. stearothermophilus* by biochemical tests (24) and analysis of the chromosomal DNA (25).

Examination of the plasmids present in this strain determined that only the 26-kb plasmid was missing. The 12-, 15-, and 108-kb plasmids were present and accompanied by a 20-kb plasmid (Fig. 3A). As a further test, plasmid preparations isolated from the α-amylase-proficient and -deficient strains of *B. stearothermophilus* were cleaved with endonuclease *Hin*dIII. The resultant fragments of DNA were analyzed by agarose gel electrophoresis. The pattern of DNA fragments was identical for the two plasmid preparations, except the 5.4-kb fragments were missing for the plasmids from the α-amylase-negative strain of *B. stearothermophilus* (Fig. 3B). In their place was a single 5.0-kb fragment of DNA. Both patterns of fragments contained the 14.5-kb *Hin*dIII fragment that comprises the majority of the 26-kb plasmid (Fig. 2). Apparently, a 6-kb segment of the α-amylase-coding region of the 26-kb plasmid was spontaneously lost, yielding an otherwise identical 20-kb plasmid unable to code for α-amylase. The lack of an α-amylase gene on the plasmids from the α-amylase-negative *B. stearothermophilus* was further confirmed by shotgun cloning the plasmids into *E. coli* on pBR322. Over 600 recombinants were obtained

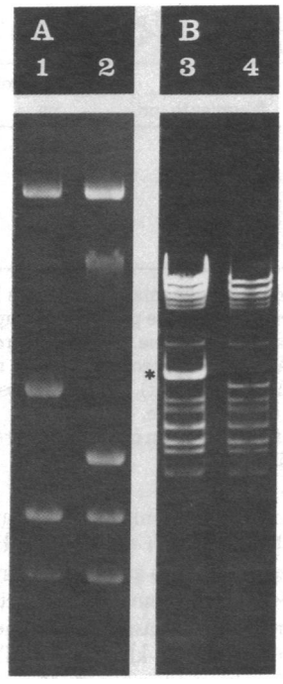

FIG. 3. Amylase-negative plasmid from *B. stearothermophilus*. (*A*) Purified plasmids from *B. stearothermophilus* strains; (*B*) digestion of purified plasmids from *B. stearothermophilus* strains with enzyme *Hin*dIII. Lanes 1 and 3, plasmids from an α-amylase-producing strain; lanes 2 and 4, plasmids from a strain unable to produce α-amylase. The asterisk indicates location of the 5.4-kb α-amylase fragment of DNA.

and none produced α-amylase (Table 2).

Finally, the possibility that there is a separate gene for α-amylase on the chromosome was addressed by hybridization of part of the 5.4-kb α-amylase DNA to *Hin*dIII-digested total and plasmid DNA from the wild-type and mutant strain of *B. stearothermophilus* (Fig. 4). The hybridization probe was a 1.7-kb *Bcl* I fragment located within the coding region for the α-amylase protein (ref. 26 and data not shown). This probe only hybridized to the DNA from the α-amylase-proficient strain at the 5.4-kb region of the gel (Fig. 4*B*). No other *Hin*dIII fragment of DNA was found to contain the gene for α-amylase. Additionally, no hybridization to the total or plasmid DNA from the mutant strain was detected, even after extended autoradiographic exposure (120 hr). Taken together, these results demonstrate the gene for α-amylase is present exclusively on a plasmid in *B. stearothermophilus* with no gene located on the chromosome.

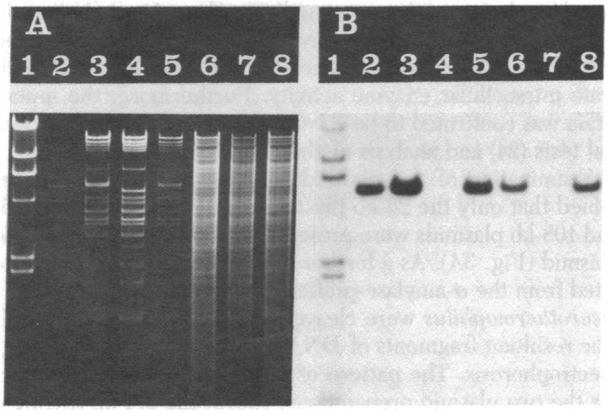

FIG. 4. Hybridization analysis of *B. stearothermophilus* plasmid and chromosomal DNA. All of the DNAs were digested with enzyme *Hin*dIII and separated on 1% agarose. (*A*) The pattern of DNA in the gel; (*B*) 48-hr autoradiograph of Southern-hybridized α-amylase DNA. Lane 1, bacteriophage λ DNA; lane 2, chimeric α-amylase plasmid from *E. coli*; lanes 3 and 5, plasmids from the α-amylase-producing strain; lane 4, plasmids from the strain of *B. stearothermophilus* unable to produce α-amylase; lanes 6 and 8, total cellular DNA from the α-amylase-producing strain; lane 7, total cellular DNA from the strain of *B. stearothermophilus* unable to produce α-amylase. The bacteriophage λ DNA in lane 1 in *B* was terminally labeled with [γ-^{32}P]ATP after *Hin*dIII cleavage.

DISCUSSION

Cloning of the gene for α-amylase from *B. stearothermophilus* into *E. coli* provided frequency data that suggested there were multiple copies of the gene in the *Bacillus*. Subsequent analysis showed the elevated frequency of the cloning event was due to the plasmid-borne genes for the α-amylase protein. *B. stearothermophilus* contains four plasmids, but only one codes for the gene for α-amylase. The role of the other plasmids and the remainder of the 26-kb plasmid remains unknown. Plasmids have been found in *B. stearothermophilus* that code for resistance to streptomycin and tetracycline (27), but they are smaller than any of the plasmids described in this paper. Furthermore, the α-amylase-proficient strain is sensitive to streptomycin and tetracycline at 2 and 0.2 μg/ml, respectively (data not shown), so segments of the *B. stearothermophilus* plasmid coding for resistance to streptomycin or tetracycline are not present in this strain of *B. stearothermophilus*. The presence of a plasmid-borne gene for α-amylase did not preclude the existence of a chromosomal gene for α-amylase in *B. stearothermophilus*. Presence of a plasmid-borne and chromosomally located gene for α-amylase could allow the *Bacillus* to overproduce an essential gene under proper conditions while also maintaining the gene on a genetically stable element, the chromosome. This is apparently not the case because the α-amylase-negative *B. stearothermophilus* was readily isolated and resulted from a plasmid deletion. The hybridization experiment confirmed that the loss of α-amylase activity in the mutant strain resulted from the elimination of the α-amylase coding region. No additional unexpressed α-amylase gene was present in the chromosome. Therefore, the lack of α-amylase synthesis was not due to the simultaneous loss of a plasmid-borne α-amylase gene and a *trans*-acting regulatory element for a chromosomal α-amylase gene. The α-amylase mutant was obtained at a higher frequency (*ca.* 1%) than expected for a spontaneous deletion. This elevated rate suggests the involvement of inverted repeat sequences (28). The α-amylase deletion observed here may represent the transposition of the α-amylase gene (Tn*amyl*) out of the plasmid.

Existence of a naturally occurring plasmid-borne gene for α-amylase joins a rapidly growing list of "accessory" genes that are present on plasmids. Other plasmid-borne genes include the genes for nitrogen fixation (*nif*) and nodulation proficiency (*Nod*$^+$) in fast-growing *Rhizobium* species (29–31), genes for tumor induction in the plant pathogen *Agrobacterium tumefaciens* (32, 33), genes for crystal toxin in the insect pathogen *Bacillus thuringenisis* (34), and genes for hydrogenase in autotrophic bacterium *Alcaligenes eutrophus* (35). Paradoxically, these genes could be considered expendable due to their localization on plasmids; yet they represent genes that in many ways are synonymous with the ecological niche the bacterium occupies. Such is the nature of the α-amylase gene from *B. stearothermophilus*, a bacterium isolated from a compost pile in Japan. Rapid fermentation within compost generates ample heat, thus providing an environment ideal for a thermophilic bacterium that produces a thermostable α-amylase. Loss of the plasmid responsible for the thermostable α-amylase should represent a

significant enough handicap to the bacterium to expect, teleologically, that the gene for α-amylase be an integral part of the chromosome or at least located on a megaplasmid (>450 kb) similar to the plasmids that contain the *nif* and *Nod*$^+$ genes in *Rhizobium* (29–31). Alternatively, timely increases in the copy number of plasmids containing genes important to the survival within an ecological niche could allow a bacterium to increase key gene products by amplification of gene copy numbers as well as by increasing rates of transcription and translation.

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