Cloning and expression of an α -amylase gene from *Streptomyces thermoviolaceus* CUB74 in *Escherichia coli* JM107 and *S. lividans* TK24

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A gene coding for a thermostable extracellular α -amylase, carried by a 5.7 kb *Bam*HI chromosomal DNA fragment isolated from *Streptomyces thermoviolaceus* strain CUB74, was cloned into *Escherichia coli* JM107 using, as a cloning vector, the high-copy-number plasmid pUC8. *E. coli* containing a recombinant plasmid pQR300 expressed the amylase gene and exported the enzyme into the periplasmic space and the culture medium. The amylase protein expressed by *E. coli* had the same molecular mass (50 kDa) as that expressed by the *Streptomyces* parent strain, which suggests that the enzyme is processed similarly by both strains. The amylase gene was also cloned into *Streptomyces lividans* TK24 using pIJ702 as vector. The enzyme was stable at 70 °C when CaCl₂ was present.

Introduction

α -Amylases are secreted endoenzymes that randomly cleave α (1,4) linkages in starch, generating shorter chains of glucose units which can eventually be taken up and utilized by many organisms. α -Amylase is found widely amongst *Streptomyces* species. Recently, α -amylases have been isolated and characterized from *Streptomyces hygroscopicus*, *S. venezuelae*, and *S. limosus*. *S. hygroscopicus* SF-1084 produces an extracellular α -amylase which is used industrially to hydrolyse starch to maltose. The gene specifying the enzyme has been isolated and sequenced (Hoshiko *et al.*, 1987; McKillop *et al.*, 1986). The amylase gene of *S. limosus* has also been isolated and sequenced. Its amino acid sequence showed regions with up to 80% homology to mammalian and invertebrate α -amylases (Long *et al.*, 1987) but not to other bacterial, fungal or plant α -amylases. The gene for an α -amylase from *S. venezuelae* has recently been cloned and characterized (Virolle *et al.*, 1988). The predicted amino acid sequence of this enzyme shows 75% identity with the *S. limosus* α -amylase.

High hydrolysis temperatures are desirable in the starch industry because starch granules cannot be attacked by α -amylase unless they have been ruptured by heat (gelatinization). Since the enzymes of thermophilic bacteria are generally heat-stable (Ljungdahl, 1979), we are investigating amylases produced by thermophilic *Streptomyces* strains.

Cloning and expression of *Streptomyces* genes have been mostly carried out within *Streptomyces* (Bibb *et al.*, 1983). However, a few examples of expression of cloned *Streptomyces* genes in *Escherichia coli* have been reported. These include genes for antibiotic resistance (e.g. Vara *et al.*, 1985; Gil *et al.*, 1985; Schupp *et al.*, 1983), a gene for an extracellular endoglycosidase H (Robbins *et al.*, 1981), and a few examples of genes for biosynthetic enzymes (Meade, 1985; Hercomb *et al.*, 1987).

In this paper, we report the isolation of an α -amylase gene from a thermophilic streptomycete, *S. thermoviolaceus* strain CUB74, and its cloning and expression in *E. coli* and *S. lividans*.

Methods

Bacterial strains and plasmids. The characteristics of *E. coli* JM107 and *S. lividans* TK24 are shown in Table 1. *S. thermoviolaceus* strain CUB74 was obtained from Dr A. McCarthy (University of Liverpool, UK). Plasmids pUC8, 18 and 19 were used for cloning in *E. coli* whereas pQR1 (R. Barallon & J. M. Ward, unpublished) and pIJ903 (Lydiat *et al.*, 1985) were used as shuttle vectors for cloning in both *E. coli* and *Streptomyces* strains. pQR1 was constructed by ligating pBR325 and pIJ702 that had been digested with *Pst*I. pIJ702 was also used for cloning in *S. lividans*.

Culture media and antibiotics. *E. coli* strains were grown at 37 °C in nutrient broth (Oxoid) or on nutrient agar (Oxoid nutrient broth solidified with 2%, w/v; Difco agar); both media were supplemented with ampicillin (500 µg ml⁻¹), IPTG (40 µg ml⁻¹), X-gal (80 µg ml⁻¹) and potato starch (1%, w/v; Sigma, type S-4251) when necessary. *S.*

Table 1. *Bacteria and plasmids*

Bacterial strain or plasmid	Genotype or relevant characteristics	Reference
<i>E. coli</i> JM107	<i>endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB) (F' traD36, proAB, lacI^q ZΔM15)</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>S. lividans</i> TK24	Str-6, SLP2 ⁻ SLP3 ⁻	Hopwood <i>et al.</i> (1983)
pUC8, pUC18, pUC19	Ap ^r , <i>lacZα</i>	Vieira & Messing (1982)
pQR1	pIJ702/pBR325 hybrid; Ts ^r Cm ^r Tc ^r	R. Barallon & J. M. Ward, unpublished
pIJ702	Ts ^r Mel ^r	Katz <i>et al.</i> (1983)
pIJ903	Ts ^r Ap ^r	Lydiat <i>et al.</i> (1985)

thermoviolaceus CUB74 was grown at 50 °C in half-strength nutrient broth or on half-strength nutrient agar; *S. lividans* TK24 was grown on R2YE (solid) or YEME (liquid) media with and without sucrose (Hopwood *et al.*, 1985). These media were supplemented with 1% (w/v) potato starch in order to visualize any amylase activity on plates, and thioestrepton (50 µg ml⁻¹) was added when required.

Isolation of total DNA and plasmids. Total DNA was prepared by a slightly modified method of that described by Chater *et al.* (1982). Mycelia, harvested from an overnight liquid culture of CUB74, were resuspended in 20 ml Birnboim buffer (25 mM-Tris/HCl, pH 7.5, 10 mM-Na₂EDTA, 0.9%, w/v, glucose, 2 mg lysozyme ml⁻¹). After adding SDS to a final concentration of 1% (w/v), 4 ml pronase solution (10 mg ml⁻¹; pre-self-digested at 37 °C for 2 h) was mixed with the preparation. This was then mixed with 4 ml TE buffer (10 mM-Tris/HCl, pH 7.5, 1 mM-EDTA) plus RNAase (20 µg ml⁻¹) and incubated at 37 °C for 1 h. Then 5 ml of the same buffer was added and incubation was continued for a further 2 h at the same temperature. Finally, 1 ml 5 M-NaCl was added, 20 ml of ethanol was layered on and the DNA was spooled with a flame-blunted Pasteur pipette. The DNA was resuspended in 18 ml TE buffer and purified on a caesium chloride/ethidium bromide gradient if necessary.

Plasmid isolation on a large scale was done by the method of Bibb *et al.* (1977). Small-scale preparation of plasmid DNA was performed as described by Birnboim & Doly (1979).

Cloning and expression of the amylase gene in *E. coli*. All restriction endonuclease digestions and ligations were performed as described by Maniatis *et al.* (1982). Agarose gels (1%, w/v) were used to separate the DNA bands.

Chromosomal DNA of *S. thermoviolaceus* CUB74 was digested to completion with *Bam*HI and ligated to pUC8 that had been cut with the same restriction enzyme. The genomic library was then used to transform competent *E. coli* JM107. All ampicillin-resistant (Ap^r) colonies obtained were replicated on to starch plates using sterile velvet pads.

Restriction endonuclease mapping and subcloning. The following restriction endonucleases were used: *Eco*RI, *Hind*III, *Bam*HI, *Pst*I, *Bgl*II, *Xho*I, *Sph*I, *Kpn*I, *Sma*I, *Sal*GI, *Bcl*I and *Sac*I. λ DNA digested with *Hind*III was used as a size marker. The map of the insert is shown in Fig. 1. In order to locate the amylase gene (*amy*) on the insert, pQR300 (pUC8 plus insert) was double digested to completion with *Bam*HI/*Bgl*II, extracted with phenol/chloroform and then religated. For the same purpose, the 1.7 kb *Bam*HI-*Sph*I fragment was extracted from an agarose gel (Girvitz *et al.*, 1980) and cloned into pUC18 that had been cut with the same restriction enzymes. In addition, and in order to determine whether the amylase gene is expressed from the *lacZ* promoter on pUC8 or from its own promoter, cloning of the whole

insert in the opposite orientation to the *lacZ* promoter was performed by digesting pQR300 with *Bam*HI and religating it. The orientation of the insert was determined by isolating and mapping plasmids from both amylase-negative and amylase-positive colonies. For the same purpose, the whole insert was subcloned in the *Bgl*II site of pQR1, which lacks the *lacZ* promoter.

For the cloning and expression in *S. lividans*, the amylase gene carried by the 5.7 kb *Bam*HI fragment was cloned into the shuttle vector pQR1 that had been cut with *Bgl*II. Recombinant plasmids (pQR308 and pQR309) were isolated from *E. coli* and used to transform *S. lividans*. The high-copy-number streptomycete vector pIJ702 was also used for subcloning. The plasmid was cut with *Bgl*II and *Sph*I and ligated to the 1.7 kb *Bam*HI-*Sph*I DNA fragment carrying the amylase gene. The recombinant plasmid construct obtained was called pQR311. The low-copy-number plasmid pIJ903 was also used for cloning in both *E. coli* and *S. lividans*. A 1.7 kb *Bam*HI-*Hind*III DNA fragment from pQR307, generated by partial *Hind*III digestion and complete *Bam*HI digestion, was inserted between the *Bam*HI and *Hind*III sites of pIJ903. The resulting plasmid was called pQR319.

Preparation of *S. lividans* protoplasts and their transformation were performed as described by Hopwood *et al.* (1985).

Preparation of enzyme, protein determination and SDS-PAGE. Total extracellular enzymes (concentrated 100-fold) were prepared from *E. coli* and *Streptomyces* cultures by precipitating with ammonium sulphate (90% saturation). The precipitate was resuspended in 20 mM-Tris/HCl, pH 7.5, 5 mM CaCl₂, dialysed twice (1 h each) against the same buffer at room temperature and once overnight against the same buffer containing 50% (v/v) glycerol at 4 °C, and finally stored at -20 °C.

The Bio-Rad Protein Assay was used to determine the protein concentration as described by the supplier. Approximately 80–100 µg of protein was subjected to 10% SDS-PAGE (Fig. 2) for molecular mass determination (Laemmli, 1970). The proteins were denatured by mixing with 50 µl loading buffer (5%, v/v, mercaptoethanol, 3.4%, w/v, SDS, 15%, v/v, glycerol, 0.01%, w/v, bromophenol blue, 47 mM-Tris/HCl, pH 6.8) and boiling at 100 °C for 3 min, and were run in duplicate. They were renatured by washing half the gel twice with 2.5% (w/v) Triton X-100, 1 h each, and then rinsing it in 20 mM-Tris/HCl, pH 7.5, 5 mM-CaCl₂ for 25 min at room temperature. The position of the enzyme activity was located by sandwiching the reactivated gel against a 1% (w/v) agarose gel made in the Tris/HCl buffer and containing 0.5% potato starch. The sandwich was incubated overnight at 37 °C between two glass plates and under a weight. Starch hydrolysis zones were detected by soaking the agarose gel in 0.05% (w/v) I₂, 2.65% (w/v) KI (Siggins, 1987). The other half of the protein gel also contained molecular mass markers (from Sigma) and was stained with Coomassie Brilliant Blue R.

Amylase assay and paper chromatography. Enzyme units were calculated by carrying out a modified Somogyi assay (Nelson, 1944) in which 500 μ l of an enzyme preparation was mixed with 500 μ l of 1% (w/v) soluble starch (in the same Tris/HCl buffer plus 5 mM-CaCl₂). After 1 h incubation at 50 °C, 100 μ l was removed from the mixture and added to 900 μ l of distilled water. Then 1 ml of a mixture of reagent A and reagent B as specified by Nelson (1944) was added and mixed. The mixture was heated at 100 °C for 20 min, cooled and 1 ml of the arsenomolybdate reagent was added. After complete evolution of CO₂, the mixture was added to 10 ml distilled water and the absorbance read at 600 nm. One unit of amylase was defined as the amount of enzyme which releases 1 mg reducing sugar from soluble starch in 1 h at 50 °C and pH 7.5. A standard curve was made using maltose.

The product of starch hydrolysis by amylase was analysed by paper chromatography using 2-propanol/water (7:3, v/v) as solvent and silver nitrate as a stain (Robyt & French, 1963).

Results

Cloning and expression of the amylase gene in *E. coli*

Transformation of *E. coli* JM107 by the *Bam*HI library of *S. thermophilaceus* CUB74 DNA in pUC8 resulted in more than 10000 Ap^r colonies, 64% of which were colourless on X-gal plates and presumed to be recombinants. After replicating on starch plates, one colony showed extensive amylase activity and a zone of starch hydrolysis was observed with or without iodine staining. This amylase-positive colony was found to have a recombinant plasmid, pQR300, containing an insert of 5.7 kb (Fig. 1). pQR300 was used to transform *E. coli* and 100% of the colonies obtained were amylase positive. On starch plates, pUC8-containing *E. coli* colonies (control) produced no hydrolysis zones. Approximately 40% of the amylase activity expressed by *E. coli* JM107(pQR300) was present in the culture supernatant (extracellular fraction); the remainder (60%) was located in the periplasm/cytoplasmic fraction (Table 2). Alkaline phosphatase was used as a periplasmic marker.

Surprisingly, IPTG, added to liquid medium, induced the amylase production by *E. coli* JM107(pQR300) but not substantially (by 16% only); on plates, larger hydrolysis zones were obtained in the presence of IPTG. Transformation of *E. coli* JM107 with pQR303 (Fig. 1), where the 5.7 kb *Bam*HI fragment was cloned in the opposite orientation to the *lacZ* promoter, yielded amylase-negative colonies only. Furthermore, cloning of the 5.7 kb *Bam*HI fragment into the *Bgl*II site of pQR1 to give the plasmids pQR308 and pQR309 resulted in amylase-negative *E. coli* colonies. The *Bgl*II site is in the *mel* operon, which is not expressed in *E. coli*. It was therefore concluded that the expression of the amylase gene was from the *lacZ* promoter in the pUC8 derivatives and not from its own promoter. The low level of induction by IPTG may be explained by the high copy

Fig. 1. Restriction enzyme map of pQR300 and plasmids obtained from subcloning experiments. The construction of these plasmids is described in Methods. The arrows represent the orientation of the amylase gene (*amy*) in relation to the *lacZ* promoter P_{lacZ} on pUC8 and pUC18, the *mel* promoter (P_{mel}) on pQR1 and pIJ702 (in the case of pQR308, pQR309 and pQR311), and the ampicillin promoter (P_{amp}) on pIJ903 (in the case of pQR319). B, *Bam*HI; P, *Pst*I; Bg, *Bgl*II; S, *Sph*I; H, *Hind*III.

number of the plasmid, which titrates out a proportion of the *lacI* repressor even in strains containing the *lacI*^a allele (Stewart *et al.*, 1986).

The *Streptomyces* origin of the amylase gene was confirmed by probing a *Bam*HI bulk digestion of *S. thermophilaceus* CUB74 total DNA (Southern, 1975) with radioactively labelled pQR300 (data not shown). A single 5.7 kb *Bam*HI fragment of CUB74 showed homology with the probe.

Mapping and subcloning

The map of the insert and the plasmids resulting from subcloning are shown in Fig. 1. It was found that the amylase gene is carried by a 1.7 kb *Bam*HI–*Sph*I fragment (pQR307) and starts from the side of the *Bam*HI site as deduced from the lack of α -amylase expression from *E. coli* harbouring pQR303. Plasmids

Fig. 2. SDS-PAGE. Approximately 80 µg of *E. coli* and *S. thermoviolaceus* CUB74 crude supernatants (concentrated 100-fold) and 100 µg of *S. lividans* supernatants were loaded on the gel. Protein samples were run in duplicate on the same gel. An agarose gel containing potato starch was overlaid on the reactivated half of the protein gel and stained with iodine as described in Methods. The other half of the gel, which also contained molecular mass markers, was stained with Coomassie Brilliant Blue R. (a) (Activity stain only): A, *E. coli* JM107(pQR300); B, *E. coli* JM107(pUC8) (control); C, *S. thermoviolaceus* CUB74. (b) A, *S. thermoviolaceus* CUB74, B, *S. lividans* TK24(pIJ702) (control); C, *S. lividans* TK24(pQR311). The molecular mass markers were bovine serum albumin (66 kDa), egg albumin (45 kDa) glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and trypsinogen, PMSF treated (24 kDa).

Table 2. Localization of amylase expressed by *E. coli* JM107(pQR300)

E. coli JM107(pQR300) was grown in the low-phosphate medium of Neu & Heppel (1964) to an OD₄₅₀ of 1.0 and fractionated by the method of Minton *et al.* (1983). Alkaline phosphatase was assayed by the method of Torriani (1966); amylase was assayed as described in Methods. This experiment was repeated three times and the results obtained were consistent, varying by only 3–5%.

Fraction	Percentage of enzyme activity	
	Alkaline phosphatase	Amylase
Supernatant	9	43.2
Periplasm/cytoplasm	91	56.8
Membrane bound	0	0

pQR308 and pQR309 isolated from *E. coli* were used to transform *S. lividans* TK24 and thiostrepton-resistant (Ts^r) colonies were selected. These plasmid constructs

(pQR308 and 309) were extremely unstable in *S. lividans*. They rearranged and lost the amylase gene.

Cloning in *S. lividans* was successful when the *Bam*HI–*Sph*I fragment carrying the amylase gene was inserted into the high-copy-number plasmid pIJ702 which had been cut with *Bgl*II/*Sph*I and *Mel*I–Ts^r colonies were selected for. The recombinant plasmid was designated pQR311. Large amylase zones were produced by *S. lividans* TK24(pQR311) colonies whereas the control, TK24(pIJ702), produced small zones. The 1.7 kb *Bam*HI–*Sph*I fragment was also subcloned into the low-copy-number plasmid pIJ903 and the recombinant plasmid (pQR319) was introduced to *E. coli* and *S. lividans*. *E. coli* colonies containing this construct were amylase negative whereas *S. lividans* containing the construct produced large amylase zones on starch plates.

SDS-PAGE and characterization of amylase

Concentrated crude culture supernatants of *E. coli* and *S. thermoviolaceus* CUB74 were analysed on the same

gel (Fig. 2a). *E. coli* JM107(pQR300) (lane A) and *S. thermoviolaceus* CUB74 (lane C) showed a starch-degrading activity corresponding to a protein of the same size (50 kDa) after renaturation of the proteins in the SDS polyacrylamide gel. This similarity in molecular mass suggests that *E. coli* can correctly process the amylase protein to the form found in the parent *Streptomyces* strain. Coomassie staining of protein bands in the gel (results not shown) revealed comigration of the amylases from *E. coli* JM107(pQR300) and *S. thermoviolaceus* CUB74, showing that the processing of the signal peptide by *E. coli* could differ by no more than a few amino acids. This preliminary result is currently being resolved by sequencing the N-terminus of both proteins. There was no detectable amylase activity from the pUC8-containing *E. coli* control (Fig. 2a, lane B).

The amylase produced by pQR311-containing *S. lividans* also had the same size as that of CUB74, and no amylase activity was detectable in the reactivated gel system from pIJ702-containing *S. lividans* (Fig. 2b).

Paper chromatography analysis

Paper chromatography of starch digestion products showed that maltose was the major end-product and started to appear as a distinct spot at 1.5 h. Glucose, however, appeared in small amounts at 8 h. From this result, it was concluded that the enzyme is an α -amylase as the major products are maltotetraose, maltotriose and maltose, with maltose accumulating over extended periods while maltotetraose disappears.

Fig. 3. Effect of Ca^{2+} on heat stability of the enzyme. Cultures (5 ml) of *E. coli* JM107(pQR300) were grown overnight at 37 °C in nutrient broth with and without 5 mM- CaCl_2 . Samples (500 μl) of their supernatants were preincubated at 50 or 60 °C for the time indicated and then assayed at 50 °C. The 1% soluble starch solution used in this assay was made up in 20 mM-Tris/HCl pH 7.5 buffer plus 5 mM- CaCl_2 . 1, In the absence of CaCl_2 and at 60 °C; 2, in the absence of CaCl_2 and at 50 °C; 3, in the presence of CaCl_2 and at 60 °C; 4, in the presence of CaCl_2 and at 50 °C. These experiments have been repeated and the results are reproducible.

Temperature stability

The temperature stability of the enzyme was examined in the presence and absence of CaCl_2 by preincubating in the absence of substrate. In the presence of 5 mM- CaCl_2 , the amylase secreted by *E. coli* JM107(pQR300) retained

Table 3. Effect of temperature on enzyme activity

E. coli JM107(pQR300) was grown with and without 5 mM- CaCl_2 overnight at 37 °C. Samples (500 μl) of each culture supernatant were preincubated at the temperature indicated for 1 h and then assayed at 50 °C as described in Methods. The 1% soluble starch used in the enzyme assay was made up in 20 mM-Tris/HCl buffer pH 7.5 plus 5 mM- CaCl_2 . This experiment has been repeated three times and the results were within 4% of those shown below.

Temp. (°C)	Minus CaCl_2		Plus CaCl_2	
	Activity (U ml ⁻¹)	Percentage of max. activity	Activity (U ml ⁻¹)	Percentage of max. activity
4	3.62	88	2.5	88
37	4.1	100	2.84	100
50	3.64	89	2.58	90
60	0.62	15	2.52	89
70	0.52	13	2	70
80	0.1	2	0.3	11

90% of its activity at 60 °C and 70% of its activity at 70 °C after preincubation for 1 h at these temperatures (Table 3). Most of this activity was abolished above 80 °C. In the absence of CaCl₂, the amylase activity rapidly fell at temperatures above 50 °C. Fig. 3 shows that in the absence of CaCl₂, the enzyme lost 30% of its activity after 8 h incubation at 50 °C and it was totally inactive after 22 h at 50 °C and 2 h at 60 °C. It also shows that in presence of CaCl₂, the enzyme was 100% stable after 22 h incubation at 50 °C and 8 h at 60 °C.

Discussion

The α -amylase gene of *S. thermophilaceus* CUB74 has been cloned and expressed in *E. coli* and more than 40% of its product was secreted into the medium. It is the exception rather than the rule for enzymes which are extracellular in their original Gram-positive host to be exported into the culture medium when cloned in *E. coli*. In most cases, Gram-positive extracellular enzymes are periplasmic when expressed in *E. coli* (Pugsley, 1988). It is not clear at this stage whether any other protein(s) beside the α -amylase are required for the extracellular location in *E. coli*. The smallest insert still expressing amylase is 1.7 kb. DNA sequencing of this and further subcloning will reveal whether all the information for secretion beyond the periplasmic space is located in the coding sequence of the *amy* gene.

The expression of *amy* in *E. coli* was not observed when the insert was subcloned either in the opposite orientation to the *lacZ* promoter or in a vector that lacks this promoter (pQR1). This barrier to expression of *Streptomyces* genes in *E. coli* has been previously observed (Horinouchi *et al.*, 1980; Bibb & Cohen, 1982; Rodgers *et al.*, 1982). On the other hand, while the expression of amylase seemed to be possibly from the *lacZ* promoter, its production was not substantially induced by IPTG. This, in theory, might be a result of the high-copy-number of the plasmid, which may titrate out the *lacI* repressor. Hoshiko *et al.* (1987) reported the induction, in *E. coli*, of *S. hygroscopicus* α -amylase. They found that the enzyme, whose gene had been cloned in pUC12, was induced approximately sevenfold in the presence of IPTG.

The molecular mass of the secreted α -amylase of CUB74 determined by SDS-PAGE was 50 kDa, which, within the errors of the method, is the same as that of the enzyme secreted by *E. coli* and *S. lividans*. This similarity may result from the ability of *E. coli* to process the signal peptide correctly as in the original *Streptomyces* host. This result can only be proven by sequencing the N-terminus of the secreted enzyme from both strains. It is

not a general case that *E. coli* will process the signal peptide in exactly the same manner as a Gram-positive organism: e.g. the endo- β -N-acetylglucosidase H in its natural host, *S. plicatus*, is smaller than when it is expressed by *E. coli* (Robbins *et al.*, 1981); it was therefore suggested that a secondary cleavage, which occurs in Gram-positive secreted proteins, does not occur when the protein is synthesized and secreted into the periplasmic space by *E. coli* (Robbins *et al.*, 1984). In our experiments, the *S. thermophilaceus* amylase was secreted into the culture medium of *E. coli* cells containing pQR300 under conditions where alkaline phosphatase, a periplasmic enzyme, was still retained up to 91% in the periplasm/cytoplasmic fraction (Table 2). This implies a specific release of the amylase into the medium rather than a non-specific release of periplasmic proteins as has been seen when there is overproduction of periplasmic proteins (Suominen *et al.*, 1987).

In order to study the expression of amylase in *S. lividans*, low (pIJ903) (Lydiat *et al.*, 1985) and high (pQR1) copy-number shuttle vectors were used. When the *Bgl*III site of pQR1 was used for cloning, the resulting plasmid constructs (pQR308 and 309) were stable in *E. coli*. However, they were very unstable in *S. lividans* and underwent large deletions which removed the cloned DNA and a part of the vector. Similar problems were also encountered when the 5.7 kb *Bam*HI fragment was inserted in the *Bgl*III site of pIJ702. This could be due to the presence of unstable sequences carried by the insert. This was avoided by cloning a smaller DNA fragment, a 1.7 kb *Bam*HI-*Sph*I fragment, in pIJ702. The recombinant plasmid construct pQR311 was stable and expression of the amylase gene was obtained. Our results strongly suggest that the amylase gene is transcribed in the direction of *Bam*HI to *Sph*I in the 1.7 kb fragment inserted into pIJ702. This would place the amylase gene in the opposite orientation with respect to the *mel* promoter of pIJ702 in pQR311. It is not known yet whether the 1.7 kb fragment contains the promoter of the amylase gene or whether expression in *S. lividans* is caused by readthrough from promoters within the vector.

The activity expressed by the cloned α -amylase gene in *E. coli* has a surprisingly extended stability in the presence of Ca²⁺. At 50 °C, 100% of the starting activity remained after 22 h (Fig. 3). At 60 °C, this had only dropped to 75% after 22 h. From the data shown in Table 3, 70% of the starting activity remained after 1 h at 70 °C in the presence of Ca²⁺. The presence of Ca²⁺ therefore substantially increases the stability to thermal denaturation. The effect of Ca²⁺ on the thermal stability of several extracellular proteins is well known: for example many extracellular enzymes from *Bacillus* species are stabilized by binding Ca²⁺ (Normen, 1979).

The stability of the α -amylase in the presence of substrate has not been extensively tested but preliminary results (data not shown) suggest an even greater stability in the presence of starch and Ca^{2+} than for Ca^{2+} alone. This enzyme may be a candidate for constructing promoter-probe vectors or for protein fusion studies since it is easily assayed and its expression leads to the appearance of zones around colonies on starch plates even before staining with iodine. It is expressed in *E. coli* and secreted in high amounts into the culture medium. This makes its isolation easy since it avoids problems associated with cell breakage. Furthermore, the enzyme assay can be carried out at a high temperature, 60–70 °C, which eliminates background caused by any amylase activity of a mesophilic host, e.g. *S. lividans*.

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