Journal of General Microbiology (1989), **135**, 37–45. *Printed in Great Britain*

Cloning and Nucleotide Sequence of the Isoamylase Gene from a Strain of *Pseudomonas* sp.

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(*Received 27 June 1988; revised 22 August 1988; accepted 29 September 1988*)

A strain of *Pseudomonas* sp., SMP1, isolated from a soil sample collected in the Monterotondo area (Rome), secreted isoamylase activity into the culture medium. The enzyme was purified and optimal reaction and stability conditions were determined by varying pH and temperature. The chemico-physical properties of the enzyme were similar to those of the isoamylase purified in Japan more than 20 years ago from '*Pseudomonas amyloderamosa*' strain SB15. A genomic library of SMP1 was prepared in *Escherichia coli* using pUC12 as vector. Two isoamylase-producing colonies were identified out of 6300 screened. The hybrid plasmids isolated from the two clones showed common restriction patterns. The chromosomal portion of one of these plasmids (pSM257) was completely sequenced. Comparison between the deduced amino acid sequence of the isoamylase and the published sequences of other amylolytic enzymes showed the presence of conserved domains.

INTRODUCTION

The isoamylases are enzymes which hydrolyse the α-1,6 glucosidic inter-chain linkages present in starch. They are industrially useful for the production of amylose and maltose. Isoamylases are known to be present in yeast (Maruo & Kobayashi, 1951; Gunja *et al.*, 1961) and in higher plants (R-enzyme) (Hobson *et al.*, 1951). The first bacterial debranching enzyme found to hydrolyse the inter-chain linkages in both amylopectin and glycogen was isolated from a strain of '*Aerobacter aerogenes*' (*Klebsiella pneumoniae*) (Abdullah *et al.*, 1966). The enzyme turned out to be a pullulanase, which differs from isoamylase in its substrate specificity (Lee & Whelan, 1971).

Harada *et al.* (1968) isolated an isoamylase-producing strain, '*Pseudomonas amyloderamosa*' SB15, in Japan. The enzyme was purified and extensively characterized (Yokobayashi *et al.*, 1970; Harada *et al.*, 1972; Amemura *et al.*, 1980). It appeared to consist of a single high-M_r polypeptide chain (M_r 90000) which was secreted into the culture medium. Furthermore, the expression of the protein was modulated by the presence of inducers such as maltose and amylopectin (Harada *et al.*, 1968; Sugimoto *et al.*, 1974).

While a large body of information is available on the biochemical properties of the isoamylase from *Pseudomonas*, nothing is known about its genetics. In the present paper we report the isolation of a *Pseudomonas* strain which produces an isoamylase similar, if not identical, to the one described by Harada and coworkers. Furthermore we describe the cloning and the entire nucleotide sequence of the isoamylase gene.

METHODS

Plasmids and strains. The isoamylase-producing strain SMP1 was isolated as described in Results. Commercially available classification tubes (computer coding and identification system for Oxi-Ferm Tube II) from Hoffmann-La Roche were used to identify it as a *Pseudomonas* sp.

Plasmid pUC12 (Messing, 1983) was used for the construction of the SMP1 genomic library. The cloning experiments were done using *Escherichia coli* 71/18 as host.

Enzymes and reagents. Restriction enzymes, T4 DNA ligase, calf intestinal phosphatase, IPTG and X-gal were purchased from Boehringer Mannheim and used according to the manufacturer's specifications. Amylopectin and amylose were from Sigma. Commercially available isoamylase from '*Pseudomonas amyloderamosa*' strain SB15, purchased from Hayashibara, was a generous gift of ENICHEM SYNTHESIS S.p.A. (Italy). The enzyme was further purified as follows. A 500 mg portion of powder was resuspended in 5 ml 0·01 M-sodium acetate buffer (pH 4) and incubated at 40 °C for 1 h. After centrifugation to remove the insoluble materials, the supernatant was applied to an amylose column and treated as described below.

Isoamylase purification. Pseudomonas SMP1 was grown for 120 h at 30 °C under agitation (220 r.p.m.) in 10 litres of Harada minimal medium (HS) (Harada *et al.*, 1968) containing 2% (w/v) maltose as inducer. The cells were removed by centrifugation and the supernatant concentrated 10-fold using the Amicon DC2 system. The concentrated solution was applied to a column of amylose gel, prepared according to Kato *et al.* (1977), previously equilibrated with 0·01 M-sodium acetate buffer pH 4. After washing the column with the same buffer containing 0·5 M-NaCl, the isoamylase was eluted with 20% maltose in 0·01 M-sodium acetate buffer pH 4. The enzyme was concentrated and dialysed by using either YM30 ultrafiltration membranes or the Centricon 30 system (Amicon).

Protein sequencing. The amino-terminal sequence of SMP1 isoamylase was determined using a 890M Beckman Sequenator starting from a 100 μg protein sample.

Assay of isoamylase activity. This was done essentially as described by Yokobayashi *et al.* (1970). The reaction mixture, containing 2 ml 1% (w/v) amylopectin, 0·4 ml 0·2 M-sodium acetate buffer (pH 4) and 0·4 ml of the enzyme sample, was incubated for 1 h at 40 °C. A sample (0·4 ml) of the reaction mixture was then added to the same volume of a solution containing 0·2% (w/v) I, 2% (w/v) KI, 0·2% (v/v) H_2SO_4 and diluted to 20 ml with water. The solution was kept for 15 min at room temperature and the absorbance was then measured at 610 nm. One unit is defined as the amount of enzyme causing an increase in A_{610} of 0·01 in 1 h.

Protein analysis. Total cell protein extracts and periplasmic proteins were prepared as described by Peschke *et al.* (1985) and Koshland & Botstein (1980). The protein preparations were analysed on SDS-polyacrylamide (10%, w/v, acrylamide) gels (Laemmli, 1970). The resolved proteins were either stained with Coomassie blue or transferred to nitrocellulose filters. The filters were then treated with anti-isoamylase antibodies and subsequently with peroxidase-linked anti-antibodies according to the procedure of Towbin *et al.* (1979). Polyclonal antibodies against SMP1 isoamylase were prepared by immunizing rabbits according to standard procedures.

Preparation of chromosomal DNA. SMP1 was grown in 100 ml HS medium at 30 °C for 120 h. The cells were centrifuged, washed with 25% (w/v) sucrose, 0·1 M-NaCl, 0·05 M-Tris/HCl pH 7·5, and the chromosomal DNA was purified as described by Dubnau & Davidoff Abelson (1971).

Preparation of the SMP1 DNA library. Chromosomal DNA from SMP1 was partially cleaved with *Sau*3A and the fragments with an average size of 3000–4000 bp were separated on a sucrose gradient and purified (Maniatis *et al.*, 1982). pUC12 was linearized with *Bam*HI and the 5′ ends were dephosphorylated with calf intestinal phosphatase. The linearized plasmid and the chromosomal fragments were then mixed in a 2:1 ratio and incubated overnight at 4 °C in the presence of T4 DNA ligase. The ligase mixture was used to transform competent cells of *E. coli* 71/18 and the transformants were selected on 2 × YT plates (Rodriguez & Tait, 1983) containing 0·05 mM-IPTG, 0·02% (w/v) X-gal and 50 μg ampicillin (Ap) ml^{-1}. The white colonies were then transferred to LB plates supplemented with 50 μg Ap ml^{-1}.

Screening of the library. Colonies of the library were transferred to plates of M9 medium (Maniatis *et al.*, 1982) buffered at pH 6 and containing 1·5% (w/v) agar (Difco), 1% (w/v) amylopectin and 50 μg Ap ml^{-1}. After 48 h incubation at 37 °C, the plates were exposed to the vapours of an iodine solution [2% (w/v) I, 1% (w/v) KI, 25% (v/v) ethanol] and the isoamylase-producing colonies identified by the presence of a surrounding intense blue halo.

Sequence analysis. pSM257 (see Results) was digested with *Bam*HI, *Xba*I and *Sma*I restriction enzymes and the two fragments of the chromosomal insert were purified (see Fig. 2). The ends of these fragments were made blunt with a fill-in reaction (Maniatis *et al.*, 1982) and subsequently cloned into the *Sma*I site of M13mp8 (Messing, 1983) in both orientations.

The single-stranded recombinant phages were used for determining the nucleotide sequence of the isoamylase gene using the specific-primer-directed method published by Strauss *et al.* (1986). The synthetic oligonucleotides used as primers for sequencing were synthesized with a DNA Synthesizer System One Plus (Beckman).

RESULTS

Isolation of the isoamylase-producing strain and enzyme characterization

Samples of soil randomly collected in the Monterotondo area (Rome) were rinsed with sterile saline buffer and centrifuged. The supernatants were plated onto HS minimal medium and incubated at 30 °C. After the appearance of bacterial colonies, the plates were exposed to iodine

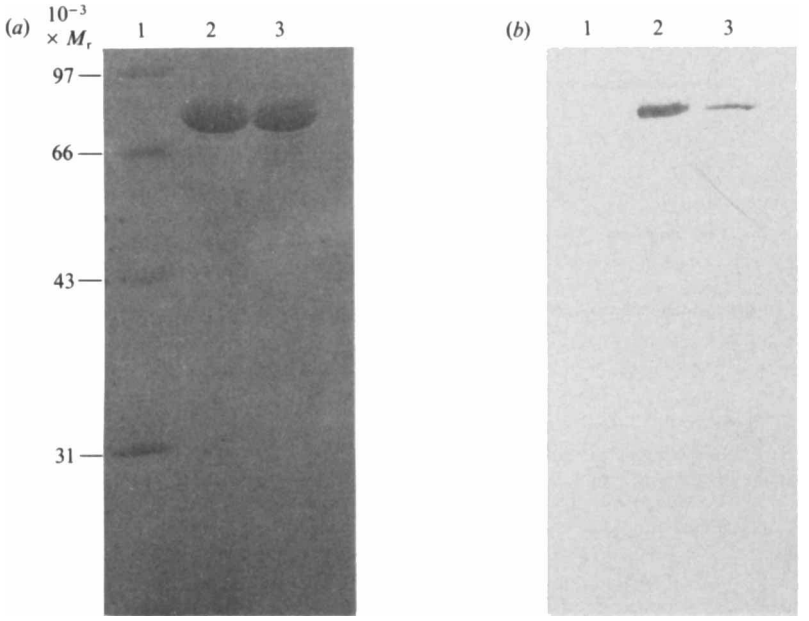

Fig. 1. Purified isoamylase preparations from '*Pseudomonas amyloderamosa*' SB15 and from *Pseudomonas* sp. SMP1. The enzymes were purified by affinity chromatography on amylose gel (see Methods) from a commercially available partially purified SB15 isoamylase preparation and from the supernatant of SMP1 culture. A 5 μg sample of each preparation was loaded onto an SDS-polyacrylamide gel. After electrophoresis the proteins were either stained with Coomassie blue (*a*) or transferred to a nitrocellulose filter and treated with specific antibodies (*b*). Lanes: 1, M_r standards; 2, SB15 isoamylase; 3, SMP1 isoamylase.

vapour. One colony, surrounded by a dark blue halo, was further characterized. The colony was formed by rod-shaped bacteria which were identified as a *Pseudomonas* species (see Methods). The strain was named SMP1.

Isoamylase activity was clearly found in the supernatants of SMP1 cultures grown in HS minimal medium. The enzyme activity, like that of '*Pseudomonas amyloderamosa*' SB15 (Harada *et al.*, 1968), was induced by maltose and maltodextrin. When *Pseudomonas* SMP1 was grown in HS medium for 120 h at 30 °C in the presence of maltose or maltodextrin, we measured 66 and 18 units of isoamylase ml^{-1}, respectively. In contrast, less than 5 units ml^{-1} were detectable when glucose, fructose or sucrose was present. The enzyme was purified from the culture supernatant as described in Methods, yielding 8 mg of a 90% pure preparation from a 10 litre culture.

When electrophoresed on an SDS-polyacrylamide gel, the enzyme preparation appeared to be formed by a major protein species of apparent M_r 90000. This protein co-migrated with the purified Harada's enzyme (Fig. 1*a*). Polyclonal antibodies against the SMP1 isoamylase preparation reacted with both isoamylases, indicating a strong structural similarity between the two enzymes (Fig. 1*b*).

The optimal pH value for SMP1 isoamylase activity ranged between 3 and 4 (not shown), as for the commercial enzyme (Yokobayashi *et al.*, 1970). A good agreement between the behaviour of the two enzymes was also found when the activities were measured at different temperatures, 55 °C being the optimum for both enzyme activities under our assay conditions. Finally, the isoamylase from SMP1 was incubated at different temperatures for 15 min and the residual enzyme activity determined. Almost 100% of the initial activity was measured after incubation at 37 °C, whereas activity was completely lost after 15 min incubation at 60 °C. Again, comparable results were obtained with Harada's enzyme.

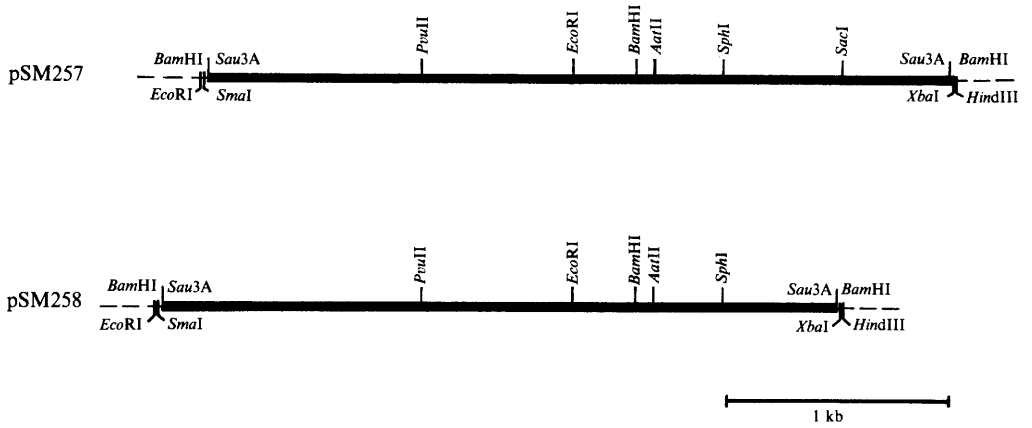

Fig. 2. Restriction maps of the recombinant plasmids harbouring the isoamylase gene. The bold lines represent the genomic inserts and the dashed lines the pUC12 vector.

Cloning of the isoamylase gene

Chromosomal DNA from strain SMP1 was partially digested with the restriction enzyme *Sau*3A. The 3000–4000 bp fragments were ligated to *Bam*HI-linearized pUC12 and the ligase mixture used to transform *E. coli* 71/18. A total of 6300 white colonies were selected on 2 × YT plates supplemented with IPTG and X-gal.

The whole collection was transferred to M9 agar plates containing 1% (w/v) amylopectin and screened for the appearance of the characteristic halo around the colonies, indicating the presence of an isoamylase-like activity. Since the SMP1 isoamylase activity is strongly inhibited at pH values above 6, the preparation of a minimal medium buffered at pH 6 was critical. More acidic media, which would be even better for enzyme activity, did not allow the growth of *E. coli* 71/18. Two colonies, named *E. coli* 71/18(pSM257) and *E. coli* 71/18(pSM258), gave a dark halo after iodine staining.

The recombinant plasmids from the two clones, named pSM257 and pSM258 respectively, were extracted and restricted with several enzymes. They appeared to carry inserts of slightly different size (approximately 3200 bp in pSM257 and 3000 bp in pSM258) but with similar restriction patterns. A physical map of both plasmids is reported in Fig. 2.

The isoamylase activity produced by *E. coli* 71/18(pSM257) was measured by growing the recombinant strain at 37 °C for 7 h in M9 medium containing 0·8% (w/v) peptone and 0·5% (w/v) maltose. The cells were then collected by centrifugation and disrupted in a French press (Aminco). The cellular debris was removed by centrifugation and the enzyme activity measured. The enzyme activity was also determined in the periplasmic space and in the culture medium. The total enzyme activity (about 2·5 units per ml of the original culture) was present in the cell extract, suggesting that the enzyme is compartmentalized in the cytoplasmic region. Further work is necessary to elucidate the location of the enzyme in *E. coli* (see Discussion).

To test whether promoter regions present on the chromosomal fragment of pSM257 were responsible for the transcription of the isoamylase gene in *E. coli*, the 3400 bp fragment obtained by *Hin*dIII and partial *Eco*RI digestions of pSM257 was ligated to the *Eco*RI–*Hin*dIII-digested pUC13. Plasmid pSM259 was obtained; it was identical to pSM257 with the exception of the orientation of the isoamylase gene with respect to the vector moiety. *E. coli* 71/18 harbouring either pSM257 or pSM259 showed identical isoamylase activity (data not shown), indicating that the transcription of the gene has its origin from a promoter located on the cloned fragment and not from a vector promoter.

Finally, to prove that this activity was indeed due to the cloned *Pseudomonas* gene, *E. coli* 71/18(pSM257) was grown in minimal medium and the total cell proteins were separated by

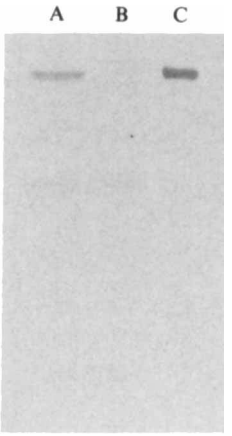

Fig. 3. Isoamylase production in *E. coli* 71/18(pSM257). Cells from *E. coli* 71/18(pSM257) and 71/18(pUC12) colonies were lysed directly in 125 mM-Tris/HCl pH 7·5, 3% β-mercaptoethanol, 3% SDS and 20% glycerol at 100 °C for 5 min and loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred to a nitrocellulose sheet and treated with isoamylase-specific antibodies. Lanes A, *E. coli* 71/18(pSM257); B, *E. coli* 71/18(pUC12); C, SMP1 isoamylase.

SDS-PAGE and analysed by immunoblotting. The anti-SMP1 isoamylase antibodies recognized a protein species in the crude extract which co-migrated with the purified enzyme (Fig. 3).

Sequencing of the isoamylase gene

The *Sau*3A chromosomal fragment of pSM257 was completely sequenced on both strands: 2635 out of 3337 bases of the fragement are reported in Fig. 4. The main features of the sequence are summarized below.

Starting with the ATG at position 229, an open reading frame (ORF) is found which can accommodate a 776-amino-acid protein, a size consistent with the expected length of the isoamylase. Confirmation that the ORF does code for the isoamylase comes from the observed identity of the amino-terminal sequence of the protein (see Methods) and residues 27 to 44 (underlined in Fig. 4) of the predicted amino acid sequence. The fact that the isoamylase sequence starts from codon 27 of the ORF also suggests that the enzyme is synthesized as a precursor with a 26-amino-acid amino-terminal segment that is removed in forming the mature isoamylase ('pre-region'). This predicted 'pre-region' has a typical leader sequence structure (Von Heijne, 1985), in agreement with the fact that the isoamylase is secreted and released into the supernatant of the *Pseudomonas* culture. Seven bases upstream from the ATG codon the sequence GGAGG is found, which is likely to be used *in vivo* as a ribosome-binding site.

In the absence of any experimental data on promoter mapping, the localization of the putative promoter of the isoamylase gene can only be speculative. We have been unable to localize any structure similar to the consensus sequence of the constitutive *Pseudomonas putida* promoters reported by Inouye *et al.* (1986). However, a sequence organization resembling that described for the *ntrA*-regulated promoters of *Pseudomonas* sp. (Johnson *et al.*, 1986) can be identified. In fact, starting at position 174 the sequence GTGGCGC is present; this matches six of the eight nucleotides of the −26 consensus box CTGGPyAPyPu. Futhermore, correctly spaced by four nucleotides, the sequence CTGTT is found, in reasonably good agreement with the −14 consensus sequence TTGCA.

Interestingly, a sequence organization resembling the structure of those promoters which are subject to maltose regulation (Chapon & Raibaud, 1985) can also be localized (double underlining in Fig. 4). In fact 129 nucleotides upstream from the ATG, the sequence GGATGT

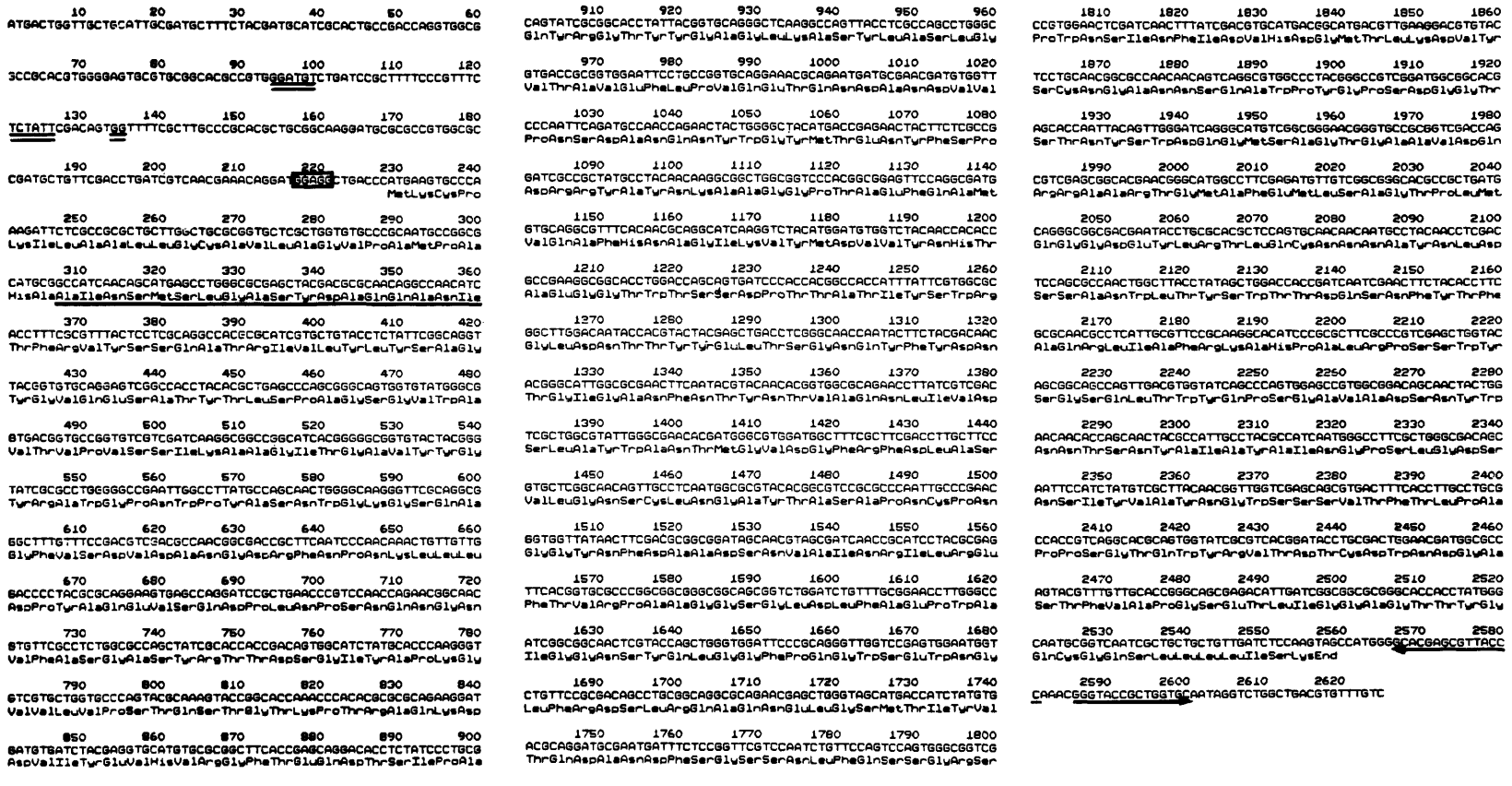

Fig. 4. Nucleotide sequence of the isoamylase gene of *Pseudomonas* sp. SMP1, and the deduced amino acid sequence of its product. The first 18 amino acids of the amino-terminal sequence of the protein are underlined. The putative ribosome binding site used for translation is boxed. A possible *malT*-regulated promoter region is indicated by double underlining (see text for explanation and other comments on promoter regions). The two arrows identify an inverted repeat region probably involved in the termination of transcription.

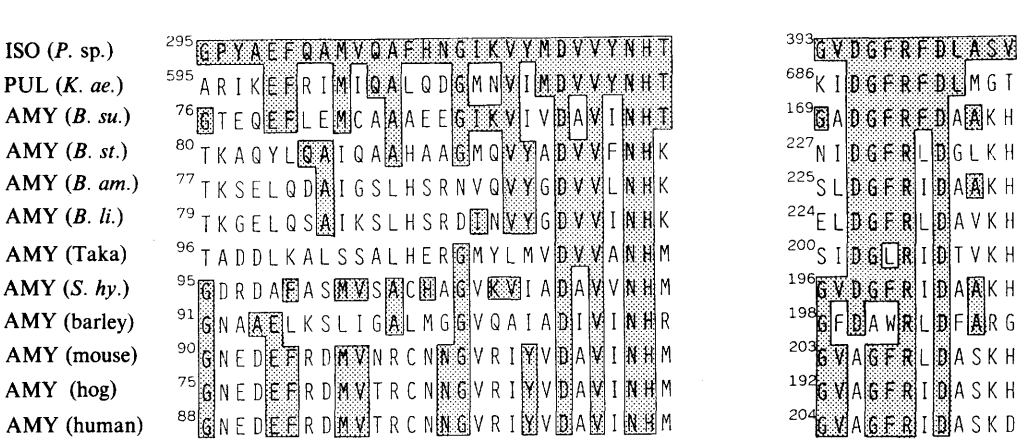

Fig. 5. Comparison of the amino acid sequences of the homologous regions in prokaryotic and eukaryotic amylolytic enzymes. Numbers refer to the amino acid position within the proteins. Regions of homology are shaded. ISO (*P.* sp.): isoamylase from *Pseudomonas* sp. SMP1 (this work); PUL (*K. ae.*): pullulanase from *Klebsiella aerogenes* (*K. pneumoniae*) (Katsuragi *et al.*, 1987); AMY (*B. su.*): α-amylase from *Bacillus subtilis* (Ihara *et al.*, 1985); AMY (*B. st.*): α-amylase from *Bacillus stearothermophilus* (Ihara *et al.*, 1985); AMY (*B. am.*): α-amylase from *Bacillus amyloliquefaciens* (Ihara *et al.*, 1985); AMY (*B. li.*): α-amylase from *Bacillus licheniformis* (Ihara *et al.*, 1985); AMY (Taka): Taka-amylase A (Ihara *et al.*, 1985); AMY (*S. hy.*): α-amylase from *Streptomyces hygroscopicus* (Hoshiko *et al.*, 1987); AMY (barley): barley α-amylase from *Hordeum vulgare* seeds (Rogers, 1985); AMY (mouse): α-amylase from mouse hepatic and salivary glands (Rogers, 1985); AMY (hog): α-amylase from hog pancreas (Ihara *et al.*, 1985); AMY (human): α-amylase from human salivary glands (Nakamura *et al.*, 1984).

is present which is located 21 bases before a Pribnow-box-like region (TCTATT). Downstream 34 nucleotides from the GGATGT sequence, two G residues are found that could represent a possible transcriptional start site. It is worth noting that in these maltose-regulated promoters the *malT* product, the positive regulator, appears to interact with the sequence GGATGA. A second possible *malT* binding site can be found at position 163 (GGATGC), although not followed by a reasonable −10 region.

Finally, regions sharing only a limited homology with the consensus sigma-70 promoter sequence of *E. coli* (Hawley & McClure, 1983) can be found in the region upstream from the ORF. A poor −35 region (ATGTCT) starting at position 96 and located 19 bp upstream from the −10 sequence TCTATT could account for the low level of expression of the isoamylase gene in *E. coli*.

Seven nucleotides downstream from the stop codon of the gene, a rho-independent terminator-like structure is present with a calculated ΔG value for secondary structure formation of −104 kJ mol^{-1} (Tinoco *et al.*, 1973). However, it must be pointed out that such a terminator would differ from the consensus rho-independent terminators in lacking the run of Ts which is normally present at the 3′ side of the stem-and-loop structure.

Comparison of the Pseudomonas SMP1 isoamylase amino acid sequence with those of other starch-hydrolysing enzymes

It has been recently shown (Rogers, 1985; Ihara *et al.*, 1985) that all known α-amylases from different sources share three different well-conserved sequence domains, spaced at similar intervals. In the case of barley α-amylase, part of the first domain is closely related to established calcium-binding sites of other proteins (Rogers, 1985). Matsuura *et al.* (1984), in their crystallographic studies on Taka amylase A, suggested that domain 1 and part of domain 3 are involved in substrate binding, whereas domain 2 and part of domain 3 participate in the hydrolysis of starch.

Searching for homologies between isoamylase, pullulanase (Katsuragi *et al.*, 1987) and α-amylases using the program MICROGENIE (Beckman), we found that domains 1 and 2 were also well conserved in both isoamylase and pullulanase (Fig. 5). However, no third domain-like sequence was identified in either enzyme. Interestingly, Matsuura *et al.* (1984) suggested that the aspartic acid conserved in the third domain of the α-amylase is required for the cleavage of the 1,4 glycosidic bond. This bond is not hydrolysed by either isoamylase or pullulanase.

DISCUSSION

We have isolated an isoamylase from *Pseudomonas* SMP1 that appears to be indistinguishable from the one purified by Harada *et al.* (1968), and have cloned the isoamylase gene in *E. coli*. The gene was located in a 3337 bp segment bounded by *Sau*3A sites which, once ligated to the plasmid pUC12, was expressed in *E. coli*.

Since the expression was unaffected by the orientation in which the chromosomal fragment was inserted into the vector, it is reasonable to suggest that the isoamylase gene was cloned with its own promoter. A promoter region resembling a maltose-regulated promoter was located upstream from the start codon of the gene. Similar sequences have been found in all *malT*-dependent promoters, including the pullulanase gene (*pulA*) of *Klebsiella pneumoniae* (Michaelis *et al.*, 1985). Since in *Pseudomonas* SMP1 isoamylase synthesis is regulated by the presence of maltose in the culture medium, this leads us to the attractive hypothesis that the mechanisms by which maltose regulates gene expression are highly conserved among many Gram-negative bacteria. An alternative promoter for the expression of the isoamylase gene could be the one identified at position 174 and similar to the *ntrA*-regulated promoters. Such promoters have been found to control the transcription of many *Pseudomonas* genes such as the *Pseudomonas putida* CPG2 gene, the *P. putida xylABC* gene and the *Pseudomonas aeruginosa* pilin genes (Johnson *et al.*, 1986). The involvement of either *malT* or *ntrA* gene products in the regulation of the isoamylase gene awaits support from experimental data.

The isoamylase was released into the culture medium by *Pseudomonas* SMP1, in agreement with the fact that the enzyme appeared to be synthesized as a precursor in the cells. We were not able to measure appreciable amounts of enzyme either in the culture medium or in the periplasmic space of *E. coli* cells, although the isoamylase-producing recombinant clones were identified by virtue of their ability to hydrolyse amylopectin present in agar plates. However, it has to be emphasized that a 10–20% efficiency in secretion would be hardly detectable because of the low level of isoamylase expression in *E. coli* 71/18(pSM257). The use of strong promoters will enable us to quantify the amount of isoamylase in the different cell compartments and to establish to what extent *E. coli* can secrete the enzyme. Nevertheless the involvement of a complex and specific secretion pathway can be envisaged, as in the case of other secreted proteins. For instance, when the *Klebsiella pneumoniae* pullulanase gene was cloned and expressed in *E. coli*, the enzyme turned out to be associated with the outer membrane (Michaelis *et al.*, 1985) but correctly secreted in the presence of other *K. pneumoniae* genes (D'Enfert *et al.*, 1987). The products of these genes were shown to play an important role in the mechanism of pullulanase translocation through the outer membrane. It would be interesting to test if similar gene products are required for isoamylase secretion.

Note added in proof. After this paper was submitted, the sequence of the isoamylase gene from '*Pseudomonas amyloderamosa*' strain SB15 was published [Amemura *et al.* (1988), *Journal of Biological Chemistry* **263**, 9271–9275]. The sequence appears to be almost identical to the one presented here, in agreement with our experimental data on the similarity of the two isoamylases.

We wish to thank Dr M. Del Bue for her contribution at the beginning of this work and Dr S. Toma for helpful discussions. The technical assistance of G. Scolla is also appreciated. This research was supported by a grant from ENICHEM SYNTHESIS S.p.A. (Italy).

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