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Molecular Cloning and Characterization of the *Streptomyces hygroscopicus* α-Amylase Gene

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We have isolated and sequenced a gene (*amy*) coding for α-amylase (EC 3.2.1.1.) from the *Streptomyces hygroscopicus* genome (H. Hidaka, Y. Koaze, K. Yoshida, T. Niwa, T. Shomura, and T. Niida, Die Stärke 26:413–416, 1974). Amylase was purified to obtain amino acid sequence information which was used to synthesize oligonucleotide probes. *amy*-containing *Escherichia coli* cosmids identified by hybridization did not express amylase activity. Subcloning experiments indicated that *amy* could be expressed from the *lac* promoter in *E. coli* or from its own promoter in *S. lividans*. The *amy* nucleotide sequence indicated that it coded for a protein of 52 kilodaltons (478 amino acids). Secreted α-amylase contained amino- and carboxy-terminal as well as internal amino acid sequences which were consistent with the nucleotide sequence. The 30-residue leader sequence showed similarities to those found in other procaryotes. The DNA sequence 5′ to the *amy* structural gene contained a sequence complementary to the 3′-terminal sequence of 16S rRNA of *S. lividans* (M. J. Bibb and S. N. Cohen, Mol. Gen. Genet. 187:265–277, 1982). The transcriptional start points of *amy* were determined by mung bean nuclease mapping, but the promoter of *amy* was not similar to the consensus sequence found in other procaryotes.

Streptomyces spp. are industrially important microorganisms which produce many secondary metabolites and secreted proteins (46). Since protein secretion often coincides temporally with secondary metabolism (8), the nucleotide sequence of the control regions of these genes may provide insights into the regulation of protein secretion and secondary metabolism of *Streptomyces* sp. Recently, genes encoding several extracellular enzymes have been cloned from *Streptomyces* species. These include the tyrosinase gene from *Streptomyces antibioticus* (20), the agarase gene from *S. coelicolor* (21), and the endoglycosidase H gene from *S. plicatus* (33). Sequencing of the *S. plicatus* endoglycosidase H gene has shown that the precursor form of the protein contains a signal sequence similar to those found in other procaryotic organisms (14). Several *Streptomyces* promoters (5, 7, 19, 45) have been studied; however, a consensus sequence has not yet been recognized. Different classes of transcriptional initiation signals have been reported which are presumably transcribed by RNA polymerase holoenzymes having different recognition specificities (45). *S. hygroscopicus* SF-1084 produces an extracellular α-amylase which is used industrially to hydrolyze starch to maltose. We would like to understand the regulation of *amy* and the properties of its products to maximize the efficiency of hydrolysis and to better understand the control of gene expression in *Streptomyces* sp.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cosmid. *S. hygroscopicus* SF-1084 was originally isolated by Hidaka et al. (13). The improved strain AA69-4 was supplied by Bioscience Laboratories of Meiji Seika Kaisha, Ltd. (Kawasaki, Kanagawa, Japan). It was isolated from *S. hygroscopicus* SF-1084 after many cycles of mutagenesis and screening. *S. lividans* 1326 was obtained from the John Innes Culture Collection. The *Streptomyces* vectors pIJ702 (20) and pIJ922 (23) were supplied by D. A. Hopwood. Plasmid vector pMS201 was constructed from pIJ922 by H. Anzai (unpublished data). Plasmid pUC12 (42) and its *Escherichia coli* host JM105 (29) were purchased from Pharmacia, Ltd. Cosmid pHC79 (16) and *E. coli* strains BHB2688 (15), BHB2690 (15), and LE392 (10) were supplied by B. Hohn.

Determination of amylase activity. α-Amylase activity was assayed by using the dye-conjugated substrate Amylase Test A Shionogi (Shionogi & Co. Ltd.) under the conditions described by the supplier. One unit of enzyme activity catalyzed an increase in A_{650} of 0.01 in 15 min.

Amino acid sequence analysis of Edman degradation. α-Amylase was initially purified from strain AA69-4 by the method of Hidaka et al. (12) and then further purified by DEAE-Toyopearl 650S (Toyo Soda Ltd.) column chromatography, eluting with a linear gradient of 0.05 to 0.50 M NaCl, and Sephacryl S200 (Pharmacia, Ltd.) gel chromatography. The final fraction was pooled and dialyzed against 50 mM triethanolamine bicarbonate buffer, pH 7.5, and lyophilized. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis indicated that the purity of the protein was >99%. Cyanogen bromide-cleaved peptides (16 mg, 340 nmol) were separated by a Nucleosil C_{18} reverse-phase column (4 by 250 mm) (Nagel Co., Ltd.), using a high-performance liquid chromatography system programmed for a linear gradient in 0.1% (vol/vol) trifluoroacetic acid (0 to 50% acetonitrile). The four peak fractions were collected and lyophilized. The smallest peptide (M_r 3,000) was purified by SDS-polyacrylamide gel electrophoresis and then eluted into 60% (vol/vol) formic acid for 16 h at room temperature. Native α-amylase (40 nmol) and cyanogen bromide-cleaved peptides (0.5 to 2.0 nmol) were loaded onto Applied Biosystems protein sequencer 470A for Edman degradation. Phenylthiohydantoin amino acids were analyzed quantita-

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TABLE 1. Amounts and localization of α-amylase produced by *E. coli* JM105(pMS120)[a]

Location	α-Amylase (U/ml)	
	−IPTG	+IPTG
Extracellular	0.5 (0.05)	3.0 (0.03)
Periplasmic	2.9 (0.09)	23.0 (0.07)
Cytoplasmic/membrane	NT	9.5 (0.84)

[a] Bacterial cells from a single colony were grown in L-broth containing 2% starch and 100 μg of ampicillin per ml in the presence or absence of isopropyl-β-D-galactopyranoside (IPTG). Supernatants (extracellular fraction) and cells were collected by centrifugation, when the cells reached early stationary phase. Cells were washed once with Tris-sucrose buffer, fractionated by the method of Talmadge et al. (38), and assayed for α-amylase activities as described in Materials and Methods. The values represent units of amylase activity per milliliter of culture. The data in parentheses are amounts of protein (milligrams) recovered from 1 ml of the broth. NT, Not tested.

tively by high-pressure liquid chromatography on a Du Pont Zorbax CN column, using a Spectra Physics SP8100 system.

Carboxy terminus analysis. The lyophilized α-amylase (2.4 mg, 50 nmol) was dissolved in 0.85 ml of 0.2 M pyridine-acetate buffer, pH 6.3; 10 μl of 5 mM norleucine was added as an internal standard. After the addition of carboxypeptidase Y (50 μg), samples (200 μl) were taken after 5, 20, 60, and 180 min of incubation at 37°C. Free amino acids were separated (32) and identified with a Hitachi 835 amino acid analyzer.

DNA preparation and manipulation. Plasmids were isolated and purified from *E. coli* and *Streptomyces* species as described previously (29). DNA fragments were purified by agarose or polyacrylamide gel electrophoresis (24).

Cloning of the α-amylase gene into cosmid vector pHC79. We synthesized two mixed DNA probes by the phosphite coupling method (25). The 5'-OH termini of the synthetic 14-mer probes were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. A genomic library of *S. hygroscopicus* AA69-4 was prepared in cosmid pHC79 (24). Partially *Sau*3AI-digested DNA of 10 to 50 kilobases (kb) was purified by agarose gel electrophoresis, ligated to dephosphorylated pHC79, and packaged in vitro (15). Bacteriophage particles were used to infect *E. coli* LE392. Ampicillin-resistant colonies were fixed onto nitrocellulose membranes and screened by hybridization to 14-mer *amy*-specific probes in 6× NET buffer containing 0.1% (wt/vol) SDS, 100 μg of denatured salmon sperm DNA per ml, and 1× Denhardt solution (43). After hybridization, filters were washed four times for 1 h in 6× NET containing 0.1% (wt/vol) SDS.

Subcloning into *Streptomyces* sp. *S. lividans* was transformed by the method described by Chater et al. (9). Since *S. hygroscopicus* AA69-4 could not be transformed by any of the established procedures, a new protocol was devised. AA69-4 was first cultivated in YMA medium (1% yeast extract, 1% malt extract, 3% soluble starch, pH 7.0) for 24 h at 28°C and subcultured in YEME medium (9) containing 34% sucrose and 4.0% glycine for 48 h at 28°C and then in YEME medium containing 50% sucrose and 4.5% glycine for 48 h. Protoplasts were prepared from the mycelium by incubation at 31°C for 30 to 45 min in P medium (31) containing 0.5 M sucrose, 1.0 mg of lysozyme per ml, and 0.5 mg of achromopeptidase per ml. Transformation was performed by the method of Thompson et al. (40) in T medium containing 0.5 M sucrose. After protoplasts were exposed to DNA, they were suspended gently in regeneration (RSH) medium containing 0.5% agar and spread on a plate of the same medium containing 1.5% agar. RSH medium contained 17.1% sucrose, 1.5% KCl, 1.0% glycose, 0.025% K_2SO_4, 0.05% sodium L-aspartate, 0.3% L-proline, 0.2% Casamino acids, 0.2% polypeptone (Daigo Eiyo Kagaku Co. Ltd.), 0.05% dextran sulfate, 0.2% yeast extract, 0.3% (vol/vol) corn steep liquor, 0.005% KH_2PO_4, and 50 mM $CaCl_2$, pH 7.0. After 20 h of growth at 28°C, transformants were selected by flooding the RSH plate with soft agar containing 0.8% nutrient broth, 1% Casamino acids, 500 μg of tyrosine per ml, 5 μg of $CuSO_4 \cdot 5H_2O$ per ml, and 50 μg of thiostreptone per ml. Transformants were replicated to a

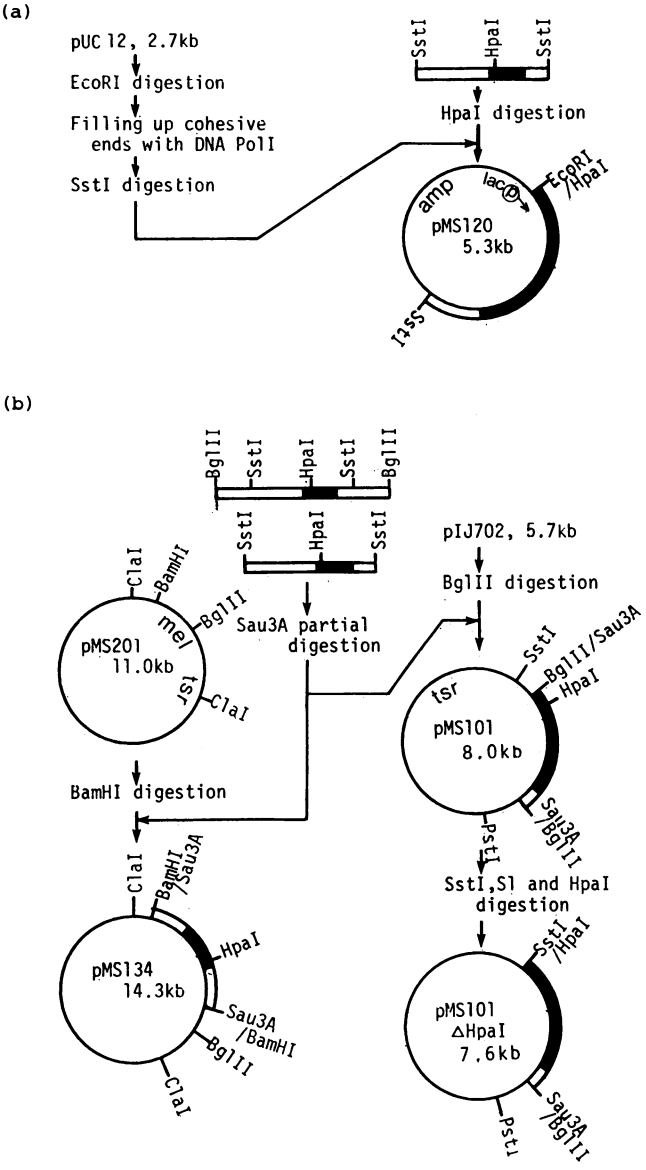

FIG. 1. Construction of recombinant plasmids. (a) Construction of pMS120. pMS120 contains the *lac* UV5 promoter derived from pUC12. The direction of transcription of the *lac* promoter is indicated by the arrow. *amp*, Beta-lactamase gene. (b) Construction of pMS101, pMS134, and pMS101ΔHpa. The construction of pMS102, pMS104, and pMS109 was similar to that of pMS101. pMS134 was constructed from pMS201, which was derived from the low-copy-number plasmid pIJ922. Insertion of fragments at the *Bgl*II, *Sph*I, or *Sst*I site was identified by insertional inactivation of the *mel* gene. *mel*, Tyrosinase gene; *tsr*, thiostreptone resistance gene.

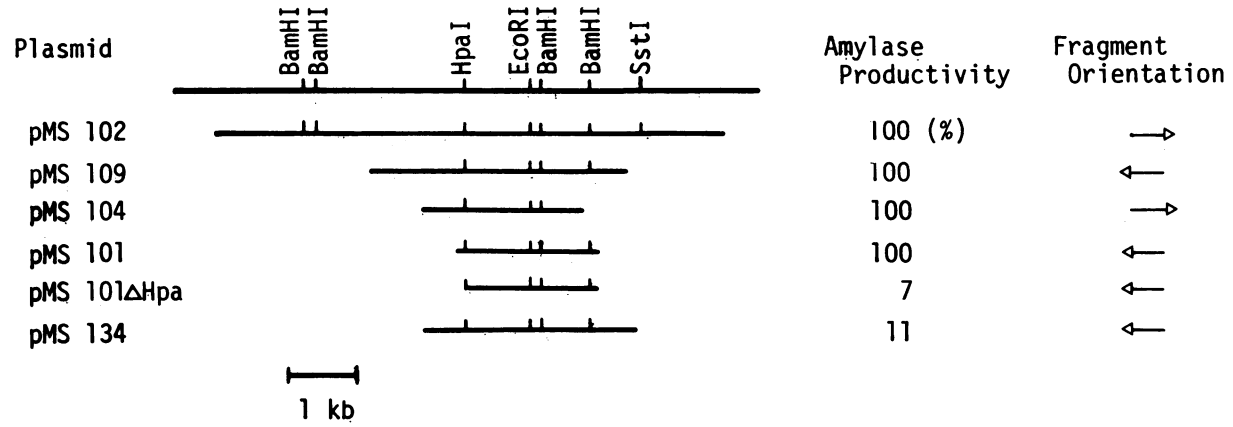

FIG. 2. Restriction cleavage maps of *amy* subclones. pMS101ΔHpa was constructed by deletion from the *Sst*I-*Hpa*I fragment of pMS101. Transcription from the *mel* promoter is indicated by the arrows. Amylase activities of the recombinant *S. lividans* strains harboring each plasmid are shown.

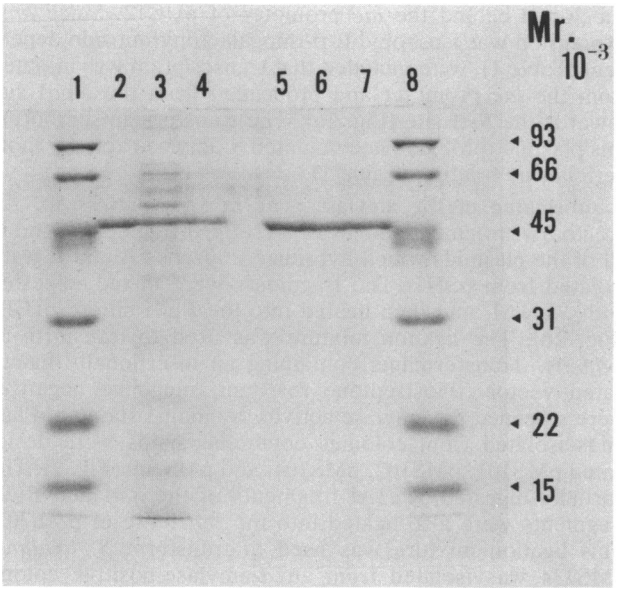

FIG. 3. Molecular weight estimation of *S. hygroscopicus* AA69-4 and *S. lividans* (pMS101) α-amylases by SDS–8% polyacrylamide gel electrophoresis. The culture supernatants were collected from *Streptomyces* species grown in YMA medium at 31°C for 2 days. The supernatant of *S. lividans* was concentrated by lyophilization (12-fold). A 2-μl portion of each sample was loaded onto the gel. Purified α-amylase from the culture supernatant of *S. lividans* (pMS101) was prepared by using MonoQ (Pharmacia, Ltd.) anion-exchange chromatography with a linear gradient elution (0.05 to 0.80 M NaCl in 10 mM Tris hydrochloride, pH 7.0, 1 mM $CaCl_2$). (Lanes 1 and 8) Mixture of molecular weight markers: phosphorylase (M_r 93,000), bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 22,000), and lysozyme (M_r 15,000); (Lanes 2 and 5) 1.7 μg of purified α-amylase from *S. hygroscopicus* AA69-4; (Lane 3) concentrated culture supernatant of *S. lividans* (pMS101); (Lane 4) culture supernatant of *S. hygroscopicus* AA69-4 (pMS101); (Lane 6) 1.7 μg of purified α-amylase from *S. lividans* (pMS101); (Lane 7) mixture of purified α-amylase (1.0 μg) from *S. hygroscopicus* AA69-4 and *S. lividans* (pMS101).

YMA plate (containing 1.5% agar), and colonies containing *amy* were detected after 48 h by halo formation, using an overlay of KI-I_2 solution (100 mM KI, 8 mM I_2).

DNA sequence analysis. DNA sequence analysis was done by the method of Maxam and Gilbert (27). Most of the DNA sequence was determined on both strands. In regions of compression in the sequence ladder, a cytosine modification reaction (1) was carried out after the Maxam-Gilbert reactions. The secondary structure of the DNA was analyzed according to Salser (37) with the aid of the GENIAS program developed by Mitsui Knowledge Industry Ltd.

Mung bean nuclease mapping (11). RNA was isolated from *S. lividans* (pMS101) and AA69-4 by the method of Kirby et al. (22). ^{32}P-labeled DNA probes containing the *amy* promoter were prepared as described below. A *Sau*3AI fragment corresponding to nucleotides (NT) 1 to 536 in Fig. 5 was subcloned in the *Bam*HI site of pUC12 to generate pMSA243. A *Hin*dIII-*Rsa*I fragment and a *Hin*dIII-*Bst*NI fragment, which contained the upstream 5′ region of *amy*, were purified from pMSA243. The fragments were dephosphorylated and 5′ ends were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled fragments were digested with *Sau*3AI. The *Sau*3AI-*Rsa*I (1 to 231 NT in Fig. 5) and *Sau*3AI-*Bst*NI (1 to 349 NT in Fig. 5) fragments were used as probes. RNA (60 μg) prepared from *Streptomyces* strains was mixed with 50 to 100 ng of the probe DNA in 30 μl of hybridization buffer {40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% [vol/vol] deionized formamide}. The samples were

TABLE 2. Amylase activities of recombinant strains of *Streptomyces* sp.[a]

Strain	Amylase activity (U/ml) with given plasmid			
	None	pIJ702	pMS101	pMS134
S. lividans	<10	<10	1,940	210
S. hygroscopicus SF-1084	840	280	11,200	NT
S. hygroscopicus AA69-4	8,600	3,700	37,700	NT

[a] Cells were grown at 31°C for 3 days in YMA medium in the presence of 5 μg of thiostreptone per ml. The culture supernatants were collected by centrifugation, and α-amylase activities were assayed as described in Materials and Methods. The values represent units of amylase activity per milliliter of culture supernatant. NT, Not tested.

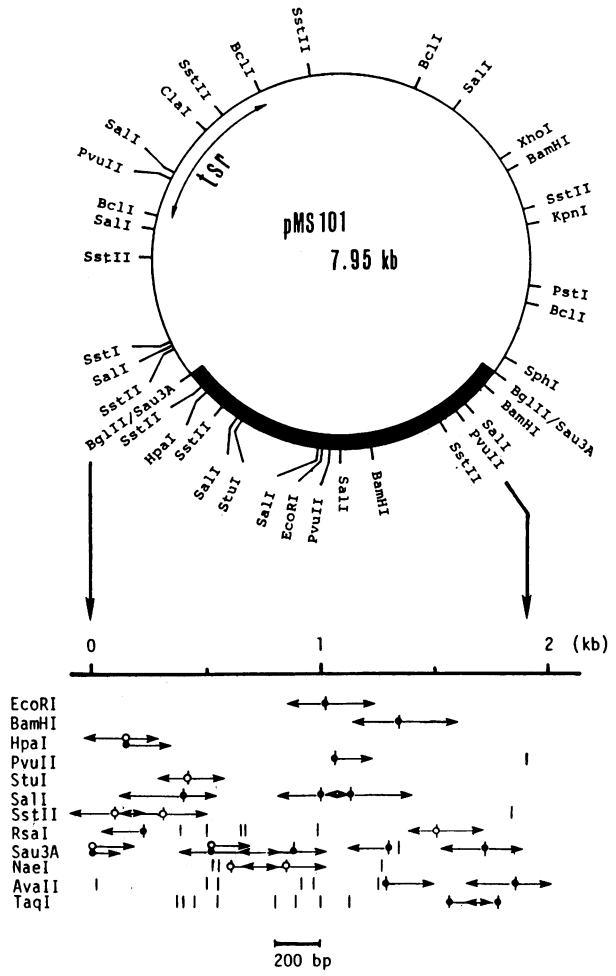

FIG. 4. Structure of plasmid pMS101 with relevant restriction sites (a) and restriction map of *amy* and sequencing strategy of the *Sau*3AI-*Sst*II (1,842-base pair [bp]) fragment (b). (a) A heavy arc indicates DNA fragment of *S. hygroscopicus* AA69-4, which contained the *amy* gene. (b) Restriction sites end labeled with ^{32}P and the sequence determined from each restriction site are presented. The closed circles indicate 5'-end-labeling sites and the open circles are 3'-end-labeling sites.

heated at 90°C for 10 min and gradually cooled over 2 h to 65°C. After 4 more hours of hybridization at 65°C, 300 μl of ice-cold mung bean nuclease digestion buffer (280 mM NaCl, 4.5 mM zinc acetate, 30 mM sodium acetate, pH 4.4, 50 μg of single-stranded salmon sperm DNA per ml) containing 175 U of mung bean nuclease was added, and samples were quickly mixed and chilled on ice. After the samples were incubated at 37°C for 40 to 60 min, 75 μl of the stop buffer (2.5 M ammonium acetate, 50 mM EDTA, 20 μg of tRNA per ml) was added. The nucleic acids were precipitated by addition of an equal volume of isopropanol, rinsed with ethanol, dried, suspended in 80% (vol/vol) formamide–10 mM NaOH–0.1% xylenecyanol–0.1% bromophenol blue, and then loaded on 8% polyacrylamide–8.3 M urea gels.

RESULTS

Amino acid sequence analysis and synthesis of oligonucleotide probes. Analysis of the native α-amylase revealed 40 amino acid residues from the NH_2 terminus beginning with NH_2-$\overset{1}{\text{Thr}}$-$\overset{2}{\text{Pro}}$-$\overset{3}{\text{Pro}}$-$\overset{4}{\text{Gly}}$-$\overset{5}{\text{Gln}}$-$\overset{6}{\text{Lys}}$-$\overset{7}{\text{Thr}}$-$\overset{8}{\text{Val}}$-$\overset{9}{\text{Thr}}$-$\overset{10}{\text{Ala}}$, etc. The corresponding mRNA nucleotide sequence (2 to 9) is CCX-CCX-GGX-CAPu-AAPu-ACX-GUX-ACX (X = A, U, G, or C; Pu = purine nucleotide). Since the third letter of a triplet codon is usually restricted to dC or dG in *Streptomyces* genes due to the high G+C content of its DNA (4, 34, 38), the A probe was synthesized by using dC and dG as the third letter. The B probe was synthesized by using all possible combinations of the codon. The A and B probes were d(CC$^{C}_{G}$ CC$^{C}_{G}$ GG$^{C}_{G}$ CAG AA) and d(CAPu AAPu ACX GTX AC), respectively.

Cloning of the α-amylase gene in *E. coli*. Probes A and B were used to identify transformants containing *amy*. Plasmid was extracted from candidates identified by a low-stringency colony hybridization screen (24). These plasmids were used in a dot hybridization protocol in which the stringency was gradually increased. At 46°C, both probes hybridized to 0.25% of the original isolates. Three of these isolates, designated pSH1, pSH2, and pSH3, contained an 8.2-kb *Sst*I fragment and an 11.2-kb *Bgl*II fragment. Further Southern hybridization experiments revealed that an *Hpa*I-*Stu*I fragment (153 to 418 NT in Fig. 5) hybridized with both probes. Expression of *amy* in *E. coli* containing the cosmids was not detected on the KI-I_2 plate. Expression was observed, however, when the *Hpa*I-*Sst*I fragment (2.7 kb) was subcloned behind the *lac* promoter of pUC12. Since *amy* expression was isopropyl-β-D-thiogalactopyranoside dependent (Table 1), we concluded that transcription was initiated from the *lac* promoter and proceeded from the *Hpa*I site towards the *Sst*I site (Fig. 1a). The transformant harboring this plasmid (pMS120) accumulated α-amylase activity in its periplasmic fraction (Table 1).

Subcloning of the amylase gene in *Streptomyces* sp. An 8.2-kb *Sst*I fragment and an 11.2-kb *Bgl*II fragment found in all of the plasmids which hybridize to probes A and B were isolated from pSH1. The fragments were mixed, digested with *Sau*3AI, and then ligated into the *Bgl*II site of pIJ702 (Fig. 1b). The ligation mixture was used to transform *S. lividans*. Transformants containing an insertionally inactivated vector (thiostreptone resistant, melanine negative) were screened for amylase activity by iodine staining. Plasmids isolated from colonies containing halos were designated pMS101, pMS102, pMS104, and pMS109 (Fig. 2). The partially digested *Sau*3AI fragments of the *Sst*I and *Bgl*II fragments were also ligated into the *Bgl*II site of pMS201. This ligation mixture was used to transform *S. lividans*; pMS134 was isolated from an α-amylase-positive colony (Fig. 1b and 2). The relative α-amylase productivities of *S. lividans* strains containing these plasmids are summarized in Fig. 2. pMS101 contained a 2.3-kb *Sau*3AI fragment which was sufficient for the expression of *amy* in *S. lividans*. The purified α-amylase from *S. lividans* (pMS101) was indistinguishable by SDS-polyacrylamide gel electrophoresis from the α-amylase produced by *S. hygroscopicus* AA69-4 (Fig. 3). When pMS101 was introduced into *S. hygroscopicus* AA69-4, α-amylase productivity was increased fourfold (Table 2).

DNA sequence. The 1,842-base pair DNA fragment containing *amy* was sequenced by the Maxam and Gilbert technique. The restriction cleavage map and sequence strategy are shown in Fig. 4. Starting from ATG codon at 264 NT, there was an open reading frame of 1,434 base pairs terminating TAG at 1,698 NT. The size of the predicted gene product (M_r 47,980) agrees well with the molecular weight of α-amylase estimated by SDS-polyacrylamide gel electropho-

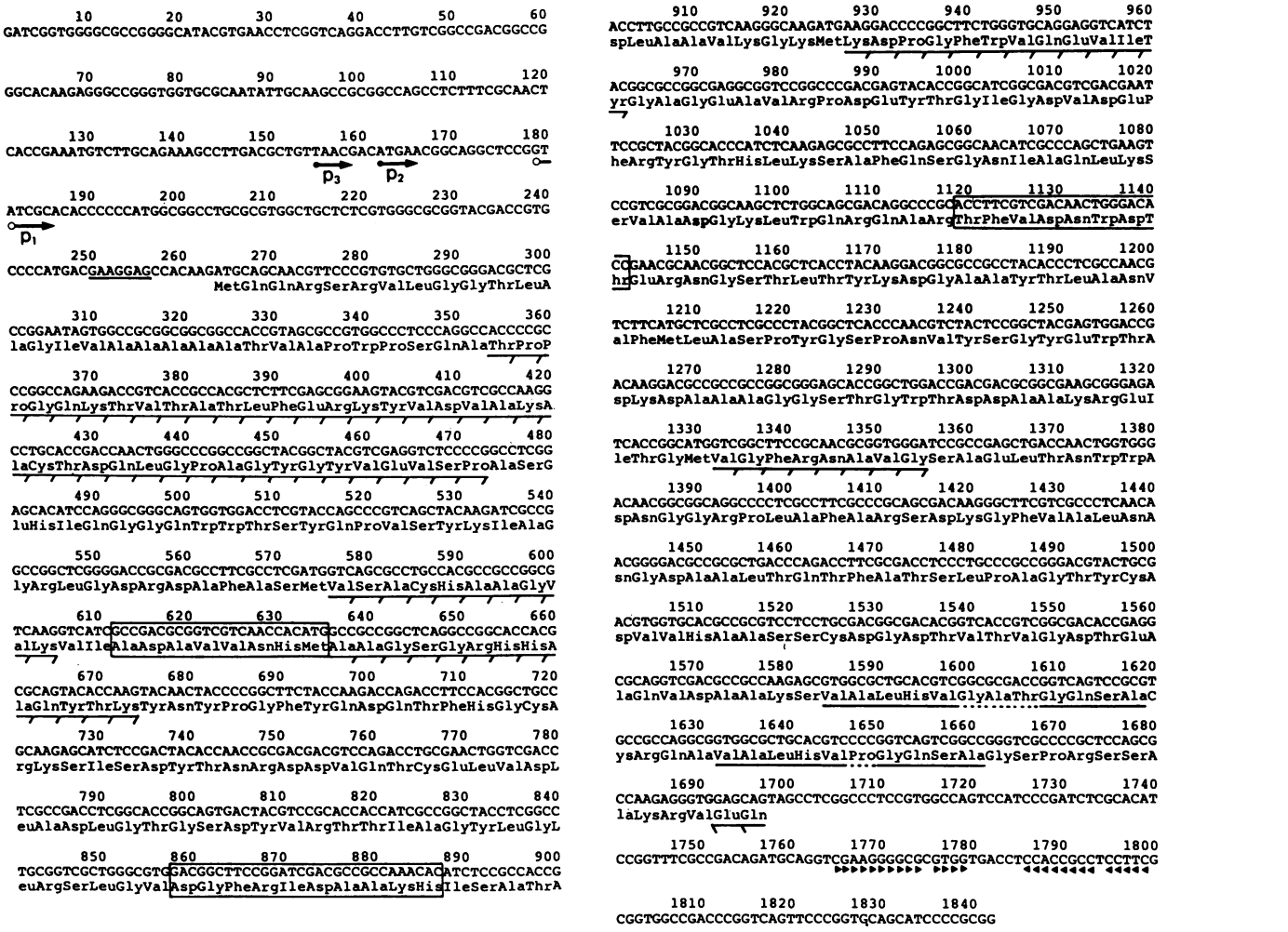

FIG. 5. DNA and amino acid sequence of *amy*. The DNA sequence is presented from *Sau*3AI (1 NT) to *Sst*II (1,842 NT). The peptide sequence data are indicated as follows: Edman degradation (→); carboxypeptidase Y (←). Conserved regions of α-amylase found in *amy* are boxed. A putative ribosomal binding site (SD) is underlined. A repeated stretch of the amino acid sequence is also underlined (residues 441 to 452 and 457 to 466). The palindromic structure of the 3′ noncoding region is shown by closed triangles (▶,◀). Transcriptional initiation sites (p_1, p_2, p_3) are indicated by arrows.

resis (M_r 47,500). The amino acid sequence deduced from the nucleotide sequence agreed with the partial NH_2-terminal amino acid sequence of α-amylase and the amino acid composition of α-amylase (13) (data not shown). The NH_2-terminal amino acid sequence of four internal peptides confirmed the nucleotide and internal reading frame of *amy* (Fig. 5). Finally, carboxy-terminal amino acid sequencing suggested the location of the 3′-translation termination site (Fig. 5). The *amy* gene also showed a codon usage typical of *Streptomyces* genes. The nonrandom condon usage reflects a preference for G or C in the third base of the triplet (4).

Mung bean nuclease mapping. The transcriptional initiation site of *amy* was determined by mung bean nuclease mapping (Fig. 6). A major and a minor protected DNA species (*amyP2*, *amyP3*) were identified with RNA preparations from *S. lividans* (pMS101) and *S. hygroscopicus* AA69-4 grown to logarithmic and early stationary phases. Two other minor DNA species (*amyP1*) which differed in length by only 2 NT were protected by RNA from late logarithmic- or early stationary-phase cultures of *S. hygroscopicus* AA69-4. These could be minor transcription start signals or an artifact of RNA degradation in the early stationary phase of *S. hygroscopicus*. The *amyP1* transcript(s) could be detected only in trace amounts in *S. lividans* and in the logarithmic phase of *S. hygroscopicus*.

DISCUSSION

Our laboratory is interested in the control of gene expression and the secretion of proteins in *Streptomyces* spp. We have chosen to study the α-amylase system, since we are interested in the overproduction of α-amylase for industrial use and have accumulated a collection of mutant strains. The *amy* gene of *S. hygroscopicus* AA69-4 was first cloned in *E. coli* on a plasmid; however, *amy* expression was not observed. This barrier to expression of *Streptomyces* genes in *E. coli* has been previously observed (3, 17, 35). Subcloning experiments showed that *amy* could be expressed from the *lac* promoter and the product was secreted into the periplasmic space. *Streptomyces* species containing the *amy* on a high-copy-number plasmid secreted increased amounts of α-amylase into the medium. The levels of expression may be

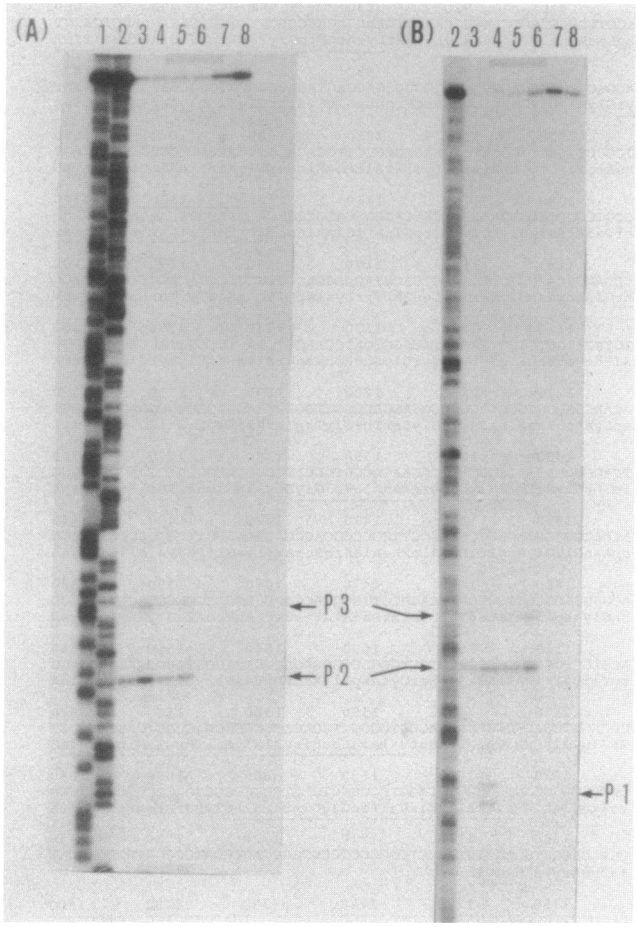

FIG. 6. Mung bean nuclease mapping analysis of *amy* promoter. Experiments were carried out as described in Materials and Methods. *Sau*3AI-*Rsa*I (1 to 231 NT) and *Sau*3AI-*Bst*NI (1 to 349 NT) fragments 5′ labeled at *Rsa*I and *Bst*NI sites were used as hybridization probes to identify the transcriptional start points. As controls, DNA probes alone or with RNA from *S. lividans* carrying pIJ702 were treated as the samples were. (A) *Sau*3AI-*Rsa*I fragment as hybridization DNA probe. (B) *Sau*3AI-*Bst*NI fragment as hybridization DNA probe. (Lane 1) Maxam-Gilbert G+A sequence reactions; (lane 2) Maxam-Gilbert C+T sequence reactions; (lane 3) fragment protected by RNA from *S. hygroscopicus* AA69-4 (logarithmic growth phase); (lane 4) fragment protected by RNA from *S. hygroscopicus* AA69-4 (early stationary phase); (lane 5) fragment protected by RNA from *S. lividans* (pMS101) (logarithmic growth phase); (lane 6) fragment protected by RNA from *S. lividans* (pMS101) (early stationary phase); (lane 7) without RNA; (lane 8) fragment protected by RNA from *S. lividans* (pIJ702) (early stationary phase).

due to a gene dosage effect, since *S. lividans* containing the *amy* on a low-copy-number plasmid produced less amylase activity. The gene dosage effect of *amy* has also been observed in *S. hygroscopicus* AA69-4 harboring pMS101. Kendall and Cullum reported the cloning and expression of an extracellular agarase gene in *S. lividans* (21). They found that the agarase was overproduced in *S. lividans* (up to 500 times more than the original producer *S. coelicolor*) and exported efficiently into the medium. Production of α-amylase by *S. lividans* containing *amy* on a high-copy-number vector was lower than that by the original producer, *S. hygroscopicus* AA69-4. *S. hygroscopicus* AA69-4 may inherently, or because it was selected as an overproducer, have much more efficient mechanisms for expression and secretion of α-amylase than *S. lividans*. We have not observed significant amounts of extracellular protease which degrade amylase in culture supernatants from either *S. lividans* or *S. hygroscopicus* (unpublished observation).

Janssen et al. reported that *Streptomyces* possessed several classes of transcriptional initiation signals (19). These signals might be recognized by different RNA polymerase holoenzymes (45). We determined the transcriptional initiation sites of *amy* cloned from a highly mutagenized strain by mung bean nuclease mapping. Two initiation sites (*amyP2* and *amyP3*) were identified with RNA preparations from *S. lividans* (pMS101) and *S. hygroscopicus* AA69-4 during logariothmic to early stationary phases. Another possible transcriptional start (*amyP1*) was observed in the early stationary phase of *S. hygroscopicus* AA69-4 and was present in trace amounts in *S. lividans* (pMS101). The −10 and −35 regions of *amyP1* show similarities to the promoter region of *S. plicatus endo*H (45) and *S. erythraues ermEP1* and *ermEP2* (5). The preceding region of *amy2* and *amyP3* possessed a weak homology to *S. fradiae appP1* (19) (Table 3).

The nature of the transcriptional termination signal of *Streptomyces* sp. has been described (6, 39). By analogy to other bacteria (36), the palindoromic structure (−32.4 kcal/mol) distal to the 3′ end of *amy* may play a role in transcriptional termination.

The 30-amino acid leader sequence of α-amylase is similar to the signal sequences of other bacteria (14, 44). It is composed of a positively charged amino terminus followed by a hydrophobic core. Two helix-breaking proline residues and a charged glutamine residue are found adjacent to the cleavage site, which was identified by amino-terminal amino acid sequence of the secreted amylase.

The amino acid sequence of the *amy* structural gene indicates several conserved features. Three regions which are conserved in other α-amylases (18, 26, 30) were also found in *Streptomyces* α-amylase (Fig. 5). The strep-

TABLE 3. Comparison of *Streptomyces* promoter sequences

Promoter	Nucleotide sequence					Reference
	−25 to −40 region	Distance (bp)[a]	−10 region	Distance (bp)	mRNA start	
amyP1	TTGACG	19	CAGGCT	3 and 5	G and A	This paper
endoH	TTGACT	21	CAGGGG	6	G	45
ermEP1	TGGACA	14	TAGGAT	5	C	5
ermEP2	TTGACG	18	GAGGAT	5	G	5
amyP2	AACTCACCGAA—ATGTCT	8	GCCTTGACGC	10	A	This paper
amyP3				3	U	
aphP1	AAGGCGCGGAACGGCGTCT	8	GCCATGATGC	5	A	19

[a] bp, Base pairs.

tomycete gene is unique in that it has a tryptophan residue (Trp291) in the third conserved region. All other α-amylases studied possess histidine in this position of the third conserved region (18, 26, 30).

A stretch of 10 to 12 amino acids close to the carboxy terminus are repeated with high homology; 9 amino acid residues are identical (Fig. 5). Repeated blocks of amino acids have also been found in other streptomycete genes, including ORF438 (2) and the *ermE* gene (41).

We are ultimately interested in knowing what controls the production of secreted α-amylase during fermentation. Nucleotide comparison of this *amy* gene isolated from overproducing or nonproducing strains with the same gene isolated from the wild-type parent should help us to determine whether transcription, translation, or secretion is rate limiting.

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