APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1994, p. 3381–3389
0099-2240/94/$04.00+0

Vol. 60, No. 9

The Gene *amyE*(TV1) Codes for a Nonglucogenic α-Amylase from *Thermoactinomyces vulgaris* 94-2A in *Bacillus subtilis*

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Received 24 November 1993/Accepted 10 July 1994

We isolated the gene *amyE*(TV1) from *Thermoactinomyces vulgaris* 94-2A encoding a nonglucogenic α-amylase (AmyTV1). A chromosomal DNA fragment of 2,247 bp contained an open reading frame of 483 codons, which was expressed in *Escherichia coli* and *Bacillus subtilis*. The deduced amino acid sequence of the AmyTV1 protein was confirmed by sequencing of several peptides derived from the enzyme isolated from a *T. vulgaris* 94-2A culture. The amino acid sequence was aligned with several known α-amylase sequences. We found 83% homology with the 48-kDa α-amylase part of the *Bacillus polymyxa* β-α-amylase polyprotein and 50% homology with Taka amylase A of *Aspergillus oryzae* but only 45% homology with another *T. vulgaris* amylase (neopullulanase, TVA II) recently cloned from strain R-47. The putative promoter region was characterized with primer extension and deletion experiments and by expression studies with *B. subtilis*. Multiple promoter sites (P3, P2, and P1) were found; P1 alone drives about 1/10 of the AmyTV1 expression directed by the native tandem configuration P3P2P1. The expression levels in *B. subtilis* could be enhanced by fusion of the *amyE*(TV1) coding region to the promoter of the *Bacillus amyloliquefaciens* α-amylase gene.

A number of thermostable enzymes have been isolated from *Thermoactinomyces vulgaris* strains. Among these were thermitase (4, 13, 14, 28, 29), a protease from the subtilisin family (2, 5, 36), and a variety of α-amylases (pullulanases). The α-amylases of strains R47 and 42, like fungal glucoamylases (1, 15, 49, 54), hydrolyze starch and pullulan (1, 54). The α-amylase of *T. vulgaris* 94-2A, however, utilizes only starch and glycogen as substrates, not pullulan. α-Amylase 1 of *T. vulgaris* 94-2A (AmyTV1) is a protein of 53 kDa and was previously shown to exhibit striking homology to Taka amylase A of *Aspergillus oryzae* for a short N-terminal sequence (22, 60, 67). Smaller peptides of 33 and 18 kDa have been shown to be products of limited AmyTV1 proteolysis (21). The AmyTV1 amylase is unusual because of its temperature optimum at 62.5°C in a low pH range (4.8 to 6), its relatively short half-life of about 5 min at 70°C, and the production of maltose and maltotriose in the hydrolysate, which lacks glucose (47).

In order to overcome problems of poor growth and product yield in *T. vulgaris* 94-2A, we cloned the gene encoding the AmyTV1 amylase and performed expression studies with *Bacillus subtilis*. The transcription start site(s) of the gene in *B. subtilis* was studied after deletion of the 5′-flanking region and after its replacement with the promoter of the *B. amyloliquefaciens* α-amylase gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The *T. vulgaris* strain used was originally isolated and described by Klingenberg et al. (29, 47). The strain 94-2A was selected for higher enzyme production (29). The EMBL3 lambda phage (12) was used for preparation of a *T. vulgaris* DNA bank in *Escherichia coli* MC1061 (6). The bacterial strains and plasmids used are listed in Table 1.

Media, culture conditions, and transformation. The media used in this study were TBY (10 g of tryptone [Difco] per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter, adjusted to pH 7.2) and NBY (10 g of peptone per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter, adjusted to pH 7.2). NBY and TBY starch agar media contained 2.3% agar and 1% insoluble starch (Maisan). Mineral salts mixture (9) was the minimal medium and contained the following ingredients, with the amounts per liter indicated: K_2HPO_4, 30 g; KH_2PO_4, 10 g; NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 100 mg; $MnSO_4 \cdot 4H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; and $CaCl_2$, 5 mg. The pH was adjusted to 6.8 to 7.0. *T. vulgaris* 94-2A was grown at 55°C, while other bacterial cultures were incubated at 37°C. The EMBL3 phage was plated on TBY starch agar by using a soft agar overlay with 1% amylopectin azure (Sigma Chemie GmbH, Deisenhofen, Germany) for identification of positive amylase clones. When they were required, the following antibiotics were added as supplements: ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), and erythromycin (50 μg/ml) for *E. coli* and chloramphenicol (5 μg/ml) and erythromycin (1 μg/ml) for *B. subtilis*.

E. coli was transformed by the method of Hanahan (20). Plasmid DNA was introduced into *B. subtilis* by using either competent cells (18) or the protoplast transformation method (7), in which the DM3 plating medium contained 0.5% insoluble starch (Maisan).

Enzyme assay. The amylase was recovered from supernatants of *B. subtilis* GSB26 cultures harboring the respective plasmid derivatives or from a culture of *T. vulgaris* 94-2A after growth at 37°C or 55°C, respectively, in 2× TBY medium. Cell-free (supernatant) samples were taken after the addition of 1 mM $CaCl_2$ assayed by automated reading in microtiter plates. Ten-microliter samples of respective dilutions in triplicate were mixed with 50 μl of amylase reagent from a diagnostic kit (Sigma Chemie GmbH), inoculated into micro-

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titer plate vials, and incubated for 10 min at 37°C. The reaction was stopped by the addition of 25 μl of 1 M $NaCO_3$ solution. The reading of A_{405} was done with an EL312e Microplate Reader (BIO-TEC Instruments). Each measure was repeated at least three times. Amylase activities are expressed as units as defined by the assay used.

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, Klenow DNA polymerase, T4 DNA kinase, RNase-free DNase, lysozyme, alkaline phosphatase, and pronase P were purchased from Boehringer GmbH, Mannheim, Germany. Avian myeloblastosis virus (AMV) reverse transcriptase and RNasin were obtained from Promega Biotec, Madison, Wis. The enzymes and reagents were used according to the manufacturer's recommendations. Antibiotics and ethidium bromide were obtained from Serva, Heidelberg, Germany. The radiochemicals [α-^{35}S]ATP and [γ-^{32}P](ATP), as well as the standard proteins (the rainbow marker), were purchased from Amersham Buchler GmbH, Braunschneig, Germany.

DNA preparation. Chromosomal DNA of *T. vulgaris* 94-2A was isolated according to standard protocols (50) with some modifications. One gram of wet cells was resuspended in 18 ml of Tris-EDTA-glucose (50 mM glucose, 25 mM Tris-HCl [pH 8], 10 mM EDTA) and was treated with lysozyme (10 mg/ml, 1 h, 37°C). Sodium dodecyl sulfate (SDS) (1%) was then added, and the incubation was continued (10 min, 65°C). Pronase P was added (1 mg/ml), and the solution was incubated for 80 min at 50°C. The nucleic acids were precipitated by ethanol and were pooled on a glass rod. The nucleic acids were dissolved in 10 ml of Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The pronase treatment was repeated (200 μg/ml, 1 h, 50°C) and was followed by phenol extraction. The aqueous phase was then treated with phenol-chloroform (1 volume of phenol-chloroform [1:1], 5 min, room temperature) and with chloroform (1 volume, 5 min, room temperature). The nucleic acids were precipitated and spooled on a glass rod and washed and dissolved in 5 ml of Tris-EDTA. The precipitate was treated with RNase (100 μg/ml, 30 min, 37°C) and was again purified by cycles of extraction with phenol, phenol-chloroform, and chloroform.

Plasmid and phage DNA from *E. coli* was prepared either as described by Sambrook et al. (50) or by using the QIAGEN plasmid minikit from DIAGEN GmbH, Düsseldorf, FRG. Single-stranded template DNA from pBS$^+$ vectors was isolated according to the method of Vieira and Messing (63).

Rapid plasmid DNA preparations from *B. subtilis* were made from 1.5-ml culture aliquots as described by Hofemeister et al. (25). Large-scale purification of plasmid DNA from *B. subtilis* was performed according to the method of Gryczan et al. (18).

RNA isolation. *B. subtilis* cells were grown in mineral salts mixture medium supplemented with Casamino Acids (0.01%), tryptophan (100 μg/ml), maltose (0.5%) as the carbon source, and chloramphenicol (5 μg/ml) for plasmid selection. Five-milliliter samples were taken from 100-ml cultures after 5, 6, 8, and 9 h of vigorous shaking (250 rpm). The RNA was prepared as described by Oelmüller et al. (42). The final RNA pellet, after DNase treatment, was dissolved in diethyl pyrocarbonate-treated water, quantified by spectrophotometry, and stored at −80°C until used.

DNA sequencing. DNA sequence analysis was performed by the dideoxy chain termination method (51). After subcloning of fragments of appropriate sizes in pBS vectors (plasmid pBS.TV7.4 or pBS.TV7.2), both strands were sequenced by using either single- or double-stranded DNA. A T7 Sequenase kit and protocols from Pharmacia LKB Biotechnology were used.

Primer extension. The method of primer extension described by Moran et al. (38) was used. The buffer used for reverse transcription was from Promega, to which the deoxynucleoside triphosphates (dNTPs) (1 mM each) and RNasin (2 U/μl) were added before use. The synthetic primer oligonucleotide used was 5′-CGACAATAAACACACCAACCCAC TGAGCAAAGCTCC-3′.

PCR, promoter deletions, and fusions. The DNA fragments amyTV-0, amyTV1, and amyTV21, stepwise deleted in the putative *amyE*(TV1) gene promoter regions, were constructed by using standard PCR and the following synthetic oligonucleotides: PTV0 (5′-GTTTGCGAGATCTGGGAATGAAAG-3′ [translation initiation sequence is underlined]), PTV1 (5′-GAG TAAACAGATCTTAAATAGTGCAAACG-3′), and PTV21 (5′-GTTGTGTGATAGATCTAGTGGCAGGGAGTTC-3′) for 5′-end priming and TV02 back (5′-GGTGCTCAGATCT CTGGGTGGATCAC-3′) for priming at the 3′-end. The cut plasmid pHP.TV3r DNA was the template. The PCR fragments, were either, after *Bgl*II cutting, cloned into the *Bam*HI site of plasmid pHP13 to give pHP.TV21, pHP.TV1, and pHP.TV0 and then sequenced in order to confirm the proposed structure or cloned into the *Bcl*I site of plasmid pGB354 to construct the respective pGB.TV derivatives for expression studies with *B. subtilis*.

The *B. amyloliquefaciens* α-amylase *amyE*(BA) gene promoter (see Fig. 5B) was fused to the ATG codon of the *amyE*(TV1) gene via PCR recombination (24). PCR-generated fragments A and B, which overlap in a sequence at the ATG translation start codons, were used. The *amyE*(BA) gene DNA (56) was used as a template to generate a 262-bp fragment A, by PCR using oligonucleotide BA1 front (5′-GCATCACT GGATCCGCCCCGCACATTCGAAAAGACTGGC-3′) for priming at the 5′ end and the hybrid oligonucleotide BA.TVatg (5′-GGGAGAGGAAACAATGAAAAAGGGAGCTTTG CTCAG-3′) for priming at the 3′ end. Fragment B was made by PCR using the *amyE*(TV1) gene DNA (on vector pHP. TV3r) as a template and the hybrid oligonucleotide TV.BAatg (5′-CTGAGCAAAGCCCCTTTTTATGTTTCCTCTCCC-3′) and oligonucleotide TV02 back for priming at the 5′ and 3′ ends, respectively. The overlapping fragments A and B were subsequently combined by a second round of PCR using oligonucleotides BA1 front and TV02 back for priming at the 5′ and 3′ ends of the hybrid fragment AB, respectively. The resulting hybrid fragment AB, after restriction endonuclease cutting by *Bam*HI or *Bgl*II at the primer BA1 front or TV02 back site, respectively, was cloned into either the *Bam*HI-cut vector pHP13 or the *Bcl*I-cut vector pGB354, to create the pHP.BRI and pGB.BRI plasmids, respectively (Table 1).

Protein sequencing. The method used for protein sequencing was described elsewhere (21, 22).

Polyacrylamide gel electrophoresis (PAGE) and zymography. Cultures were grown for 20 h at 37°C in twice-concentrated TBY. One-milliliter samples were withdrawn and centrifuged at 6,000 rpm in order to remove the cells, and 100 μl of the supernatant was mixed into three-times-concentrated loading buffer, boiled for 3 min, and immediately loaded onto 10% (wt/vol) polyacrylamide slab gels containing 0.1% SDS (32). For detection of amylase activities after PAGE, we used the following zymogram technique. The gels run under denaturing conditions were renatured by extraction with 0.02 M sodium acetate buffer (pH 6) for about 2 h. The gels were subsequently overlaid with 0.8% agarose dissolved in 0.02 M sodium acetate buffer (pH 6) containing 1% insoluble starch (Maisan). The gel was kept at 37°C in a wet chamber for a few

TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Description	Reference(s) or source
Strains		
Thermoactinomyces vulgaris 94-2A	Selected for higher enzyme production	29, 47
Escherichia coli K-12 strain MC1061		6
E. coli K-12 strain DH5α		20
Bacillus subtilis GSB26	*strR* mutant of strain QB1133 (*aroI906 metB6 sacA321 amyE*)	56, 57
Plasmids		
pBS$^+$	Cloning vector, Ap^r[a]	Stratagene
pBS.TV7.2	pBS$^+$ containing a 2.2-kb *Hin*dIII fragment[b]	This work
pBS.TV7.4	pBS$^+$ containing a 1.4-kb *Hin*dIII fragment[b]	This work
pHP13	Cloning vector, Cm^r[c] Em^r[d]	19
pHP.TV3.2	pHP13 containing the 4.5-kb fragment shown in Fig. 1	This work
pHP.TV1L	pHP13 containing a 2.2-kb *amyE*(TV1) fragment[b] in left-hand orientation to *lacZ'*P	This work
pHP.TV3r	pHP13 containing a 2.2-kb *amyE*(TV1) fragment[b] in right-hand orientation to *lacZ'*P	This work
pHP.BRI	pHP13 containing the BRI.amyTV-0 fragment[e]	This work
pGB354	Cloning vector, Cm^r	3
pGB.TV321	pGB354 containing the amyTV321 fragment[e]	This work
pGB.TV21	pGB354 containing the amyTV21 fragment[e]	This work
pGB.TV1	pGB354 containing the amyTV1 fragment[e]	This work
pGB.TV-0	pGB354 containing the amyTV-0 fragment[e]	This work
pGB.BRI	pGB354 containing the BRI.amyTV-0 fragment[e]	This work
Phage		
EMBL3		12

[a] Ap^r, ampicillin resistance.
[b] See Fig. 1.
[c] Cm^r, chloramphenicol resistance.
[d] Em^r, erythromycin resistance.
[e] See Fig. 5.

hours to detect clearing zones. One-half of the gel was stained in order to visualize protein bands with Coomassie brilliant blue G250 (50).

Computer analysis. Analysis of sequencing data for open reading frames, multiple alignment, and searches for codon usage and of amino acid composition for potential signal sequences and secondary structures were all performed by using the PC/GENE software system of IntelliGenetics Inc. (serial no. IGI2952) on an ESCOM 386 personal computer. The amino acid sequences used for the alignment were taken from the original papers or from EMBL/GenBank. The GenBank accession numbers are as follows: P00691, *Bacillus subtilis*; P00692, *Bacillus amyloliquefaciens*; P06278, *Bacillus licheniformis*; P20845, *Bacillus megaterium*; P21543, *Bacillus polymyxa*; P08137, *Bacillus circulans*; P06279, *Bacillus stearothermophilus*; and P10529 and P11763, *Aspergillus oryzae*.

Nucleotide sequence accession number. The DNA sequence data of the 2,247-bp DNA fragment reported here have been submitted to the EMBL Data Library and have been assigned the accession number X69807.

RESULTS

Cloning of the *amyE*(TV1) gene of *T. vulgaris* 94-2A. High-molecular-weight chromosomal DNA from *T. vulgaris* 94-2A was partially digested with *Sau*3A and was fractionated by sucrose gradient centrifugation in order to obtain fragments 12 to 20 kb in size. The fragmented DNA was subsequently ligated with the *Bam*HI-cut and alkaline phosphatase-treated arms of the EMBL3 phage vector and was packaged and propagated in *E. coli* MC1061 as described by Sambrook et al. (50). The plating efficiency of the phage progeny on strain

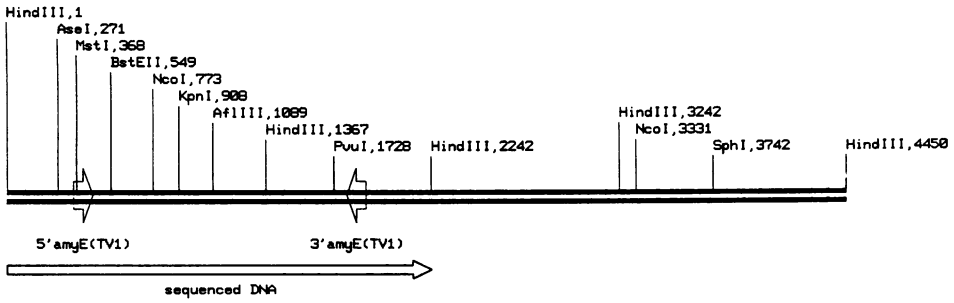

FIG. 1. Restriction map of the 4.45-kbp DNA fragment of *T. vulgaris* 94-2A cloned on plasmid pHP.TV3.2. The 5' and 3' ends of the *amyE*(TV1) gene region coding for α-amylase are marked by arrows. The DNA from *Hin*dIII nucleotide 1 up to *Hin*dIII nucleotide 2242 has been sequenced (see Fig. 2).

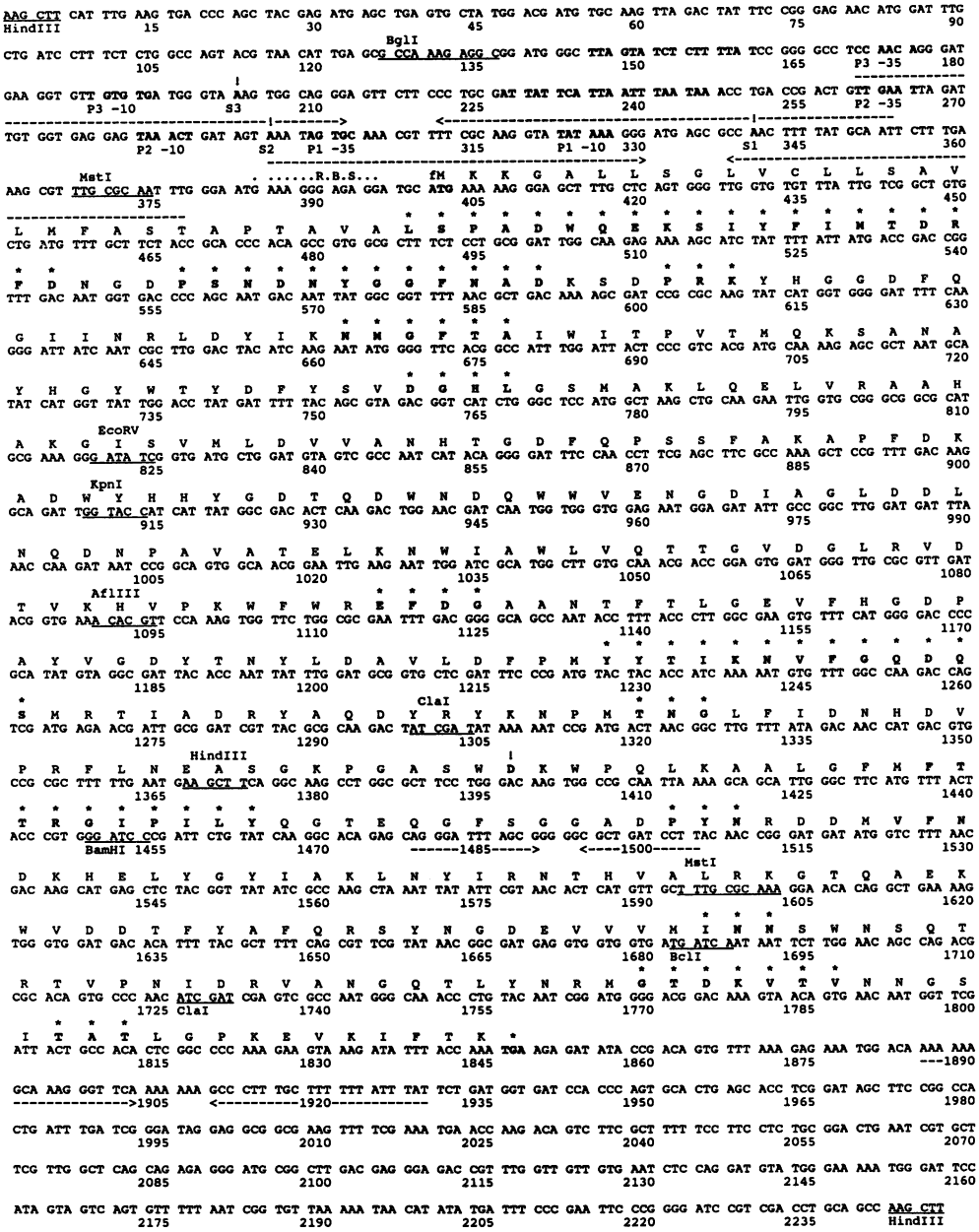

FIG. 2. The nucleotide sequence of a DNA fragment containing the promoter(s), open reading frame, and terminator regions of the *amyE*(TV1) gene of *T. vulgaris* 94-2A. The converging arrows indicate putative regions of self-complementarity. The vertical (!) symbols point to the putative transcription start sites S1, S2, and S3 (see Fig. 4). The putative promoters are shown in boldface. The amino acid sequences marked by asterisks were found to be identical to peptides sequenced from extracellular AmyTV1 protein. The N terminus of the extracellular (mature) AmyTV1 was previously (22) shown to have the amino acid sequence L-S-P-A-D-W, etc.

MC1061 was about 5×10^9 PFU/ml. About one of 200 plaques showed white zones after being plated on strain MC1061 by using soft agar containing amylopectin azure (1%) for detection of amylase expression clones. A total of six Amy^+ phage clones (w1 to w6) were isolated and found to contain DNA inserted by *Sal*I digestion. The DNA from phage w3 was purified, digested with *Eco*RI, ligated into *Eco*RI-cut vector pHP13, and transformed into *E. coli* DH5α for detection of Amy^+ colonies. After plasmid DNA isolation and transformation into *B. subtilis* GSB26, only a few (about 5%) transformants showed amylase activity, apparently because of genetic instability of the insert DNA. After screening for stable Amy^+ progeny, the plasmid pHP.TV3.2 was finally chosen for restriction mapping and subcloning experiments. The 4.5-kb DNA insert of the plasmid (Fig. 1) is composed of four *Hin*dIII fragments 1.4, 0.8, 1.0, and 1.2 kb in size. After subcloning of the *Hin*dIII fragments into the Bluescript vector pBS^+, it was shown that both the 1.4- and the 0.8-kb fragments (on plasmid pBS.TV7.2) were essential to obtain synthesis of an additional protein of 53 kDa as well as zymographic detection of α-amy-

TABLE 2. Comparison of the four highly conserved regions I, II, III, and IV in AmyTV1 of *T. vulgaris* 94-2A and of two recently identified (neopullulanase-type) α-amylases, TVA I and TVA II, from *T. vulgaris* R-47 (61)[a]

Amylases	Strain	Region I	Region II	Region III	Region IV
AmyTV	*T. vulgaris* 94-2A	**DVVANHTGD**	**DGLRVDTVKHVPKWFWREF**	**GEVFHG**	FIDN**HD**
TVA I	*T. vulgaris* R-47	**DGVFNH**???	?**GWRLDAAQY**?????????	?**EYWG**?	FLSN**HD**
TVA II	*T. vulgaris* R-47	**DAVFNHAGD**	**DGWRLDVANEVDHAFWREF**	**GEIWHD**	LLDS**HD**

[a] Boldface type indicates amino acids conserved in all three sequences.

lase activity from *E. coli* cultures after SDS-PAGE. The 1.4-kb fragment was tested for homology (i) with the DNA inserts of the other five detected EMBL3 phage clones and (ii) with chromosomal DNA of *T. vulgaris* 94-2A. The other five phage clones, w1, w2, w3, w4, and w5, which also conferred the Amy$^+$ phenotype, were cut with *Hin*dIII, blotted, and hybridized by using the 1.4-kb *Hin*dIII fragment as a probe. Only one distinct fragment of each DNA insert hybridized with the 1.4-kb probe, indicating that similar DNA inserts were present in all six EMBL3 phage clones. By Southern blotting the probe was found to hybridize with only one 1.4-kb fragment of the *Hin*dIII-digested chromosomal DNA of *T. vulgaris* 94-2A (data not shown).

For further expression studies, the 2.2-kb insert of pBS.TV7.2, after *Bam*HI cutting, was isolated and subcloned either into the *Bam*HI site of vector pHP13 to give derivatives pHP.TV1L and pHP.TV3r or into the *Bcl*I-linearized vector pGB354 to give pGB.TV321. The former contain the insert DNA in both orientations relative to the *lacZ* gene promoter.

Nucleotide sequence of the gene *amyE*(TV1). The DNA insert on plasmid pBS.TV7.2 or pBS.TV7.4 was sequenced by the dideoxy chain termination method. The sequence of the 2,247-bp DNA fragment is shown in Fig. 2. The DNA has an overall G+C content of 48.2% and exhibits an open reading frame comprising 489 codons. Homology of the deduced protein was confirmed (i) by comparison either with the already known N terminus (22) or with newly sequenced tryptic or CNBr-cut peptides from AmyTV1 and (ii) by the alignment with known *Bacillus* and other amylase sequences.

The mature AmyTV1 protein had previously been found by peptide sequencing to have the N-terminal amino acid sequence L-S-P-A-D-W (22). The deduced amino acid sequence is, however, preceded by an N-terminal extension that is likely to function as a signal peptide for amylase secretion. Since the next upstream ATG codon overlaps a putative ribosome binding site (RBS), the gene is assumed to start from the ATG codon at bp 400 (Fig. 2). The proposed signal peptide would thus be 29 amino acids in length. The sequence pattern A-V-A-/-L-S-P occurs at the proposed processing site, and the hydrophobicity profile (data not shown) has a good fit to the compilation of known signal peptides (53, 65, 66).

The deduced sequence of the amylase protein did perfectly confirm the sequences of 15 different tryptic or CNBr peptides, which are marked in Fig. 2 by asterisks. The secreted amylase is therefore proposed to consist of 453 amino acid residues and to have a calculated molecular mass of 51,661 Da. These data confirm the suggestion that we have cloned the gene encoding the AmyTV1 protein (22).

The gene *amyE*(TV) is thus proposed to comprise 483 codons, i.e., to code for a deduced precursor protein of 482 amino acids with a calculated molecular mass of 54,483 Da.

Homology of AmyTV1. The alignment of the predicted amino acid sequence of AmyTV1 with the recently published neopullulanase sequence TVA II of *T. vulgaris* R-47 (61) indicated 29% identical and 16% similar residues, as well as numerous substitutions in the conserved regions I to IV. This is also true for the conserved regions known for a second but only partially sequenced amylase, TVA I, of the same *T. vulgaris* strain (Table 2). Homologies were further screened for proteins in the SWISS and EMBL protein databases by using the CLUSTAL program. The highest homologies were detected with the 48-kDa α-amylase part of the β-α-amylase polyprotein of *B. polymyxa* (62) and to a lesser extent with Taka amylase of *A. oryzae* (60, 67). The percentages of homology (the identity values are given in parentheses) of AmyTV1 to these α-amylases were 83% (74.2%) and 50.6% (38.7%), respectively. There are only two minor gaps distinguishing AmyTV1 from the 48-kDa α-amylase of *B. polymyxa* (data not shown). The homology with Taka amylase was found to be largely limited to the N terminus of the mature proteins and to the highly conserved regions I, II, III, and IV of the amylase family (27, 40, 47, 58). Like other Taka-type amylases, the AmyTV1 protein lacks a distinct internal sequence, the so-called B domain loop, which is thought to characterize a few amylases, e.g., *B. amyloliquefaciens* and *B. licheniformis* α-amylases (64).

A dendrogram of relatedness of AmyTV1 with various *Bacillus* α-amylases, i.e., those of *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus*, *B. polymyxa*, *B. circulans*, and *B. megaterium*, as well as with Taka amylase A, is shown in Fig. 3. The homology with α-amylases of the actinomycetes

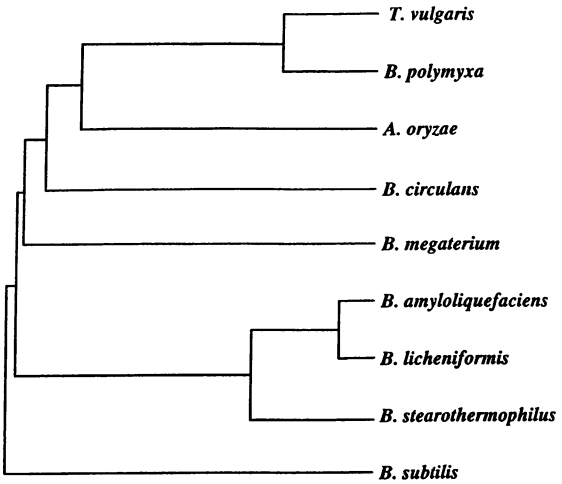

FIG. 3. Dendrogram of relatedness of AmyTV1 from *T. vulgaris* 94-2A to α-amylases from *B. polymyxa* (GenBank accession number P21543), *B. stearothermophilus* (P06279), *B. licheniformis* (P06278), *B. amyloliquefaciens* (P00692), *B. megaterium* (P20845), *B. subtilis* (P00691), *B. circulans* (P08137), and Taka amylase A of *A. oryzae* (P10529 and P11763) done by the CLUSTAL program with the following setting parameters: gap penalty, 5; window size, 10; filtering level, 2.5; and open and unit gap cost, 10.

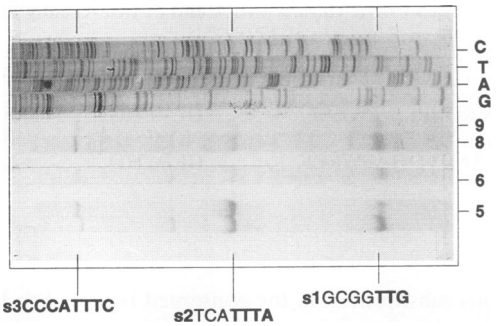

FIG. 4. Primer extension mapping of transcription initiation sites of the *amyE*(TV1) gene in *B. subtilis*. The RNA was isolated from *B. subtilis* GSB26/pHP.TV3r cultures after 5, 6, 8, and 9 h of growth. A synthetic primer was annealed either to mRNA fractions for extension or to DNA of pBS.TV7.2 used as a template for nucleotide sequencing (see Materials and Methods). The 3′ ends of the transcripts which were deduced from the position of the extension signals S1, S2, and S3 and after searching for putative promoter contact sites are marked in boldface type.

Streptomyces hygroscopicus (26) and *Thermomonospora curvata* (44) was below 20%, indicating lower relatedness.

Primer extension studies with *B. subtilis*. Certain problems with *T. vulgaris* 94-2A cultures and genetic studies of this species led us to analyze the putative gene promoter in its heterologous host by primer extension and deletion experiments. Total RNA was isolated from *B. subtilis* GSB26/pHP.TV3r cultures after growth for 5, 6, 8, and 9 h at 37°C. A synthetic oligonucleotide (see Materials and Methods) was used for primer extension (Fig. 4). Three signals were detected with distinct strengths and positions, indicating three transcription start sites, S1, S2, and S3, in *B. subtilis* (Fig. 2). The putative start sites S1 and S2 were the predominant sites used. After prolonged growth (for about 8 h), the S2 signal became weaker and S1 site usage seemed to predominate (Fig. 4). A third, more distant primer extension signal, S3, had less than 20% of the band intensity of the S1 signal.

On the basis of these data we propose that there are two, or possibly three, putative promoters in the gene upstream region (Fig. 5A).

The cooperation of the proposed *amyE*(TV1) gene promoters was attempted after stepwise removal of the upstream DNA by using oligonucleotide-primed PCR for the construction of fragments amyTV21, amyTV1, and amyTV-0 (Fig. 5A). After subcloning on vector pHP13 or pGB354, the respective plasmid derivatives were transformed into *B. subtilis* GSB26 (Table 1). After 15 h of incubation, the relative expression levels were compared by activity assay and visualization of the amylase-specific protein band from cell-free supernatant fractions of parallel cultures after SDS-PAGE (Fig. 6). The native *amyE*(TV1) gene was found on vector pGB.TV321 to direct about 15 times more amylase per volume in *B. subtilis* than from the shuttle plasmid pHP13 (constructs pHP.TV1L or pHP.13r) (Fig. 6). The two vectors differ in copy numbers by a factor of about 10 , i.e., they replicate with about 30 or 3 copies per host cell (3, 19). The amylase activities seem to correlate with the intensities of the amylase-specific protein bands (Fig. 6). In this way, we also compared the relative activities and the amylase-specific protein bands from cultures containing the distinct promoter fragments, as shown in Fig. 5, cloned on vector pGB354 (Table 1). The construct pGB.TV1, devoid of the DNA upstream of promoter P1, directs about 1/10 the AmyTV1 level directed by the constructions pGB.TV21 and pGB.TV321. The latter are about equal in their levels of amylase expression, although they are proposed to contain two putative promoters, P2 and P3 (Fig. 5A).

For comparison and in order to estimate the relative strengths of these promoters in *B. subtilis*, a 257-bp fragment

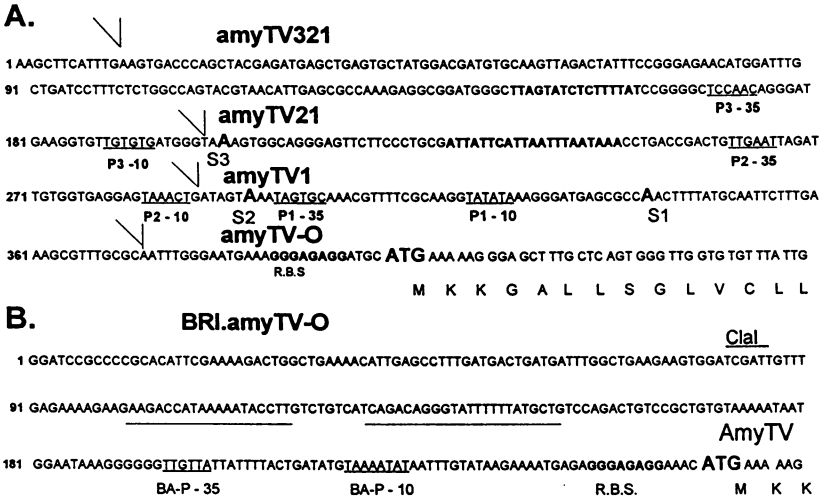

FIG. 5. Nucleotide sequences of promoter fragments used for expression studies. (A) Sequence of the DNA upstream of the *amyE*(TV1) gene containing either the entire region with the multiple promoters (amyTV321) or the sequence deletions (the positions of the 5′ ends are marked by wedges) amyTV21, amyTV1, and amyTV-0, the last of which is proposed to lack promoter activity. These fragments in continuity with the downstream *amyE*(TV1) gene sequence were cloned on vector pHP13 or pGB354 (Table 1) and were used for the expression studies shown in Fig. 6. (B) Nucleotide sequence of the PCR-generated fragment BRI.amyTV-0 composed of upstream DNA of the *B. amyloliquefaciens* α-amylase gene containing its promoter and RBS fused at the ATG codon to the coding region of the *amyE*(TV1) gene. This fragment was in continuity with the downstream *amyE*(TV1) gene DNA used to construct the plasmid derivatives pHP.BRI and pGB.BRI (Table 1). The putative promoter sites as well as the AT-rich regions are indicated relative to the transcription start sites S1, S2, and S3. The putative RBSs of the *amyE*(TV1) gene and of the *B. amyloliquefaciens* α-amylase gene *amyE*(BA) are indicated for comparison.

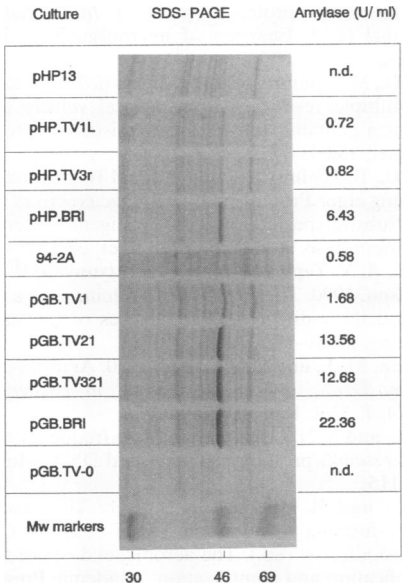

FIG. 6. AmyTV1 expression in *B. subtilis* GSB26 in relation to the promoter constructs and the cloning vector used. The amylase-specific protein band (at about 53 kDa) is visualized, after SDS-PAGE, relative to the amylase activity recovered from cell-free fluids of cultures grown for 15 or 20 h at 37°C. The intensity of the SDS-PAGE band represents the protein of 100-μl samples. The constructs contain the fragments described in the legend to Fig. 5, where the pHP and pGB plasmids contain the gene constructs on either vector pHP13 or vector pGB354 (Table 1). Lane 94-2A shows the protein bands of a 100-μl aliquot of a *T. vulgaris* 94-2A culture.

spanning the entire promoter region of the *B. amyloliquefaciens* α-amylase *amyE*(BA) gene (59) was precisely fused by PCR recombination with the coding region of the *amyE*(TV1) gene. The scheme of the resulting BRI.amyTV-0 construct is illustrated in Fig. 5B. After the BRI.amyTV-0 cassette was subcloned on vector pHP.BRI or pGB.BRI (Table 1), the level of AmyTV1 expression in *B. subtilis*, relative to that of the native *amyE*(TV1) gene, was enhanced by a factor of 2 or 8, respectively (Fig. 6).

A putative ribosome binding site and palindromes. The sequence 5′-GAAAGGGAGAGGATGC(ATG), upstream of the putative ATG initiation codon of the deduced *amyE*(TV1) gene, exhibits striking homology (13 of 16 nucleotides) with the corresponding region of the α-amylase gene *amyE*(BA) of *B. amyloliquefaciens* (Fig. 5). This region is proposed to contain an RBS (underlined) of the *amyE*(BA) gene mRNA (59). This homology was unexpected because the promoter regions of both genes are otherwise dissimilar (Fig. 5). A possible rho-independent terminator starts 28 nucleotides downstream of the proposed stop codon TGA. Its stem is formed by 44 perfectly paired nucleotides and includes a downstream extended stretch of thymidines.

DISCUSSION

We have cloned and characterized the gene *amyE*(TV1) and have found it to code for a previously isolated 53-kDa α-amylase (AmyTV1) of *T. vulgaris* 94-2A (22). The gene comprises 483 codons. On the basis of peptide sequencing, it is proposed to code for the AmyTV1 protein consisting of 453 amino acids in its mature amylase portion and for an extension of 29 N-terminal amino acids, which exhibits the character of signal peptides of extracellular proteins (65).

The variety of amylases reported in the literature for various *T. vulgaris* strains (see the introduction) posed questions about the relatedness of either the strains or the amylases in that one bacterial species. Our preliminary hybridization data obtained by using the cloned *amyE*(TV1) gene DNA and a spectrum of distinct amylase-positive clones from our phage library, as well as preliminary tests for additional pullulanolytic activities (unpublished observations), did not preclude the existence of other amylase genes in the *T. vulgaris* 94-2A strain used throughout these studies. It was thus of great interest to compare the sequences of two distinct α-amylases, TVA I and TVA II, recently identified from *T. vulgaris* strain R-47 (59). Both are distinct from AmyTV1 pullulanolytic α-amylases, which differ in their ratios of pullulan-hydrolyzing to starch-hydrolyzing activities (61). Sequence comparison of AmyTV1 with the amino acid sequence of TVA II revealed low homology with 29% identical residues. Even the amino acid sequences of the four highly conserved regions of TVA II and TVA I α-amylases differ in few residues when compared with AmyTV1 (Table 2). The distinction between the amylase AmyTV1 of *T. vulgaris* and the neopullulanase-type amylases TVA I and II of *T. vulgaris* R-47 led us to propose either variability of amylase genes among the strains or a still uncertain heterogenicity of the genus *Thermoactinomyces* (8, 43, 45).

The comparative 16S rRNA analyses of round spore-forming bacilli displayed the relationship of *T. vulgaris* to the genuine *Bacillus* species, it being closest to *B. stearothermophilus* (8, 45, 55). Indeed, TVA II was found to be homologous to the neopullulanase of *B. stearothermophilus* TRS40 (31). These facts prompted us to compare the AmyTV1 protein with other *Bacillus* α-amylases, i.e., those from *B. polymyxa*, *B. megaterium*, *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus*, *B. subtilis*, and *B. circulans*. However, the dendrogram does not confirm a pronounced relationship with the other *Bacillus* amylases, except with that of *B. polymyxa*. Sequence homologies below 20% with the α-amylases of two actinomycete species (*S. hygroscopicus* and *T. curvata*) also indicate low relatedness with AmyTV1.

The highest homology of about 82.8% (74.2% identical residues) with the 48-kDa α-amylase part of the *B. polymyxa* β-α-amylase polyprotein (62), as well as moderate homology of about 50% (38.7% identity) to the fungal Taka amylase, indicates relatedness of those α-amylases at the amino acid sequence level.

The relatively high expression level of the cloned *amyE* (TV1) gene in *B. subtilis* suggested homologies at the functional level, i.e., the codon usage by and the arrangement and structure of the putative promoters of the *T. vulgaris* 94-2A gene. However, minor differences were detected in codon usage of the gene *amyE*(TV1) (data not shown) compared with the overall usage of codons in *Bacillus* genes (35, 54). Moreover, the putative RBSs in the leader regions of the transcripts of the α-amylase genes *amyE*(BA) of *B. amyloliquefaciens* and *amyE*(TV1) of *T. vulgaris* 94-2A were found to have striking homologies (Fig. 5). Consequently, we also attempted to study the *amyE*(TV1) gene promoter in *B. subtilis* by primer extension and sequence deletions. Three transcription start sites, S1, S2, and S3, are proposed (Fig. 4). Site S2 is fairly similar to bacterial vegetative (σ^A) promoters (37, 38). Its deduced consensus regions P2 −10 (TAAACT) and P2 −35 (TTGAAT), as well as spacing by 17 bp exhibit pronounced homology. The more upstream gene promoter boxes P3 −10 (TGTGTG) and P3 −35 (CCAACA) neither exhibited

marked sequence homologies with known *B. subtilis* promoters (37) nor fitted the proposed spacing of the contact sites. Both putative promoter regions contain AT-rich sequences upstream of their −35 boxes, as several bacterial promoters do (17, 38). A third primer extension signal, S1, had a strength similar to that of S2 but may originate from processing of a primary transcript. This suggestion is supported by the facts that its proposed P1-10 box TATAAA lies unusually distant from site S1 and that the upstream sequences lack homology to known bacterial promoters (Fig. 5A).

The functionality of these promoter sites was studied by sequence deletions and constructs containing the promoter of the *B. amyloliquefaciens* α-amylase gene (33, 59) fused to the coding region of the *amyE*(TV1) gene (Fig. 5). The activity of the secreted amylase and the intensity of the amylase-specific protein bands were evaluated. In summary, we found no evidence for the contribution of the putative P3 region to the level of AmyTV1 expression in *B. subtilis* compared with the cassette containing only promoters P2 and P1 in tandem. The proposed P1 promoter alone with fragment amyTV1 was shown to result in about 1/10 the amylase level directed by the tandem promoter P2P1 of construct amyTV21 (Fig. 5). We thus state that there is a discrepancy between the nearly equal intensities of the primer extension signals for sites S2 and S1, on one hand, and the low level of amylase expression of constructs containing the promoter P1 separated from the tandem promoter P2P3, on the other hand. We thus propose that the transcription site S1 requires elements of the tandem upstream promoter P2P3 for higher efficiency. These promoter interactions are often a feature of dual or triple promoters, which usually respond to distinct sigma factors of several *B. subtilis* and *E. coli* genes and operons (10, 16, 48).

The promoter of the *B. amyloliquefaciens* α-amylase gene, fused to the promoterless amyTV-0 gene cassette, was used to evaluate the relative efficiency of the amyTV321 promoter. The levels of AmyTV1 assayed from the supernatant of *B. subtilis* cultures were compared. Although we could not exclude transient activities in growth dependency after 15 h of growth, the levels of AmyTV1 expression (secretion) were two- and eightfold higher with the BRI.amyTV-0 gene cassette in vectors pHP.BRI and pGB.BRI, respectively. This result indicates the relative weakness of the native *amyE*(TV1) promoter(s) in *B. subtilis*. The result also demonstrates the possibility of enhancing AmyTV1 expression significantly by changing the promoter and probably the copy number of the cloning vectors used. Further studies are in progress to verify the promoter activities. Preliminary data from these studies indicate that the signal peptide in relation to the promoter also affects the level of AmyTV1 secretion in *B. subtilis*.

ACKNOWLEDGMENTS

We are grateful to Andreas Czihal and Helmut Bäumlein for help in establishing the EMBL3 lambda gene bank, to Andreas Polley for his help throughout these studies, and to Barak Askari and Manfred Klaas for proofreading the manuscript. We are also indebted to Mauno Vihinen, who for the first time discovered the striking sequence homology of AmyTV1 with the *B. polymyxa* amylase.

This work was supported by a grant from the Bundesministerium für Forschung und Technologie (Vorhaben 0319689B7).

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