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# Cloning, Nucleotide Sequence, and Enzymatic Characterization of an α-Amylase from the Ruminal Bacterium *Butyrivibrio fibrisolvens* H17c

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**A *Butyrivibrio fibrisolvens* amylase gene was cloned and expressed by using its own promoter on the recombinant plasmid pBAMY100 in *Escherichia coli*. The amylase gene consisted of an open reading frame of 2,931 bp encoding a protein of 976 amino acids with a calculated $M_r$ of 106,964. In *E. coli*(pBAMY100), more than 86% of the active amylase was located in the periplasm, and Tn*phoA* fusion experiments showed that the enzyme had a functional signal peptide. The *B. fibrisolvens* amylase is a calcium metalloenzyme, and three conserved putative calcium-binding residues were identified. The amylase showed high sequence homology with other α-amylases in the three highly conserved regions which constitute the active centers. These and other conserved regions were located in the N-terminal half, and no similarity with any other amylase was detected in the remainder of the protein. Deletion of approximately 40% of the C-terminal portion of the amylase did not result in loss of amylolytic activity. The *B. fibrisolvens* amylase was identified as an endo-α-amylase by hydrolysis of the Phadebas amylase substrate, hydrolysis of γ-cyclodextrin to maltotriose, maltose, and glucose and the characteristic shape of the blue value and reducing sugar curves. Maltotriose was the major initial hydrolysis product from starch, although extended incubation resulted in its hydrolysis to maltose and glucose.**

Members of the genus *Butyrivibrio* are among the most numerous and nutritionally versatile of rumen bacteria. In particular, strains of *Butyrivibrio fibrisolvens* have been described that are cellulolytic (1, 21, 51), xylanolytic (12, 23), amylolytic (10), pectinolytic (68), lipolytic (20), and proteolytic (11, 54). This ability to ferment a wide range of macromolecules is considered to enable *B. fibrisolvens* to be the most dominant rumen bacterium under adverse nutritional conditions. In winter, *B. fibrisolvens* is the most abundant starch- and cellulose-fermenting bacterium in the high-arctic Svalbard reindeer (43) and is unusual in its capacity to utilize both carbohydrate substrates whereas other cellulolytic species are unable to do so. *B. fibrisolvens* is also the most prevalent bacterium in the rumen of semi-starved Zebu cattle in Kenya (35).

Starch is an important component of the ruminal diet, and its digestion is essential for maximum productivity. High-starch diets are, however, associated with a number of digestive disorders. For example, lactate acidosis is associated with a proliferation of the amylolytic species *Streptococcus bovis* and is thought to occur as a result of the rapid fermentation of starch leading to an accumulation of lactic acid (53). A knowledge of the nature and regulation of amylolytic enzymes could therefore assist in controlling these digestive disorders and improving the efficiency of starch digestion. Relatively few amylases of ruminal bacteria have been characterized. Those that have been studied include amylases from *S. bovis*, *Clostridium butyricum* (24, 65), and *Ruminobacter amylophilis* (formerly *Bacteroides amylophilis*) (38). In addition, Cotta (10) has carried out a general investigation of amylases from the major starch-utilizing bacteria found in the rumen, including *B. fibrisolvens*.

α-Amylases are widely distributed among both prokaryotes and eukaryotes, and substantial nucleotide and amino acid sequence information is available. No molecular studies, however, of the amylases from ruminal bacteria have been reported. We report here the cloning and nucleotide sequence of a gene coding for an amylase, 1,4-α-D-glucan-4-glucanhydrolase (EC 3.2.1.1.), from *B. fibrisolvens* H17c. The hydrolytic properties of this enzyme were characterized, and the secretion and locations of the cloned amylase in *Escherichia coli* were examined. The derived amino acid sequence for the encoded polypeptide was compared with sequences for amylases of prokaryotic and eukaryotic origin.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The rumen bacterium *B. fibrisolvens* H17c (12) was used. Cloning and genetic manipulations were carried out in the *E. coli* C600 (69), LK111 (71), and K12G6MD3 (50), a gift from J. Preiss, Department of Biochemistry, Michigan State University. The *phoA E. coli* strain CC118 (34) was used for the Tn*phoA* fusion experiment. Phage λb221*rex*::Tn*phoA*cI857 *P*am3 (18) was a gift from C. Manoil, Department of Genetics, University of Washington. The *E. coli-Bacillus subtilis* shuttle vector pEB1 has been described previously (30).

**Media and growth conditions.** Litner soluble starch, starch azure, α-, β-, and γ-cyclodextrins (Schardinger dextrins), and linear malto-oligosaccharides (glucose [G1], maltose [G2], maltotriose [G3], maltotetraose [G4], maltopentaose [G5], and maltohexaose [G6]) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Raw corn starch was obtained from BDH (Poole, England), and Phadebas reagent was obtained from Pharmacia (Uppsala, Sweden). *E. coli* strains were grown in Luria broth with 5 mM $CaCl_2$ and 0.1 mg of ampicillin per ml for the selection of transformants.

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When *E. coli* G6MD3 was used, cultures were supplemented with 0.05 mg of diaminopimelic acid per ml because of the *asd* deletion present in this strain. The growth medium (M10) for *B. fibrisolvens* H17c was as previously described (54).

**Screening for amylolytic activity and characterization of clones.** Construction of the *B. fibrisolvens* gene library has been described by Lin et al. (30). The gene bank was transformed into *E. coli* LK111, and ampicillin-resistant transformants were selected on 0.5% starch azure-plus-ampicillin plates. Colonies producing clear halos were selected for further study. The preparation of plasmid DNA and restriction endonuclease mapping of the clones were done by using standard techniques (32). *B. fibrisolvens* chromosomal DNA was prepared as described by Berger et al. (1), and Southern hybridization using the cloned DNA as a probe was used to confirm that the insert DNA originated from *B. fibrisolvens*. The probe was labeled by using a nonradioactive digoxigenin DNA labeling kit (Boehringer Mannheim).

**Preparation of cell extracts and cell fractionation.** Cell extracts were prepared from 24-h, 100-ml *E. coli* cultures. Cells were harvested, rinsed with saline, and resuspended in 2 ml of 50 mM sodium phosphate buffer containing 5 mM $CaCl_2$ (pH 6.8). The cell suspension was disrupted by sonication on ice (30-s bursts for 3 min) using an MSE (Soniprep 150) sonicator. The extract was clarified by centrifugation for 15 min at 27,000 × *g* at 4°C. Periplasmic and cytoplasmic extracts were prepared from mid-stationary-phase *E. coli* C600(pBAMY100) cultures (200 ml) by the osmotic shock procedure of Willis et al. (67). β-Galactosidase and β-lactamase activities were assayed by the methods of Pardee et al. (44) and Sykes and Nordstrom (55), respectively.

**Amylase and protein assays.** Amylolytic activity was determined at 45°C, using 0.5% soluble starch as a substrate in 50 mM sodium phosphate buffer (pH 6.8) containing 5 mM $CaCl_2$. Release of reducing sugar from soluble starch was measured with 3,5-dinitrosalicylic acid (2). The activity of 1 U of amylase was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per min under the specified conditions, using glucose as the standard. A water-insoluble cross-linked blue starch polymer (8) commercially available as the Phadebas amylase test (Pharmacia Diagnostics) was used as a qualitative assay. Protein concentration was measured by the dye-binding method of Bradford (4), using bovine serum albumin (BSA) as a standard.

**Tn*phoA* mutagenesis.** Transposon insertions into *amyA* (pBAMY100) were made by using λ::Tn*phoA* (34) according to the protocol of Gutierrez et al. (18), with adaptations reported by Scholle et al. (49).

**Western immunoblotting and activity gels.** Cell extracts of amylase-phosphatase fusion proteins prepared from *E. coli* CC118 were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (29). Western blots were processed as described by Rybicki and von Wechmar (47). Rabbit antiserum raised against a commercial preparation of alkaline phosphatase was used as a probe. Blots were developed by using goat anti-rabbit alkaline phosphatase antibody-enzyme conjugate (Miles Laboratories, Cape Town, South Africa) and 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium substrate solution. Amylase activity gels were performed with crude extracts of *E. coli* G6MD3 cells containing the various clones and fractionated on 10% SDS-PAGE gels. Amylolytic activity bands were detected in situ after electrophoresis and renaturation of proteins according to Lacks and Springhorn (28).

**Nucleotide sequence determination and protein sequence comparison.** DNA fragments from pBAMY100 were subcloned into vectors pBluescript SK or KS (Stratagene, San Diego, Calif.). The exonuclease III method was used to generate overlapping deletions (22). Sequencing was carried out by the chain termination method, using a Sequenase kit (version 2.0) from U.S. Biochemical Corp., Cleveland, Ohio. The sequence of the entire pBAMY200 subclone was determined from both strands. Sequence data was analyzed by using the Genetics Computer Group Inc. software package (version 6.2). The TFASTA subroutine was used to screen the GENEMBL (release 65.0), Swiss Protein (release 15.0), NBRF-N (release 36.0), and NBRF-P (release 25.0) data bases for sequences having similarity to the amino acid sequence of the *B. fibrisolvens* amylase.

**Analysis of blue value, reducing sugar, and hydrolysis products.** Iodine blue values were determined by a modification of the method of Mountfort and Asher (39). A sample of crude enzyme extract was added to a 50 mM sodium phosphate–5 mM $CaCl_2$ (pH 6.8) buffer containing 1% soluble starch, the volume was adjusted to 3 ml, and the mixture was incubated at 45°C. Samples (0.1 ml) were withdrawn and mixed with 0.4 ml of HCl, and 0.1 ml of the mixture was added to 1 ml of iodine stain (50 mg of $I_2$ and 0.5 g of KI liter$^{-1}$). The $A_{620}$ was measured, and the blue value was calculated. Two additional 0.1-ml samples were used to determine the amount of reducing sugar liberated and the products of hydrolysis. High-pressure liquid chromatography (HPLC) was used to identify the products of hydrolysis of soluble starch, amylose, amylopectin, pullalan, α-, β-, and γ-cyclodextrins, and malto-oligosaccharides. Samples were loaded on a Beckman HPLC system equipped with a model 156 refractive index detector and a Waters C18 separation column, using double-distilled water as the eluant.

**Nucleotide sequence accession number.** The nucleotide sequence reported has been assigned GenBank accession number M62507.

## RESULTS

**Isolation, location, and origin of the amylase gene.** Two amylase-positive *E. coli* LK111 transformants were detected from the approximately 7,500 colonies screened. Restriction enzyme analysis showed that the plasmids from these clones had identical 5-kbp insert fragments, and a plasmid designated pBAMY100 was chosen for further study. A restriction map of pBAMY100 and subclones was constructed (Fig. 1). The 3.52-kbp *Cla*I-*Sac*I fragment was subcloned in both orientations by using the pBluescript SK and KS sequencing vectors (pBAMY200 and pBAMY300, respectively). Both subclones retained amylolytic activity. Construction of a 120-bp *Hpa*I-*Hpa*I deletion (pBAMY210) resulted in the loss of amylolytic activity. Exonuclease III deletions from the *Sac*I site (pBAMY220, -230, and-240) retained amylolytic activity, while pBAMY250 lost activity. Two *Eco*RI fragments (1.2 kbp and 250 bp) internal to the pBAMY100 insert hybridized to two *B. fibrisolvens* H17c chromosomal fragments of the same size, confirming the origin of the cloned DNA fragment (data not shown).

**Nucleotide sequence of the *B. fibrisolvens amyA* gene.** The nucleotide sequence of the 3,523-bp *Cla*I-*Sac*I fragment from pBAMY200 revealed a single large open reading frame (ORF) (Fig. 2) which encoded a protein of 976 amino acids with a calculated $M_r$ of 106,964. This protein is approximately double the molecular size of the majority of prokaryote and eukaryote amylases (for reviews, see references 14

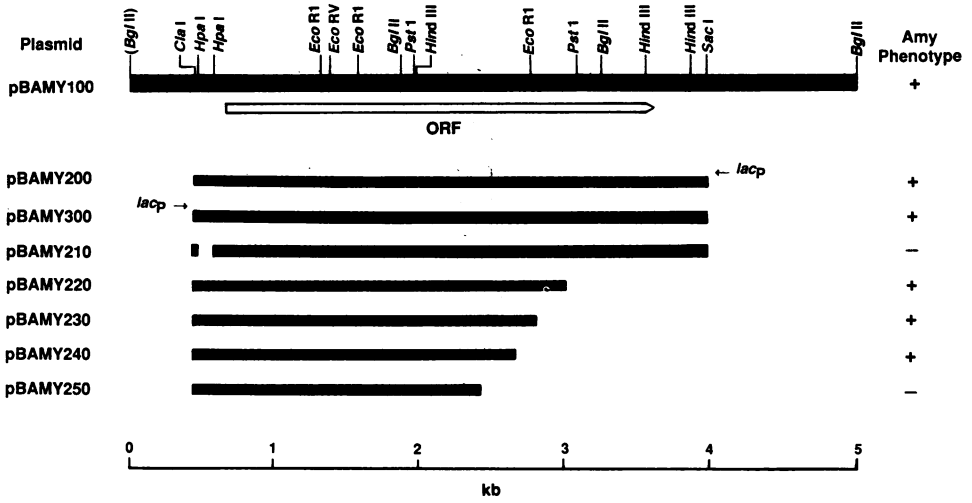


FIG. 1. Restriction endonuclease map of insert DNA of pBAMY100 and subclones encoding the *B. fibrisolvens* H17c amylase. The ORF and direction of transcription are indicated by an open arrow; $lac_P$ with an arrow indicates the direction of transcription of the vector *lac* promoter.

and 62), although a number of similarly sized enzymes have been described (see Discussion). A potential ribosome binding sequence (GGAGG) was present 4 bp upstream of the most likely ATG translational initiation codon at position 211. A putative promoter region (TTGACG-N17-TATAAT) with strong homology to the *E. coli* ($\sigma^{70}$) consensus promoter (19) was located 113 bp upstream of the ATG start codon (Fig. 2). A 10-bp inverted repeat sequence, which could form a Rho-dependent terminator ($\Delta G = -11.0$ kcal [ca. −46 kJ]/mol [48]), was located 301 bp downstream of the putative TAA stop codon. Several inverted repeat sequences of unknown function were present upstream and within the ORF.

**Analysis of the putative signal sequence and the expressed polypeptides.** In *B. fibrisolvens*, the amylase enzyme is either cell associated or secreted (10), and thus the corresponding gene would be expected to contain a leader sequence. Sequence analysis of the N-terminal region of the *B. fibrisolvens* amylase revealed a putative 33-amino-acid signal sequence that showed several features characteristic of signal peptides (64). A cluster of positively charged amino acids adjacent to the fMet residue at the N terminus (two Lys and two Arg) was followed by a region rich in hydrophobic amino acids (Fig. 2). There were two Ala residues at positions −3 and −1 with respect to the most likely cleavage site calculated according to the rules of von Heijne (64). The ability of this region to function as a signal sequence in *E. coli* was confirmed by Tn*phoA* analysis. Seven different Tn*phoA* insertions along the length of the *amyA* gene carried on pBAMY100 (Fig. 2) conferred alkaline phosphatase activity to the *E. coli* CC118 *pho* strain. The exact positions of the Tn*phoA* insertions were confirmed by nucleotide sequencing using a primer complementary to the start of the *phoA* gene. As the PhoA protein encoded by Tn*phoA* lacks its own signal peptide and is inactive unless exported from the cytoplasm (34), it was concluded that the AmyA protein provided the signal sequence for protein export. The amylolytic activity of all of the fusion proteins was tested; two fusion proteins, PhoA6 and PhoA7, retained amylolytic activity. A Western blot of the amylase-phosphatase fusion proteins obtained by using antibodies to alkaline phosphatase is shown in Fig. 3A. The predicted increase in molecular size of the fusion proteins from PhoA1 to PhoA7 was 57, 77, 82, 99, 107, 112, and 127 kDa, respectively. Polypeptides with apparent size values that corresponded closely to these predicted values were detected. An additional protein with an apparent $M_r$ of approximately 48,000 was observed in all lanes of the Western blot. This protein corresponded to the size of the phosphatase moiety and might possibly be due to instability of the fusion proteins. These results indicated the presence of a single signal peptide and ORF up to the last Tn*phoA* insertion. In vitro protein synthesis and analysis using a prokaryotic DNA-directed translation kit (Amersham) and pBAMY100 DNA were attempted without success, suggesting that additional components or membrane-bound factors could be required for correct transcription or translation (data not shown).

The results of an amylase activity gel of the crude enzyme extracts from *E. coli* G6MD3 containing pBAMY100 or deletion plasmids pBAMY220, -230, and -240 are shown in Fig. 3B. *E. coli* G6MD3(pBAMY100) produced an active amylase with an apparent $M_r$ of approximately 100,000, in close agreement with the calculated $M_r$ of 103,257 after signal peptide cleavage. *E. coli* G6MD3 containing plasmids pBAMY220, -230, and -240, encoding proteins with predicted C-terminal deletions of 197, 260, and 315 amino acids, respectively, produced correspondingly smaller polypeptides, all of which retained amylolytic activity even though approximately 40% of the C terminus had been deleted. The *E. coli* G6MD3(pBluescript) control did not show any amylolytic activity. Additional active amylase bands of lower molecular size were also detected in all extracts and may have been either due to proteolysis of the amylase in the crude extract or the result of posttranslational proteolysis in *E. coli*. This latter mechanism has been suggested for the endo-β-1,4-glucanase of *B. fibrisolvens* (21).

**Localization of amylolytic activity in *E. coli*.** The cellular location of the *B. fibrisolvens* amylase enzyme in *E. coli* cells was determined by using the full-length plasmid pBAMY100 and the shortened plasmid pBAMY220. The results with both plasmids were very similar, with the periplasmic fraction containing approximately 86% of the amylolytic activity

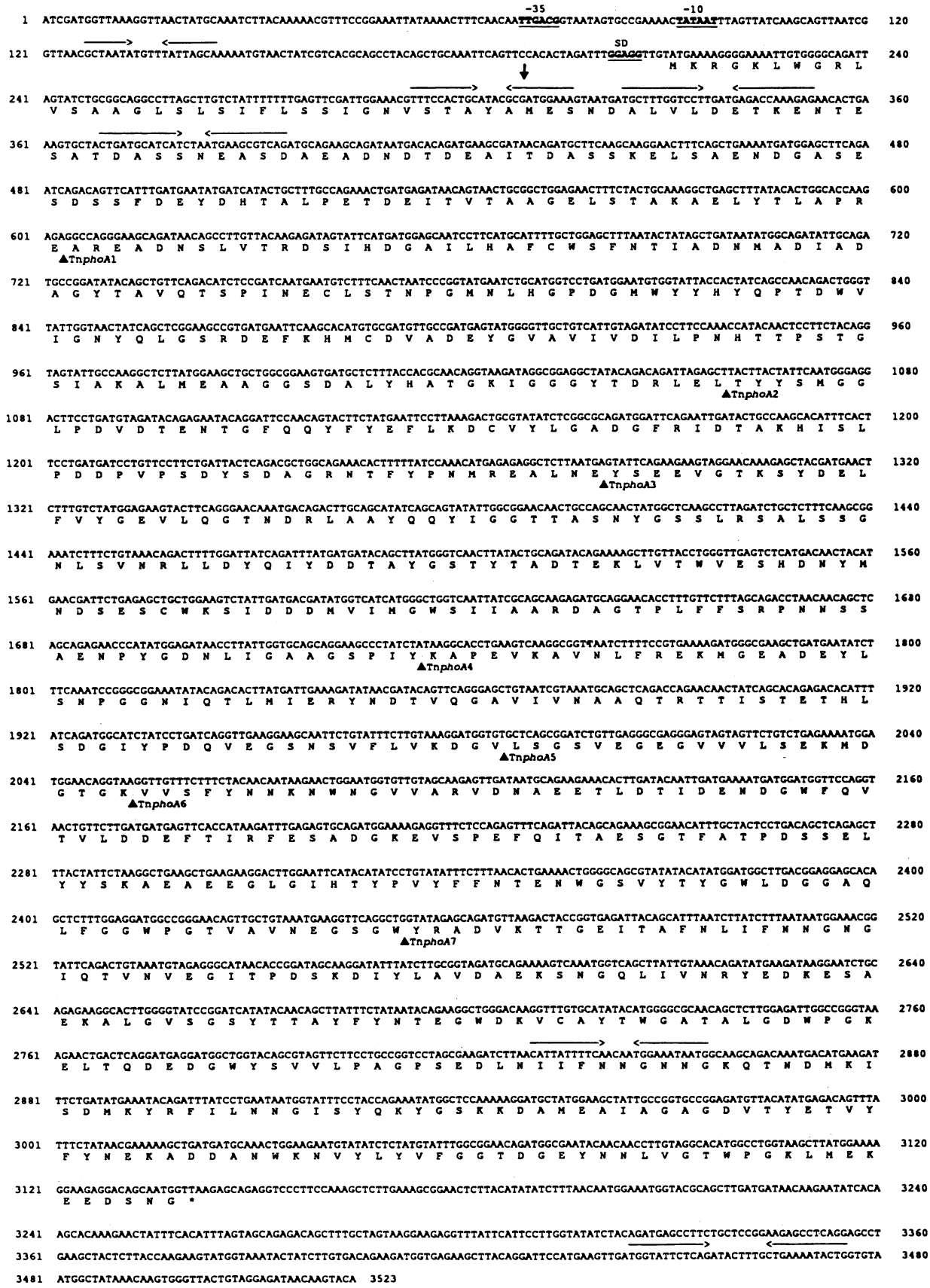


FIG. 2. Nucleotide sequence of the *B. fibrisolvens* H17c *amyA* structural gene. The predicted amino acid sequence of the amylase is given below in single-letter code. Consensus promoter sequences at −10 and −35 and the Shine-Delgarno (SD) sequence are underlined and in bold. Inverted repeat sequences are shown by converging arrows. A downward arrow indicates the putative cleavage site of the signal peptide. Tn*phoA* insertions, as determined by nucleotide sequencing, are indicated by upward arrowheads.

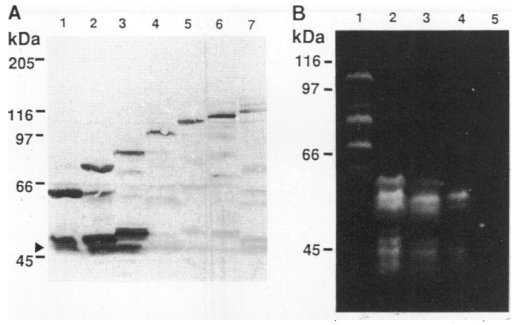


FIG. 3. (A) Western blot of amylase-phosphatase fusion proteins, using antibodies to alkaline phosphatase as the probe. Lanes 1 to 7 represent Tn*phoA* insertions 1 to 7, respectively. The 48-kDa protein common to all insertions is indicated by an arrowhead. The molecular size standards indicated were rabbit muscle myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). (B) SDS-PAGE of amylolytic activity of extracts from *E. coli* G6MD3 containing pBAMY100 (lane 1), pBAMY220 (lane 2), pBAMY230 (lane 3), pBAMY240 (lane 4), and vector pBluescript SK (lane 5). Molecular size standards indicated were as for panel A.

(Table 1), indicating that the C terminus was not involved in the secretory process in *E. coli*. Only low levels of amylolytic activity were detected in cytoplasmic and supernatant fractions. The location of β-galactosidase (a cytoplasmic enzyme) and β-lactamase (a periplasmic enzyme) confirmed the correct fractionation of the cell components.

**Characterization of the amylolytic activity.** *B. fibrisolvens* amylolytic activity expressed in *E. coli* C600 was dependent on the presence of $CaCl_2$ both in the growth medium and in the assay buffers. In *E. coli*, the synthesis of the amylase was constitutive. The amount and location of the enzyme were similar to those shown in Table 1 irrespective of whether maltose, glucose, or starch was included in the growth medium. The *B. fibrisolvens* amylase cloned in *E. coli* had a pH optimum of 6.8 and was stable between pH 5.5 and 7.5. Although the optimum temperature for amylolytic activity was 50°C, the thermal stability of the enzyme decreased above 45°C and was completely lost at 52°C (data not shown). Preincubation of the enzyme (20 min) to determine temperature stability was carried out in the absence of the substrate. It has been shown for other amylases (5, 26) that the substrate protects the enzyme against thermal denaturation.

The relative rates of hydrolysis of soluble starch, amylose, amylopectin, and glycogen were determined by the release

TABLE 1. Localization of amylolytic activity in *E. coli* C600(pBAMY100) and *E. coli* C600(pBAMY220)

| Plasmid | Fraction | Activity (U)[a] | | |
|---|---|---|---|---|
| | | Amylase | β-Galactosidase | β-Lactamase |
| pBAMY100 | Supernatant | 12.8 (7) | 55.2 (1) | 59.6 (4) |
| | Periplasmic | 159.0 (87) | 584.3 (15) | 1,410.0 (94) |
| | Cytoplasmic | 11.8 (6) | 3,198.4 (84) | 22.0 (2) |
| pBAMY220 | Supernatant | 9.3 (11) | 56.0 (2) | 66.0 (4) |
| | Periplasmic | 75.2 (86) | 479.0 (14) | 1,369.0 (93) |
| | Cytoplasmic | 3.2 (3) | 2,876.0 (84) | 41.0 (3) |

[a] One unit of activity was determined as the release or hydrolysis of 1 μmol of substrate $min^{-1}$. Numbers in parentheses represent the percentage of enzymatic activity in the respective fractions.

TABLE 2. α-Amylolytic activity against the Phadebas substrate of extracts of *E. coli* G6MD3 transformants

| Plasmid | Amino acids deleted[a] | Sp act (U)[b] |
|---|---|---|
| pBAMY100 | 0 | 70 |
| pBAMY220 | 197 | 22 |
| pBAMY230 | 260 | 20 |

[a] Number of amino acids deleted from the C terminus.
[b] Determined as the hydrolysis of 1 μmol of glucosidic linkage $min^{-1}$ μg of $protein^{-1}$ at 45°C.

of reducing sugars and were 100, 65, 58, and 14%, respectively. Pullulan and α- and β-cyclodextrins were not hydrolyzed but γ-cyclodextrin was, indicating that the enzyme had endoamylolytic activity. This was confirmed by hydrolysis of the Phadebas substrate (8). A decrease in amylase reactivity against the Phadebas substrate was detected with subclones containing deletions of the amylase C terminus (Table 2). The complete enzyme showed no ability to hydrolyze or adsorb to raw corn starch granules under the conditions used (65) (data not shown).

**Analysis of the amylolytic hydrolysis products.** *E. coli* has been shown to contain an α-amylase (MalS) which has been shown to degrade small malto-oligosaccharides (15). Whereas the cell extract of *E. coli* C600 containing the pBluescript vector degraded starch and amylose very slowly (<0.1% of the activity of the *B. fibrisolvens* amylase), G6 was hydrolyzed at an appreciable rate, resulting in a pattern of polysaccharide products similar to that described previously (15) (Fig. 4A); the appearance of G7 and G8 was probably due to the polymerization action catalyzed by amylomaltase (MalQ). The cell extract of *E. coli* G6MD3 containing the pBluescript vector, however, did not hydrolyze starch, amylose, or G6 (Fig. 4B). This strain has a deletion in the *malA* region encoding *malQ* and *malT*, the latter coding for a positive regulator protein for maltose operons, including *malS*. The *B. fibrisoluens* amylase was therefore expressed in this strain of *E. coli* for analysis of the hydrolysis products. We initially investigated the hydrolysis of G6 by using a crude enzyme extract of *E. coli* G6MD3 (pBAMY200); G6 was hydrolyzed into mainly G3, with some G2 and a little G1 and G4 (Fig. 4C). Starch degradation was monitored by measuring the reduction in the blue value and production of reducing sugars as a function of time (Fig. 5A). The shape of the blue value and reducing sugar curves confirmed that the *B. fibrisolvens* amylase was an endoamylase (24). At the point where the blue value decreased to almost zero, the proportion of reducing sugars in the reaction mixture was 27%. HPLC analysis showed that the initial hydrolysis product was G3, with smaller amounts of G2 and G4 (Fig. 5B). Very little G1 was detected; trace amounts of G5 and G6 were also produced but disappeared rapidly. After extended incubation (24 h), the amount of G3 decreased with a concomitant increase in G1 and G2. As the amylase seemed to be active against G3, the hydrolysis of G3 was examined; this was found to be slow, with 32% remaining after 24 h and with G1 and G2 as the digestion products. No amylolytic activity could be detected with G2 as the substrate (results not shown). Hydrolysis of amylose and amylopectin yielded products qualitatively similar to those from starch, except that with amylopectin and starch, two unidentified compounds (probably branched oligosaccharides) were also detected eluting between G3 and G4 and between G4 and G5 on the HPLC profile. This finding

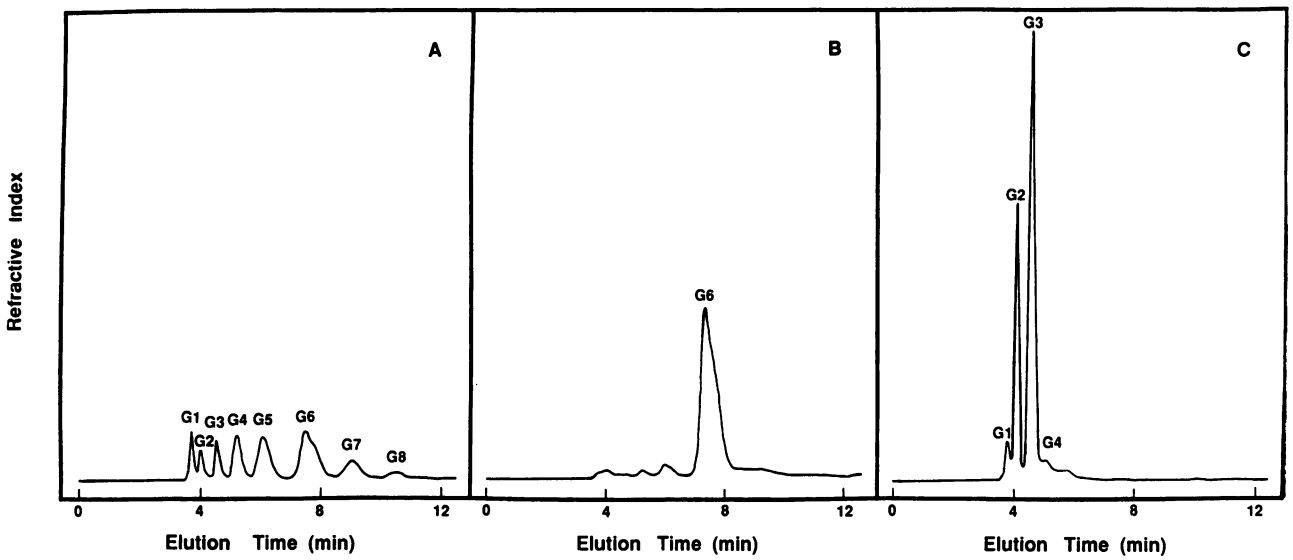


FIG. 4. HPLC analysis of hydrolysis products of G6, using extracts of *E. coli* C600(pBluescript SK) (A), *E. coli* G6MD3(pBluescript SK) (B), and *E. coli* G6MD3(pBAMY200) (C). Incubation with 1% G6 was for 2 h at 45°C.

suggested that the enzyme was unable to hydrolyze the α-1,6 branch points, which is characteristic of endoamylases (62).

**Comparison with other amylases.** The TFASTA subroutine, based on the Pearson and Lipman algorithm, was used to screen nucleotide sequence data bases for amylases with amino acid sequence similarity to the *B. fibrisolvens* amylase. The greatest similarity was found with a limited number of α-amylases from both prokaryotes and eukaryotes. Alignment of the amino acid sequence of the *B. fibrisolvens* enzyme with sequences of other α-amylases for greatest homology (Fig. 6A) showed the presence of three highly conserved domains (regions I, II, and IV) (46). A fourth conserved domain (region III) (40) was less clear but could be present at one of the two positions (353 to 356 or 374 to 377). Region IV is characterized by a highly conserved Phe residue as the first amino acid of the region; the *B. fibrisolvens* and *B. subtilis* amylases, however, are exceptions in that they have a Trp residue in this position. Close inspection of the amino acid sequences of α-amylases from diverse genera indicated the conservation of four additional regions, A, B, C, and D, situated between the N terminus and region I and one additional region, E, between regions I and II (Fig. 6B). Region C of the *B. fibrisolvens* amylase included a WYXXYQP sequence, which is similar to the WWXRYQP motif shown to be involved in substrate binding in porcine pancreatic amylase (6). No discernible homology with any amylases was detected in the C-terminal region. From the three-dimensional structure of porcine pancreatic amylase, Buisson et al. (6) proposed two important catalytic residues, Asp-197 and Asp-300, in regions II and IV, respectively, and four calcium-binding residues, Asn-100 in region I, Asp-159 and Asp-167 in region E, and His-201 in region II. Similarly, Matsuura et al. (36) suggested for *Aspergillis oryzae* (Taka A) amylase that Glu-230 of region III and Asp-297 of region IV were the catalytic residues. Residues corresponding to these catalytic residues are clearly present in the *B. fibrisolvens* amylase (Fig. 6A). Three of the four calcium-binding residues corresponding to those identified for porcine pancreatic amylase (6) were present in regions I, II, and E and were conserved. The residue which could not be identified

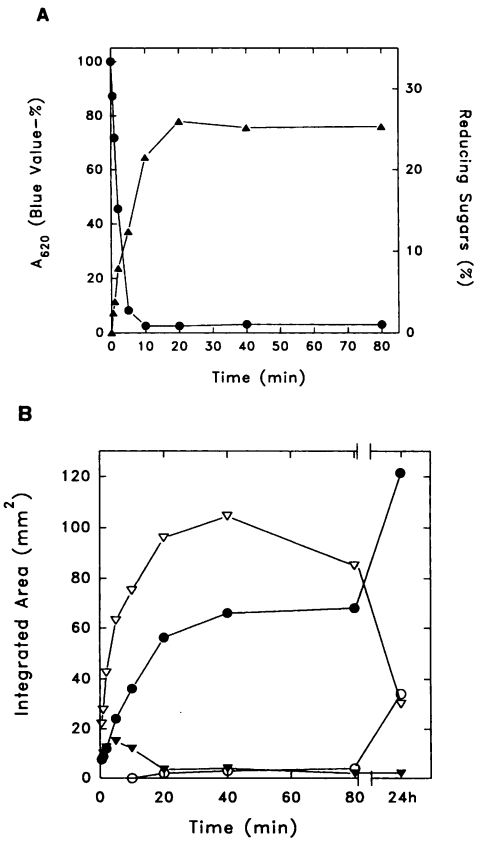


FIG. 5. (A) Kinetics of starch hydrolysis in an extract of *E. coli* G6MD3(pBAMY200). The blue color values (●) and the amount of reducing sugars (▲) were measured by the $I_2$-KI method and the 3,5-dinitrosalicylic method, respectively. (B) HPLC analysis of the hydrolysis products. G1 (○), G2 (●), G3 (▽), and G4 (▼) are illustrated; G5 and G6 were not included since they were present in only trace amounts. The break in the time axis indicates that the incubation period extended to 24 h.

6A

| | I | II | III | IV |
|---|---|---|---|---|
| | • •• | • • • •• | • | •• |
| B.f | 239 DILPNH | 317 GADGFRIDTAKH | 353 EALN/<br>374 EVLQ | 441 WVESHD |
| TAA (59) | 117 DVVAN***H*** | 199 SIDGLRIDTV***KH*** | 230 e***VLD*** | 292 FVEN***Hd*** |
| A.h (17) | 81 DVVLNH | 187 GIKGFRVDAVKH | 221 EVIT | 288 FAITHD |
| B.a (57) | 98 DVVLNH | 244 SLDGFRIDAAKH | 261 EYWQ | 323 FVENHD |
| B.s (70) | 97 DAVINH | 169 GADGFRFDAAKH | 208 EILQ | 264 WVESHD |
| PPA (45) | 96 DAVIN***H*** | 190 GVAGFRL***d***AS***K***H | 233 ***E***V***I***D | 295 FVDN***Hd*** |

6B

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | ** * * | ** ** ** | ** *** | * * * ** * | * * * ** ** |
| B.f | 150 ILHAFCW | 170 DAGYTAVQTSP | 200 WYYHYQP | 215 QLGSRDEFKHMCDVADEYGVAV | 279 DRLELTYYSMGGLPDV |
| B.s (70) | 52 ILHAWNW | 72 DAGYTAIQTSP | 99 WYWLYQP | 114 YLGTEQEFKEMCAAAEEYGIKV | 173 DRWDVTQNSLLGLYDN |
| A.h (17) | 64 ILHAFNW | 84 GAGYKQVLISP | 103 WWARYQP | 118 PLGNKQDLEQLIAAMQARGIAV | 212 YWRLCGGAGDKGLPDL |
| S.h (25) | 39 TATLFER | 60 PAGYGYVEVSP | 80 WWTSYQP | 94 RLGDRDAFASMVSACHAAGVKV | 161 NRDDVQTCELVDLADL |
| S.l (31) | 37 TAVLFEW | 58 PAGYGYVQVSP | 78 WWTSYQP | 92 RLGDRAAFKSMVDTCHAAGVKV | 159 NRANVQNCELVGLADL |
| S.v (63) | 37 TAVMFEW | 58 PAGYGYVQVSP | 78 WWTSYQP | 92 RLGDRTAFKNMIDTCHAAGVKV | 161 DRANVQNCELVQLPDL |
| D.m ( 3) | 31 MVHLFEW | 52 PNGYAGVQVSP | 74 WWERYQP | 88 RSGNEEQFASMVKRCNAVGVRT | 159 DANEVRNCELVGLRDL |
| M.m (60) | 28 IVHLFEW | 49 PKGFGGVQVSP | 73 WWERYQP | 87 RSGNEDEFRDMVTRCNNVGVRI | 165 DAYQVRNCRLTGLLDL |
| H.s (41) | 28 IVHLFEW | 49 PKGFGGVQVSP | 73 WWERYQP | 86 RSGNEDEFRNMVTRCNNVGVRI | 168 DATQVRDCRLSGLLDL |
| PPA (45) | 13 IVHLFEW | 34 PKGFGGVQVSP | 58 ***WW***ERYQP | 72 RSGNENEFRDMVTRCNNVGVRI | 153 DPYQVRDCQLVGLLDL |

FIG. 6. Amino acid sequence alignment using the TFASTA program of prokaryotic and eukaryotic amylases with *B. fibrisolvens* amylase. Catalytic residues (6, 36) are denoted in bold lowercase, substrate-binding residues (6, 36) are shown in bold italic uppercase, and calcium-binding residues (6) are underlined. Abbreviations: TAA, *A. oryzae* (Taka A amylase); B.f, *B. fibrisolvens*; A.h, *Aeromonas hydrophila*; B.a, *Bacillus amyloliquefaciens*; B.s, *B. subtilis*; PPA, *Sus scrofta domestica* (pig pancreatic amylase); S.h, *Streptomyces hygroscopicus*; S.l, *S. limosus*; S.v, *S. venezuelae*; D.m, *Drosophila melanogaster*; M.m, *Mus musculus*; H.s, *Homo sapiens*. References are given in parentheses. (A) Comparison of the previously recognized conserved regions I to IV. Residues identical in all sequences are indicated by a solid circle above the sequences. (B) Additional conserved regions A to E reported in this study. Regions A to D are situated between the N terminus and region I, and region E is between regions I and II. Asterisks indicate the locations of residues that are the same or have undergone conservative changes in 7 of the 10 amylase sequences.

was Asp-159, which is often found in mammalian but not in bacterial amylases (Fig. 6B). As well as the catalytic and calcium-binding residues, possible additional substrate-binding residues of porcine and Taka A amylases were present in regions I, II, III, IV, and C (Fig. 6).

## DISCUSSION

We have shown that the gene cloned from *B. fibrisolvens* H17c codes for a large polypeptide ($M_r$ 106,694) with α-amylolytic activity. This polypeptide is approximately twice the size of typical microbial α-amylases (50,000 to 60,000 Da) (62). Other large enzymes with α-amylolytic activity usually have additional properties. These include the G6-amylase of *Bacillus* sp. strain H-167 of 102,597 Da (52), the G4-amylase of a *Micrococcus* sp. of 118,000 Da (27), and the amylase from *Bacillus polymyxa* of 127,314 Da (61). The *Bacillus* sp. strain H-167 and the *Micrococcus* sp. amylases have a specific exoamylolytic activity associated with them, whereas the *B. polymyxa* amylase has both α- and β-amylolytic activities. The finding that approximately 40% of the C-terminal region of the *B. fibrisolvens* amylase was not essential for amylolytic activity raised the question as to the function of this region. Deletion of this region resulted in a slower rate of hydrolysis of insoluble substrate (Table 2), and it is possible that this region is associated with efficient substrate binding although the enzyme did not bind to or hydrolyze raw corn starch.

The *B. fibrisolvens amyA* gene appeared to be expressed in *E. coli* from a promoter located on the cloned fragment. Expression of the *amyA* gene was independent of orientation with respect to the vector *lacZ* gene (pBAMY200 and pBAMY300). A putative *B. fibrisolvens amyA* gene promoter very similar with both the sequence and spacing of the −35 and −10 regions of the *E. coli* consensus promoter was situated within a *Hpa*I fragment upstream of the 5′ end of the gene. *Hpa*I deletion of this sequence did not remove the ribosome binding site or the start codon but resulted in a loss of expressed enzymatic activity, indicating that the *B. fibrisolvens* promoter was functional in *E. coli*. The first 33 amino acids of the amylase ORF had features characteristic of signal peptides (64). Although longer than signal peptides generally found in gram-negative bacteria (9), this signal peptide was functional in *E. coli* since both the amylase and the AmyA-PhoA fusion proteins were exported to the periplasm. Leader sequences of between 31 and 44 residues have been reported previously for gram-positive bacterial proteins (66), and the *B. fibrisolvens* amylase signal peptide is the same length as that from the xylanase gene of *B. fibrisolvens* (33).

Most α-amylases studied in detail are calcium metalloenzymes and require calcium for optimal stability and activity (14, 62). Calcium was a strong activator of the *B. fibrisolvens* amylase and had to be included for optimum activity in both the growth media and assay buffers. The ability of the *B. fibrisolvens* amylase to hydrolyze the Phadebas substrate and the characteristic shape of the blue value and reducing sugar curves enabled the enzyme to be identified as an endoamylase. Hydrolysis of γ-cyclodextrin to G3, G2, and G1 confirmed the endo-α-amylolytic activity of the enzyme. α- and β-cyclodextrins were not hydrolyzed by the *B. fibrisolvens* amylase, but the reason why these substrates were not cleaved is unclear. These substrates have, however, been shown to inhibit the *R. amylophilis* α-amylase (38) and the sweet potato β-amylase (58), and likewise may be inhibitory to the *B. fibrisolvens* enzyme. G3 was the main degradation product during the initial stages of substrate hydrolysis, although after extended incubation G3 was hy-

drolyzed to G2 and G1. This hydrolysis profile suggested that the *B. fibrisolvens* endoamylase, like some exo-α-amylases, has a preference for removing G3 units. The action of the *B. fibrisolvens* amylase, however, appears to be different from that of the exo-α-amylases, which produce specific malto-oligosaccharides from soluble starch (7, 16, 56), in that it has no specific exo activity. This is illustrated by the rapid disappearance of the blue color in starch digests and the presence of oligosaccharides other than G3 after hydrolysis. This saccharifying-type activity of the *B. fibrisolvens* amylase has been described for α-amylases from *B. subtilis* (13, 37) and for a few α-amylases from ruminal bacteria (10, 65). Ruminal amylases that have been characterized also have G1, G2, and G3 as their main α-amylolytic products (10, 24, 38, 65). The *B. fibrisolvens* amylolytic activity is also similar to the maltogenic α-amylase of *A. oryzae*, which is used commercially in the production of high-maltose-content syrups (42).

Amino acid sequence comparison of the *B. fibrisolvens* amylase with other amylases showed that the four conserved regions commonly found in α-amylases were present. Three putative calcium-binding residues (6) were also observed, which was not surprising since the *B. fibrisolvens* amylase required calcium ions for activity. In addition, five regions of sequence similarity with prokaryotic and eukaryotic amylases were found, of which three regions (A, C, and E) are associated with substrate and calcium binding and of which two regions (B and D) are associated with protein folding (6). The overall amino acid similarity between the *B. fibrisolvens* H17c amylase and other amylases was limited to the N-terminal half of the enzyme, with a 20 to 30% similarity to both prokaryotic and eukaryotic amylases in this region. No similarity in the C-terminal half was found with any previously determined amylase sequences. Buisson et al. (6) have concluded from the crystal structure of porcine pancreatic α-amylase that domains A and B, comprising the first 407 amino acids, contain the calcium-binding and catalytic residues. The function of domain C, a distinct globular region comprising the remaining 89 amino acids, is unclear. Long et al. (31) found that the amino acid sequence of the *Streptomyces limosus* amylase had a high degree of similarity with sequences of mammalian and invertebrate amylases and have suggested a common evolutionary origin. The similarity between the N-terminal region of the *B. fibrisolvens* amylase with that of amylases from both *S. limosus* and eukaryotes is interesting, as it supports this view.

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