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Characterization of the *Butyrivibrio fibrisolvens glgB* Gene, Which Encodes a Glycogen-Branching Enzyme with Starch-Clearing Activity

ELAINE RUMBAK,[1] DOUGLAS E. RAWLINGS,[1] GEORGE G. LINDSEY,[2] AND DAVID R. WOODS[1]*

Departments of Microbiology[1] and Biochemistry,[2] University of Cape Town, Rondebosch 7700, Cape Town, South Africa

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A *Butyrivibrio fibrisolvens* H17c *glgB* gene, was isolated by direct selection for colonies that produced clearing on starch azure plates. The gene was expressed in *Escherichia coli* from its own promoter. The *glgB* gene consisted of an open reading frame of 1,920 bp encoding a protein of 639 amino acids (calculated M_r, 73,875) with 46 to 50% sequence homology with other branching enzymes. A limited region of 12 amino acids showed sequence similarity to amylases and glucanotransferases. The *B. fibrisolvens* branching enzyme was not able to hydrolyze starch but stimulated phosphorylase *a*-mediated incorporation of glucose into α-1,4-glucan polymer 13.4-fold. The branching enzyme was purified to homogeneity by a simple two-step procedure; N-terminal sequence and amino acid composition determinations confirmed the deduced translational start and amino acid sequence of the open reading frame. The enzymatic properties of the purified enzyme were investigated. The enzyme transferred chains of 5 to 10 (optimum, 7) glucose units, using amylose and amylopectin as substrates, to produce a highly branched polymer.

Glycogen is a branched homopolysaccharide of α-1,4-linked glucose subunits which are α-1,6 linked at the branch points. It represents a form of stored carbon for *Escherichia coli* and many other prokaryotes and provides a readily metabolized substance for endogenous metabolism and possibly for survival under starvation conditions. Biosynthesis of bacterial glycogen from glucose-1-phosphate involves at least three enzymes: ADP-glucose pyrophosphorylase (EC 2.7.7.27), glycogen synthase (EC 2.4.1.21), and the branching enzyme (α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase [EC 2.4.1.18]), products of the *glgC*, *glgA*, and *glgB* genes, respectively (for reviews, see references 40–42). The *glgB* gene product catalyzes the synthesis of α-1,6-glucosidic linkages in glycogen. A number of reports have dealt with the properties and action of bacterial branching enzymes (7, 12, 24, 54), and the enzyme from *E. coli* has been purified nearly to homogeneity (7). Branching enzyme activity has also been reported in a number of other bacteria (48, 54, 60), fungi (35), higher plants, and animals (42).

The *E. coli glg* genes have been cloned on a single genomic fragment, and their nucleotide sequences have been determined (3, 4, 29, 38). This gene cluster also encodes the degradative enzyme glycogen phosphorylase, GlgY(P), and contains an additional open reading frame, *glgX*, which has sequence similarity to the *E. coli* branching enzyme, as well as to glucan hydrolases and transferases (43, 58). The gene order for these enzymes is *glgY-glgA-glgC-glgX-glgB*. The *glg* genes have been reported to have no selectable phenotype, and two different strategies have therefore been utilized for their isolation and cloning. Indirect selection was used by Okita et al. (38) to clone the *glg* genes by cotransformation with the selectable *asd* (aspartate semidehydrogenase) gene which is adjacent to the *glg* gene cluster. Kiel et al. (27) isolated the *glgB* gene from the cyanobacterium *Synechococcus* sp. strain PCC7942 (*Anacystis nidulans*) by using the *glgB* gene from *E. coli* as a hybridization probe. The *A. nidulans* gene was sequenced and shown to have a 46% overall amino acid sequence similarity to the *E. coli* branching enzyme (25). It has been shown that branching enzymes from some bacteria (especially the enteric bacteria) cross-reacted with antibodies raised against branching enzymes from other bacteria (17, 21), thereby suggesting similarities in overall structure.

We used direct selection to isolate the *glgB* gene from the ruminal bacterium *Butyrivibrio fibrisolvens* by selecting for zones of clearing on starch azure plates. This gene was characterized, and its nucleotide sequence was determined; the gene product was purified to homogeneity, and the catalytic activity was characterized. The amino acid sequence was compared with that of previously sequenced enzymes of glycogen metabolism.

MATERIALS AND METHODS

Materials. Potato amylose (amylopectin free), potato amylopectin (amylose free), rabbit muscle phosphorylase *a*, rabbit liver glycogen, pullulanase (from *Enterobacter aerogenes*), and isoamylase (from *Pseudomonas amyloderamosa*) were obtained from Sigma Chemical Co., St. Louis, Mo. [^{14}C]glucose-1-phosphate was purchased from New England Nuclear, Stermage, United Kingdom; DEAE-cellulose was from Whatman, Clifton, N.J.; and Sephadex G-100 was from Pharmacia, Uppsala, Sweden. All of the other chemicals used were of analytical grade.

Bacterial strains, plasmids, and growth conditions. The ruminal bacterium *B. fibrisolvens* H17c (15) was used. Cloning and genetic manipulations were carried out with *E. coli* LK111 (*lacI lacZ* ΔM15 derivative of *E. coli* K514) (59) and K12 G6MD3 [Hfr *his thi* Str^s Δ(*malA-asd*)] (47), a gift from J. Preiss, Department of Biochemistry, Michigan State University. *E. coli-Bacillus subtilis* shuttle vector pEB1 has

* Corresponding author.

been described previously (32). *B. fibrisolvens* H17c was grown in M10 medium as described by Strydom et al. (51). *E. coli* strains were grown in Luria-Bertani medium, and 0.1 μg of ampicillin ml^{-1} was added for selection of transformants. When *E. coli* G6MD3 was used, the medium was supplemented with 50 μg of diaminopimelic acid ml^{-1} because of the *asd* deletion present in this strain. For isolation of the branching enzyme, 0.6% glucose was added to the media.

Screening of a *B. fibrisolvens* H17c genomic library and characterization of clones. Construction of the *B. fibrisolvens* gene library has been described previously (32). The gene bank was transformed into *E. coli* LK111, ampicillin-resistant transformants were selected on 0.5% starch azure plates containing ampicillin, and colonies producing clear halos were selected for further study. Preparation of plasmid DNA and restriction endonuclease mapping of the clones were carried out by standard techniques (33). *B. fibrisolvens* chromosomal DNA was prepared as previously described (5), 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 labelled by using a nonradioactive digoxigenin DNA labelling kit (Boehringer Mannheim).

Nucleotide sequencing. DNA fragments were subcloned in Bluescript vectors (Stratagene, San Diego, Calif.), and exonuclease III was used to generate two sets of overlapping deletions opposite in polarity (20). Complete sequencing of both strands was carried out by the chain termination method of Sanger et al. (46) with a Sequenase kit (version 2.0) from U.S. Biochemical Corp., Cleveland, Ohio. The nucleotide and deduced amino acid sequences were analyzed by using the Genetics Computer Group Inc. software package (version 6.2). The TFASTA subroutine was used to screen the GenBank (release 65.0), EMBL (release 24.0), Swiss Protein (release 15.0), NBRF-N (release 36.0), and NBRF-P (release 25.0) data bases for sequences similar to the amino acid sequence of the *B. fibrisolvens* branching enzyme.

In vitro transcription and translation. A prokaryotic DNA-directed in vitro transcription and translation kit (no. N380; Amersham) was used as specified by the manufacturer. The resulting proteins were analyzed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) (30). Pharmacia low-molecular-mass standards were used as markers.

Determination of branching enzyme activity. (i) Assay 1. Cells from 24-h, 100-ml *E. coli* G6MD3(pBGB100) cultures were harvested, rinsed with saline, and suspended in 2 ml of 0.1 M sodium citrate buffer, pH 7.0. The cell suspension was disrupted by sonication on ice (30-s bursts for 3 min) by using an MSE (Soniprep 150) sonicator and clarified by centrifugation for 15 min at 27,000 × *g* at 4°C. Samples were stored at −20°C, and the cell extracts were used to assay for branching enzyme activity. The basis of the assay used is the stimulation caused by the branching enzyme preparation to the unprimed synthesis of α-1,4-glucan polymer from glucose-1-phosphate by rabbit muscle phosphorylase *a* (10). The method used was essentially as described previously (21), except that the [^{14}C]glucose-1-phosphate concentration used was 370 nM and the reaction was initiated by addition of phosphorylase *a* rather than by glucose-1-phosphate.

(ii) Assay 2. Branching enzyme action against α-1,4-glucans was determined by monitoring the decrease in absorption of the α-glucan–iodine complex described by Boyer and Preiss (7). The absorbance of the iodine complex was measured at the λ_{max} for the natural α-glucan. One unit of branching enzyme activity was defined as the amount of enzyme that caused a decrease of 20% in the absorbance of the α-glucan–iodine complex in 1 min at 30°C. The average degrees of polymerization of the unit chains before and after the action of branching enzyme on amylopectin were determined (7). The unit chain lengths were separated by using a Beckman high-pressure liquid chromatography system equipped with a model 156 refractive index detector and a Waters C18 column.

Protein concentration was measured by the dye-binding method of Bradford (8) by using bovine serum albumin as the standard.

Purification of the branching enzyme. All work was carried out at 4°C unless otherwise stated. Assay II was used to determine enzymatic activity at the various stages of purification. Cells of 24-h, 200-ml *E. coli* G6MD3(pBGB200) cultures were harvested, rinsed with saline, and suspended in 20 ml of 10 mM Tris-HCl–50 mM citrate, pH 7.4. The cells were passed through an Aminco French pressure cell at 1.1 × 10^5 kPa, and the extract was clarified by centrifugation (20,000 × *g*, 30 min). The crude extract was absorbed onto a Whatman DE 52 column (2.5 × 15 cm) equilibrated in 10 mM Tris-HCl, pH 8.0. After the column was washed with 2 column volumes of this buffer, the branching enzyme was eluted with a linear gradient (0 to 0.3 M, 300 ml each) of NaCl in the same buffer. Fractions (2 ml) were collected and analyzed by SDS-PAGE, and those containing the branching enzyme were pooled. This branching enzyme pool was concentrated by ultrafiltration and applied to a Sephadex G-100 column (2.5 × 90 cm) equilibrated in 10 mM Tris-HCl–20 mM NaCl, pH 7.4. Fractions (0.5 ml) were collected and analyzed as described above.

Amino acid sequence and chemical analysis. Sequence analysis was performed on a gas-liquid solid-phase sequencer (9). The amino acids were identified by an isocratic high-pressure liquid chromatography system on a 3υ Lichrospher C18 (Bishoff) column (3 × 250 mm). The amino acid composition was determined.

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned GenBank accession no. M64980.

RESULTS

Isolation, location, and origin of the *glgB* gene. From approximately 7,500 colonies screened, seven *E. coli* G6MD3 transformants that produced a distinct halo around the colonies on starch azure plates were detected (Fig. 1). Similar halos were produced by the colonies when they were plated onto starch plates stained with I_2-KI, as well as on Phadebas substrate plates (data not shown). Restriction enzyme analysis showed that the plasmids from two of these clones had identical 5-kbp insert fragments and encoded a *B. fibrisolvens* α-amylase (44). Restriction enzyme mapping indicated that the plasmids from the other five clones had different-size overlapping inserts, and of these, plasmid pBGB100 was chosen for further study. A restriction map of the 5.8-kbp insert fragment on pBGB100 was constructed (Fig. 2). The 3.25-kbp *Eco*RV-*Eco*RV fragment was subcloned into the Bluescript SK sequencing vector in both orientations, pBGB200 and pBGB300. The orientation of pBGB200 was opposite to that of the vector *lac* promoter. Both subclones retained the ability to produce halos on starch plates, suggesting that an endogenous promoter was present on the DNA insert fragment. A smaller 2.5-kbp *Eco*RI-*Eco*RV fragment, pBGB210, which retained starch-

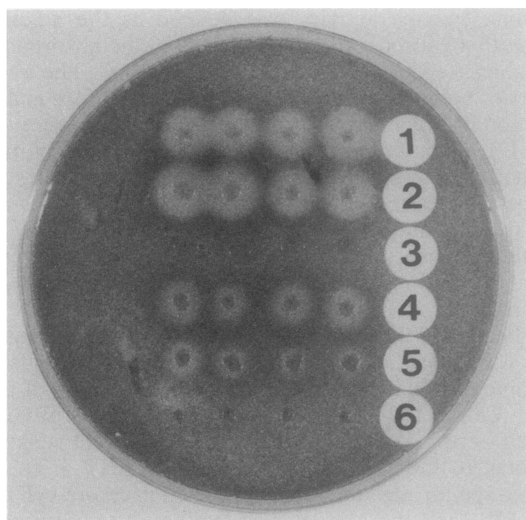

FIG. 1. Starch azure plate showing a comparison of the zones of clearing produced by the *B. fibrisolvens* branching enzyme (pBGB100) and a *B. fibrisolvens* α-amylase (pBAMY100) expressed in *E. coli* G6MD3. Rows: 1 and 2, *E. coli*(pBAMY100); 3, *E. coli* (pBluescript); 4 and 5, *E. coli*(pBGB100) and *E. coli*(pBGB200), respectively; 6, *E. coli*(pBluescript).

clearing activity, served to localize the position of the *glgB* gene. This plasmid contained the smallest fragment to code for an active gene, as exonuclease III deletions from either the 5′ or the 3′ ends (pBGB220 and pBGB230, respectively) resulted in loss of enzyme activity. The 3.25-kbp *Eco*RV fragment from pBGB100 hybridized to a fragment of an *Eco*RV digest of *B. fibrisolvens* chromosomal DNA of the same size, confirming the origin of the cloned DNA fragment (data not shown).

Nucleotide sequence of the *glgB* gene. The nucleotide sequence of the 2,500-bp *Eco*RI-*Eco*RV fragment from pBGB200 contained an open reading frame (ORF) encoding a protein of 639 amino acids with a calculated M_r of 73,875 (Fig. 3). A potential ribosomal binding sequence (GAGGG GG) was situated 6 bp upstream of the most likely ATG initiation codon at position 439. No sequence similar to either the σ^{70} or the σ^{54} *E. coli* consensus promoter sequence (18) could be found upstream of the initiation codon. An incomplete unidentified ORF of 134 amino acid residues terminated at a TAA codon 35 bp upstream of the putative *glgB* start codon. An 18-bp inverted repeat sequence including a region of six T residues was located 21 bp downstream of the putative *glgB* stop codon (Fig. 3). This sequence has the potential to form an mRNA stem-loop structure with a ΔG of −16.55 kcal (−69.26 kJ)/mol (45) and could serve as a rho-independent terminator in *E. coli*.

Detection of the translation product and comparison of the amino acid sequence with branching enzymes. An in vitro *E. coli* cell-free transcription-translation system was used to determine the M_r of the protein expressed by plasmid pBGB200 and its derivatives. SDS-PAGE of the in vitro translation products showed (Fig. 4A) that a protein with an apparent M_r of approximately 71,000 and some degradation products were produced from both pBGB200 and pBGB300 (lanes 1 and 2). The apparent M_r of this protein is in close agreement with the calculated M_r of 73,875 of the expected amino acid sequence of the *glgB* gene. A protein with a similar apparent M_r was also produced from pBGB210, the smallest insert coding for an active enzyme (lane 3). The higher concentration of protein produced when pBGB300 was used (lane 2) may reflect an increased level of transcription, as both the *lacZ* promoter of the vector and the *glgB* promoter were being transcribed in the same direction. The 71-kDa protein was absent when the Shine-Dalgarno ribosomal binding site and the ATG initiation codon of *glgB* were deleted (pBGB220, lane 4), and the apparent M_r of the protein was reduced when a C-terminal deletion of the *glgB* polypeptide was used (pBGB230, lane 5). The protein with an apparent M_r of 30,000 present in all lanes, including that of the Bluescript vector (lane 6), corresponded to the β-lactamase polypeptide. A dominant protein with the same apparent M_r of 71,000 was present in the in vivo translation products of *E. coli* G6MD3 carrying plasmids pBGB100, pBGB200, pBGB300, and pBGB210 (Fig. 4B, lanes 1 to 4, respectively). This protein was not present in *E. coli* G6MD3(pBluescript) (lane 5). High levels of expression of the *glgB* gene product appeared to be lethal. Although pBGB300 had the highest protein level in vitro, only low yields of protein were observed in vivo (Fig. 4A, lane 2, and B, lane 3).

The TFASTA subroutine based on the algorithm of Pearson and Lipman was used to compare the deduced amino acid sequence of the *B. fibrisolvens glgB*-encoded polypep-

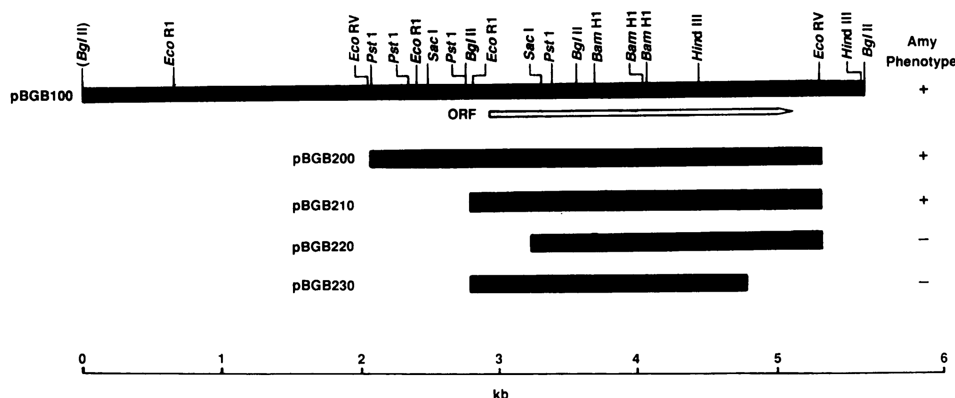

FIG. 2. Restriction endonuclease map of the insert DNA on pBGB100 and derivatives thereof encoding the *B. fibrisolvens* H17c branching enzyme. The ORF and direction of transcription are indicated by an open arrow, and the Amy phenotype indicates the presence or absence of starch-clearing zones produced by clones. pBGB300 is not shown, as it is identical to pBGB200 but opposite in orientation.

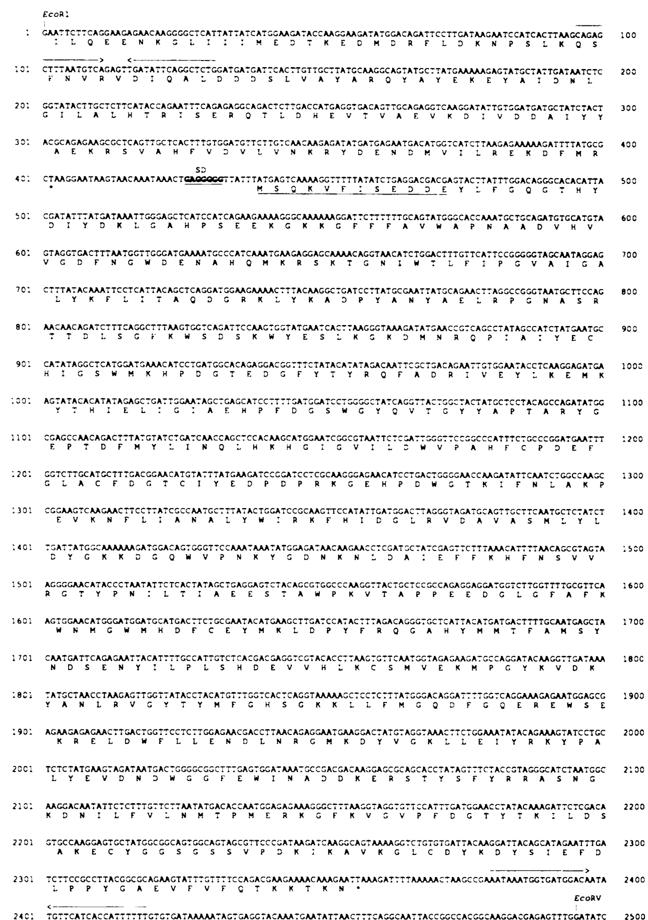

FIG. 3. Nucleotide sequence of the *B. fibrisolvens* H17c *glgB* structural gene. The predicted amino acid sequence is given below in single-letter code. The putative Shine-Dalgarno (SD) sequence is underlined and in boldface, the amino acids determined by sequence analysis are underlined, and the inverted repeat sequences are shown by converging arrows. The nucleotide and amino acid sequences of a second incomplete ORF are indicated upstream of the *glgB* gene.

tide with sequences in several data bases. The amino acid sequence was more similar to that of the glycogen branching enzymes rather than to those of the amylolytic enzymes (1, 16, 22, 23, 28, 52, 57). Alignment of the amino acid sequences of branching enzymes from *A. nidulans* (25), *Bacillis stearothermophilus* (26), and *E. coli* (4) with that of the *B. fibrisolvens* enzyme revealed similarities of 50, 46, and 46%, respectively (Fig. 5A). The *E. coli* and *A. nidulans* branching enzymes had approximately 100 additional amino acid residues at the N terminus. Some similarity to one of the conserved regions of amylases and glucanotransferases (37) was detected in a region of the N terminus (Fig. 5B), a region known to have contacts with the α-1,4-glucan substrate (36). Similarity in this region to the *glgX* gene from the *E. coli* glycogen gene cluster was also found, and this region is particularly well conserved among the branching enzymes (Fig. 5A).

Branching enzyme activity in crude extracts. Since the *glgB* gene product had greater sequence similarity to branching enzymes than to amylases, the branching enzyme activity expressed by the *glgB* gene in glycogen deletion strain *E.*

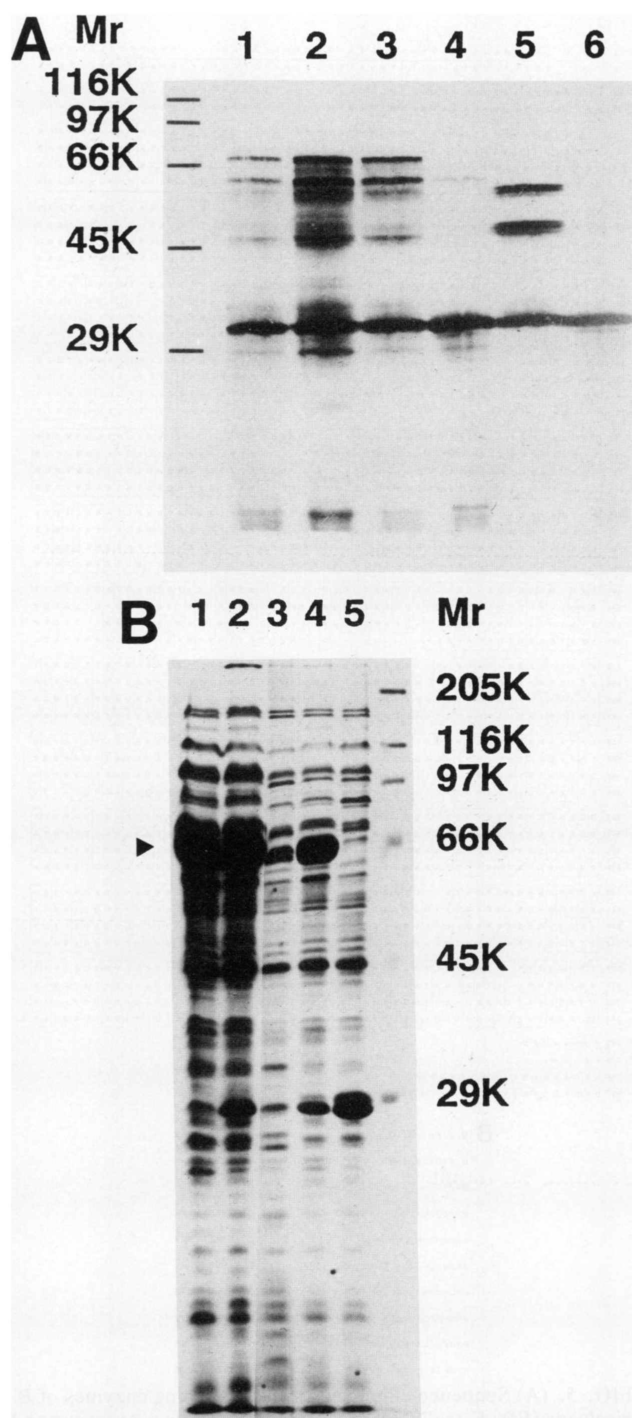

FIG. 4. SDS-PAGE analysis of in vitro (A)- and in vivo (B)-expressed proteins encoded by the cloned *B. fibrisolvens glgB* gene. (A) Lanes: 1, pBGB200; 2, pBGB300; 3, pBGB210; 4, pBGB220; 5, pBGB230; 6, pBluescript. (B) Lanes: 1, *E. coli*(pBGB100); 2, *E. coli* (pBGB200); 3, *E. coli*(pBGB300); 4, *E. coli*(pBGB210); 5, *E. coli* (pBluescript). Maps of the pBGB subclones are shown in Fig. 2. The molecular size (M_r) standards indicated were rabbit muscle myosin (205,000), β-galactosidase (116,000), phosphorylase *b* (97,400), bovine serum albumin (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000). The arrowhead indicates the position of the *glgB* gene product.

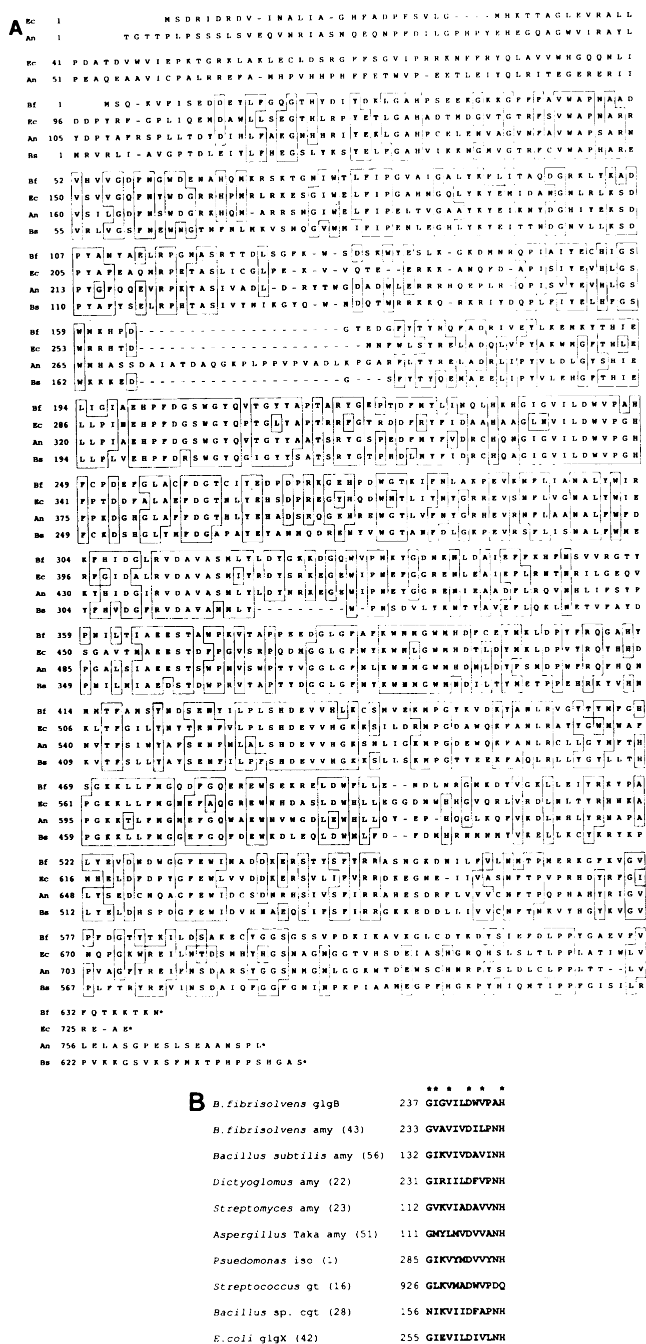

FIG. 5. (A) Sequence alignment of the branching enzymes of *B. fibrisolvens* (Bf), *E. coli* (Ec) (4), *A. nidulans* (*Synechococcus* sp.) (An) (25), and *B. stearothermophilus* (Bs) (26). Similarity was maximized by introducing gaps (dashes). Identical amino acids in three or four of the sequences are boxed. The number on the left refers to the first amino acid in each line. Asterisks indicate the ends of the proteins. (B) Alignment of the *B. fibrisolvens* branching enzyme with amylases over 12 amino acid residues. Abbreviations: amy, amylase; iso, isoamylase; gt, glucanotransferase; cgt, cyclodextrin glucanotransferase. The number to the left of each sequence refers to the amino acid position relative to the N terminus. Identical or conservative residue changes in 8 of 10 sequences are indicated by asterisks.

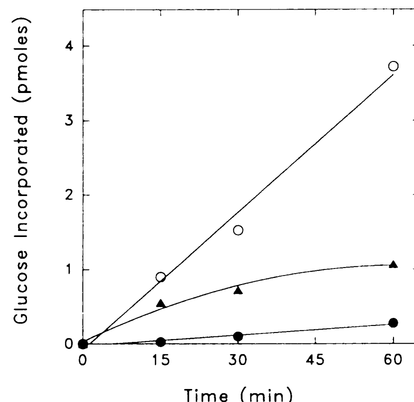

FIG. 6. Formation of [^{14}C]glucan catalyzed by the simultaneous action of crude cell extracts of the *B. fibrisolvens* branching enzyme expressed in *E. coli* G6MD3 and phosphorylase *a* (○), heat-denatured branching enzyme (▲), and phosphorylase *a* only (●). The reaction mixtures used (assay 1) are described in the text.

coli G6MD3 was determined (Fig. 6). Plasmid pBGB200 was chosen for study, since the *glgB* gene is expressed from its own promoter. Whereas cell extracts of *E. coli* G6MD3 (pBluescript) were unable to stimulate phosphorylase *a*-mediated incorporation of [^{14}C]glucose into an α-1,4-glucan polymer, *E. coli*(pBGB100) extracts containing the *glgB* gene product caused a 13.4-fold increase in this incorporation. Heat denaturation of the *E. coli* G6MD3(pBGB200) cell extract by incubation for 2 min at 100°C did not abolish the stimulated incorporation of glucose, and approximately 30% of the original activity was retained. This residual activity may have been due to the presence of a small amount of endogenous glucan primer in the crude extract (7). Since the *glgB* gene-containing clones were originally detected by the ability to clear starch plates, the cell extract was incubated with soluble starch to determine the hydrolytic activity of the expressed enzyme. Measurement of the reducing sugars released (6) showed that the extract had no detectable amylolytic activity towards soluble starch.

Purification of branching enzyme. A simple two-step procedure resulted in purification of the branching enzyme to apparent homogeneity (Fig. 7). Fractions 66 to 94 from the DEAE-cellulose column, containing the branching enzyme (Fig. 7a and b), were pooled and loaded on the G-100 column. Both SDS-PAGE (Fig. 7d) and nondenaturing PAGE (data not shown) indicated that only one protein band was eluted in the major peak from the Sephadex G-100 column (Fig. 7c). The graph of enzymatic activity followed the pattern of absorbance exactly. The purification procedure resulted in a final yield of approximately 33% of the activity and a 5.4-fold increase in specific activity (Table 1). The enzyme was stable for several months at 4°C.

Amino acid sequence analysis and hydrolysis. The amino acid sequence of the first 12 residues of the purified protein was determined. This showed that the N-terminal amino acid sequence was identical to that deduced from the nucleotide sequence (Fig. 3), confirming the putative translational start position of the *B. fibrisolvens glgB* gene. The amino acid composition (data not shown) agreed with the deduced amino acid composition.

Activity of purified branching enzyme. The branching enzyme was active between pHs 5 and 9, with an optimum pH of 7.2 in 10 mM Tris-HCl–50 mM citrate buffer. The enzyme

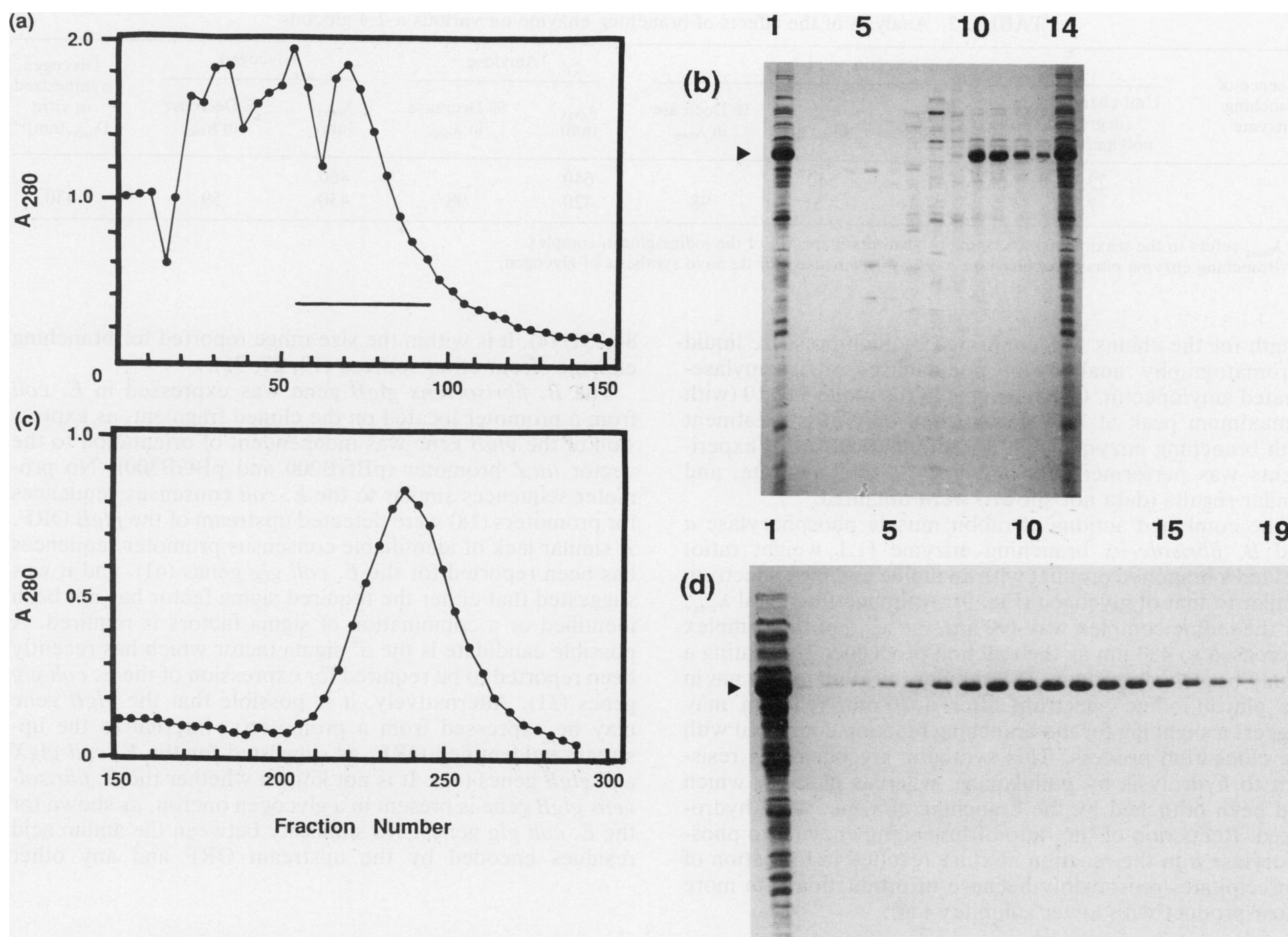

FIG. 7. Purification of the branching enzyme from *E. coli* G6MD3(pBGB200). (a) DEAE-cellulose chromatography of the crude cell extract in 10 mM Tris-HCl (pH 8). Protein eluted from the column was determined by measuring the A_{280} of every fourth fraction. The bar indicates fractions that contained branching enzyme activity. (b) SDS-PAGE of every eighth fraction of protein eluted from the DEAE column. Lanes: 1, cell extract; 2 to 13, fractions 6 to 94. Fractions 66 to 94, containing the branching enzyme (arrowhead), were pooled and applied to a Sephadex G-100 column. (c) Sephadex G-100 chromatography (in 10 mM Tris-HCl–20 mM NaCl [pH 7.4]) of the pooled fractions from a DEAE-cellulose column. Protein eluted was determined as described for panel a. (d) SDS-PAGE of every fourth fraction of protein eluted from the Sephadex G-100 column. Lanes: 1, cell extract; 2 to 19, fractions 202 to 270. Fractions 210 to 270 were pooled. The branching enzyme is indicated by the arrowhead.

had a broad pH range for maximal activity, with 90% of the activity present between pHs 6.8 and 8.0. Optimal branching activity was obtained at 37°C. The pH and temperature optima are similar to those reported for other bacterial branching enzymes (7, 27). The branching enzyme activity was unaffected by NaCl (up to 2 M) or dithiothreitol (1 mM), but the activity was completely inhibited by 0.1 mM mercuric chloride. The action of the *B. fibrisolvens* branching

TABLE 1. Purification of the *B. fibrisolvens* branching enzyme from *E. coli* G6MD3(pBGB200)

Fraction	Vol (ml)	Amt of protein (mg)	Activity (U)[a]	Sp act (U/mg)	Yield (%)
Crude extract	20	215.69	53,900	249.89	100
DEAE-cellulose	50	45.42	43,575	761.22	64.2
Sephadex G-100	43	13.00	17,459	1,343.03	32.4

[a] Units were calculated by using assay 2 (see Materials and Methods).

enzyme was tested on various natural α-glucans. Reactions were measured by monitoring the shift in the absorbance spectrum of the α-glucan–iodine complex and were terminated only when constant values were reached. A 98% decrease in the absorbance at the λ_{max} of the α-glucan–iodine complex for both amylose (640 nm) and amylopectin (540 nm) was observed (Table 2). A shift in the maximum wavelength of the iodine complex to 420 nm indicated that a large increase in the number of branch points had occurred. Similarly, the branching enzyme caused a decrease of 59% in the λ_{max} at 460 nm and a shift of the maximum wavelength of absorption of the rabbit liver glycogen-iodine complex from 460 to 430 nm.

To investigate the effect of the branching enzyme on amylopectin, the changes in the average chain length and the percentage of α-1,6 bonds were determined (Table 2). The degree of polymerization (as determined by the ratio of glucose to reducing glucose equivalents) of amylopectin changed from 22.7 to 7.1, and simultaneously the percentage of α-1,6 bonds increased from 4.4 to 19.3%. This average

TABLE 2. Analysis of the effects of branching enzyme on various α-1,4-glucons

Presence of branching enzyme	Amylopectin				Amylose		Glycogen		Glycogen synthesized in vitro (λ_{max}[nm])[b]
	Unit chain length (degree of polymerization)	% α-1,6 bonds	λ_{max} (nm)[a]	% Decrease in λ_{540}	λ_{max} (nm)	% Decrease in λ_{640}	λ_{max} (nm)	% Decrease in λ_{460}	
−	22.1	4.4	540		640		460		
+	7.1	19.3	420	98	420	98	430	59	430

[a] λ_{max} refers to the maximum wavelength of absorption spectra of the iodine-glucan complex.
[b] Branching enzyme plus phosphorylase *a* (assay 1) was used for de novo synthesis of glycogen.

length for the chains was confirmed by high-pressure liquid chromatography analysis of pullulanase- or isoamylase-treated amylopectin. Chain lengths in the range 5 to 10 (with a maximum peak at 7) were detected only after treatment with branching enzyme (Fig. 8). An identical set of experiments was performed with amylose as the substrate, and similar results (data not shown) were obtained.

The combined actions of rabbit muscle phosphorylase *a* and *B. fibrisolvens* branching enzyme (1:1 weight ratio) yielded a branched product with an iodine complex spectrum similar to that of glycogen (Fig. 9). Although the initial λ_{max} for the iodine complex was 490 nm, the λ_{max} of the complex decreased to 430 nm as the reaction proceeded, indicating a highly branched product. The shoulder present at 470 nm in the glucan-iodine spectrum after a 30-min reaction may suggest a slight lag for the branching reaction compared with the elongation process. This synthetic glycogen was resistant to hydrolysis by pullulanase, whereas glucans, which had been branched by the branching enzyme, were hydrolyzed. Reduction of the ratio of branching enzyme to phosphorylase *a* in the reaction mixture resulted in formation of a precipitate, presumably because of production of a more linear product with lower solubility (56).

DISCUSSION

We have shown that the gene isolated from a *B. fibrisolvens* H17c gene bank by selection for starch-clearing activity codes for a polypeptide with an M_r of 73,875 with branching enzyme rather than amylolytic activity. Although this polypeptide is smaller than the *E. coli* branching enzyme (M_r, 84,231) (4), it is within the size range reported for branching enzymes from other sources (19, 27, 35).

The *B. fibrisolvens glgB* gene was expressed in *E. coli* from a promoter located on the cloned fragment, as expression of the *glgB* gene was independent of orientation to the vector *lacZ* promoter (pBGB200 and pBGB300). No promoter sequences similar to the *E. coli* consensus sequences for promoters (18) were detected upstream of the *glgB* ORF. A similar lack of identifiable consensus promoter sequences has been reported for the *E. coli glg* genes (41), and it was suggested that either the required sigma factor has not been identified or a combination of sigma factors is required. A possible candidate is the σ^s sigma factor which has recently been reported to be required for expression of the *E. coli glg* genes (31). Alternatively, it is possible that the *glgB* gene may be expressed from a promoter(s) internal to the upstream unidentified ORF, as suggested for the *E. coli glgX* and *glgB* genes (41). It is not known whether the *B. fibrisolvens glgB* gene is present in a glycogen operon, as shown for the *E. coli glg* genes. No similarity between the amino acid residues encoded by the upstream ORF and any other

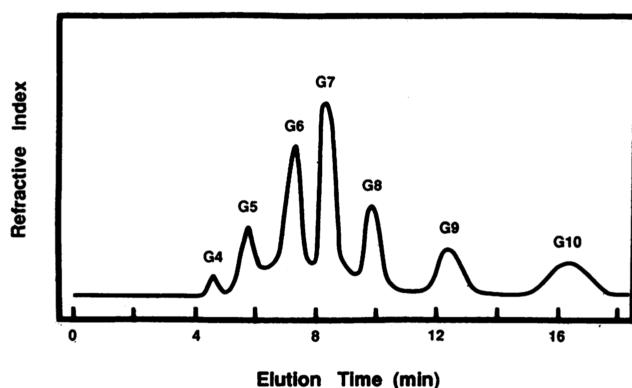

FIG. 8. High-pressure liquid chromatographic analysis of unit chains debranched from amylopectin by isoamylase or pullulanase after the action of the branching enzyme. G4 to G10 represent numbers of glucose residues present in the unit chains. The control, debranched native amylopectin, showed no glucose units released within the elution time measured.

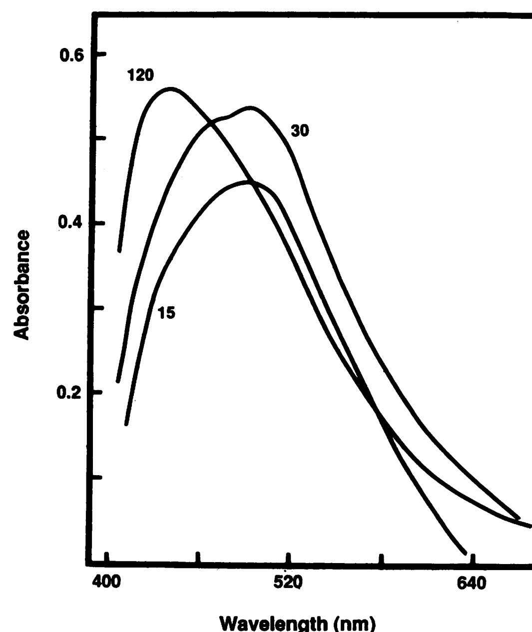

FIG. 9. Absorption spectra of the iodine-glucan complex of the α-glucan formed de novo by the purified branching enzyme and phosphorylase *a* (ratio of 1:1). In vitro synthesis of glycogen was as described for assay 1, except that 50 mM glucose-1-phosphate was used. The iodine-glucan absorption spectra of samples were measured after incubation for 15, 30, and 120 min at 30°C.

sequence reported in the data bases could be found. The amino acid sequence of the *B. fibrisolvens* branching enzyme showed similarity to those from *E. coli* (4), *Synechococcus* sp. (25), and *B. stearothermophilus* (26) over the entire length of the polypeptide.

The high degree of conservation between branching enzymes of bacteria that belong to vastly different groups is remarkable, particularly when it is considered that glycogen storage is not essential for growth and as such is absent from many species of bacteria. Since *B. fibrisolvens* has been reported to be the most prevalent ruminal bacterium under adverse nutritional conditions (34, 39) and since carbohydrate storage is thought to play an important role in cell survival under such conditions (50), it is tempting to speculate that *B. fibrisolvens* stores glycogen or a glycogenlike polysaccharide as a survival strategy. Storage of glycogen in butyrivibrios has not been reported, although several other ruminal bacteria are known to produce a glycogenlike polysaccharide (14, 49, 55). Attempts to isolate glycogen from *B. fibrisolvens* were unsuccessful, although an anthrone-positive polysaccharide that could not be digested with α-amylase was isolated.

The simple two-step procedure for purification of the *B. fibrisolvens* branching enzyme to homogeneity was largely due to the high level of expression of the *glgB* gene product in the *E. coli* cell extract, i.e., 18.6% of total protein, calculated from the relative specific activities. The abundance of the *B. fibrisolvens* branching enzyme in *E. coli* extracts is probably due to a combination of the high-copy-number plasmid vector and an efficient *glgB* promoter and ribosomal binding site. Because of the initial concentration of the branching enzyme in the crude extract, the increase in specific activity obtained on purification was not high (5.4-fold) compared with increases obtained for purification of the *E. coli* B (754-fold; 7) and *Neurospora crassa* (226-fold; 35) branching enzymes.

The *B. fibrisolvens* branching enzyme exhibited activity towards a wide range of α-1,4-glucans, as has been shown for other branching enzymes (7, 54, 60). With amylopectin as the substrate, the *B. fibrisolvens* branching enzyme transferred oligosaccharide chains 5 to 10 glucose units long, with 7 as the preferred unit size. Branching enzymes from liver (11), muscle (53), and *E. coli* B (7) have been reported to exhibit similar chain length specificity. The product obtained from branching amylopectin had a maximum wavelength of 420 nm for the iodine complex, characteristic of highly branched glycogen with comparatively short chain lengths (2). This λ_{max} for the iodine complex is similar to that obtained from the glycogenlike polysaccharide isolated from the ruminal bacterium *Bacteroides succinogenes* (49). The percentage of α-1,6 bonds introduced into amylopectin by the branching enzyme was 19.33%, which is higher than the 9.6% reported for *E. coli* B (7) and the 8% reported for *Arthrobacter globiformis* (60). De novo biosynthesis of a glycogenlike polysaccharide using the *B. fibrisolvens* branching enzyme in conjunction with rabbit muscle phosphorylase *a* resulted in production of a branched glucan that resembled native glycogen in its iodine-staining properties. This synthetic branched polysaccharide, like native glycogen, was resistant to pullulanase hydrolysis, although the branches formed by branching native α-1,4-glucans were readily hydrolyzed by pullulanase. This supports the hypothesis (7, 19) that the enzymes involved in α-glucan polysaccharide synthesis act in a coordinate rather than a successive fashion.

The ability of the *B. fibrisolvens* branching enzyme to produce halos on starch azure and Phadebas substrates can be explained by the ability of the *B. fibrisolvens* branching enzyme to produce highly branched structures, which are more soluble than the relatively unbranched substrates. Although the Phadebas substrate, an insoluble starch incorporating a covalently cross-linked blue dye, is considered to be specific for starch hydrolysis (13), increased branching of the Phadebas substrate would result in increased solubility (56) without hydrolysis of the dye-substrate linkage. The soluble substrate would then diffuse to equilibrium over the entire plate, thereby producing a halo (Fig. 1). An increase in absorbance was also obtained with the Phadebas substrate in liquid assays as a result of the increased solubility, due to branching enzyme activity, of the dye–cross-linked-starch complex (results not shown). The *B. fibrisolvens* branching enzyme displayed no hydrolytic activity towards soluble starch, as no release of reducing sugars could be detected. Caution must therefore be exercised in using substrates utilizing the solubility of dye-linked starch to assay amylolytic activity.

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