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Regulation and Cloning of the Gene Encoding Amylase Activity of the Ruminal Bacterium *Streptococcus bovis*

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***Streptococcus bovis* is an important starch-degrading ruminal bacterium that has been implicated as being important in the etiology of a number of ruminal pathologies associated with diets high in grains. Previous studies with *S. bovis* have shown that amylase production was influenced by the growth substrate, but the nature of this regulation was not determined. The current study was conducted to better describe the regulatory phenomena and gain a better understanding of the molecular characteristics of this activity. Nutritional experiments demonstrated that the presence of starch or the starch-derived disaccharide maltose was required for maximum amylase production. Subsequent time-course experiments showed that amylase synthesis was induced by maltose and repressed by glucose, cellobiose, and fructose, while inulin and lactose had little effect on enzyme accumulation. The effects of the added antibiotics rifampin and tetracycline were consistent with transcriptional control of amylase synthesis. Analysis of *S. bovis* cells grown on glucose or maltose showed that they contained similar low levels of cyclic AMP, indicating that it was unlikely that regulation of amylase synthesis was mediated through a mechanism involving this nucleotide. The amylase gene from *S. bovis* JB1 was cloned and expressed in *Escherichia coli*. The amylase produced in *E. coli* was of lower molecular weight than that synthesized by *S. bovis* and had catalytic characteristics different from those of *S. bovis* amylase. When the gene was introduced back into *S. bovis* JB1, only one form of amylase activity was detected, indicating that the entire gene was present on this insert. The use of the amylase gene as a genetic probe for identification of *S. bovis* strains is discussed.**

Digestion in ruminant livestock is largely accomplished by a dense population of microorganisms that inhabit the rumens of these animals. During normal rumen digestion, the numbers and activities of these organisms are balanced such that stable ruminal fermentation that generates all the nutrients required by the host animal results. This balance can be perturbed by abrupt changes in feeding regimens, causing both acute and chronic ruminal disfunction. This is particularly true when animals are switched from forage diets to grain diets (1, 18, 19, 35). In practice, this can occur when animals are fed diets high in grains such as those given to feedlot beef cattle and high-producing dairy cattle. Many of the problems associated with these diets have been attributed to the proliferation of the ruminal bacterium *Streptococcus bovis* (11, 16).

S. bovis is a normal inhabitant of the rumen, generally present at about 10^7 cells per ml compared with over 10^{10} total bacterial cells per ml of rumen fluid but can be more numerous for short periods immediately after feeding (10, 30). Strains can grow on glucose, sucrose, lactose, and starch and its hydrolysis products (maltose and maltooligosaccharides) but cannot utilize pentoses (xylan hydrolysis products) or uronic acids (pectin hydrolysis products) (32). Although *S. bovis* ferments cellobiose, the ability to utilize cellulodextrins (possible cellulose hydrolysis products) appears to be limited (25). While the spectrum of carbohydrates that *S. bovis* can ferment is somewhat limited, this species is able to grow extremely rapidly when provided with a suitable energy source for growth. Growth rates in batch culture are frequently about 2 h^{-1} (doubling time of approximately 21 min), and growth rates in continuous cultures greater than 3 h^{-1} (doubling time of 14 min) have been reported (6, 29). During normal, stable ruminal fermentation, the concentration of soluble carbohydrates is maintained at low levels, restricting the growth rate of microorganisms. These conditions favor the growth of species other than *S. bovis* that exhibit higher affinities for available growth substrates and lower maintenance energy expenditures (28, 29). This condition changes when large quantities of starch are fed to animals unadapted to a high-grain diet. When this occurs, the numbers of *S. bovis* can increase in a matter of a few hours from 10^7/ml to nearly 10^{10}/ml, and ruminal pH drops to 4.0 to 4.5 (11, 16). While undoubtedly not the sole organism involved in the generation of low ruminal pHs and resultant rumen disfunctions, clearly the rapid growth and production of lactic acid by this species are important contributors to these conditions.

S. bovis is among the most active amylase-producing ruminal bacteria. Cultures growing in starch-containing media rapidly convert starch into maltooligosaccharides, which are then fermented (6, 7). Its ability to assimilate these hydrolytic products is superior to those of the other species of ruminal bacteria examined, both in batch and continuous cultures (7, 31). Previous work showed that the amylolytic activity of *S. bovis* is the endo-splitting variety such as that of α-amylase (6, 36). Production of amylase varied with the growth substrate, being produced in large quantities in maltose and starch cultures but in much smaller quantities in glucose-grown cultures, suggesting that the synthesis of activity was probably regulated (6). The objective of the current study was to further examine the characteristics of the amylolytic activity by examining the nutritional regulation of amylase production and describe the cloning and expression of the gene in *Escherichia coli* and *Bacillus subtilis*. The information collected in this and other studies

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should be useful in devising strategies to control the overgrowth of *S. bovis* and the associated problems.

MATERIALS AND METHODS

Organisms, growth, and sampling. *S. bovis* JB1 (27), K27FF4 and 21-09-6C (gifts from N. O. Van Gylswyk, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden), and SC101 (gift of D. A. Odelson, Department of Biology, Central Michigan University, Mt. Pleasant, Mich.) were grown in batch cultures in a complex, Trypticase-yeast extract-containing medium (routine growth medium [RGM] [8]). For experiments examining the influence of energy source on the production of amylase, *S. bovis* JB1 was grown in RGM with various carbohydrates and sampled at various time intervals for determination of amylase activity. Culture samples were centrifuged (10,000 × *g*, 20 min, 4°C), and cells were resuspended in an equal volume of 100 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 6.8). Cells and cell-free supernatant fluid samples were analyzed immediately for amylase activities. The addition of tetracycline (5 μg/ml) or rifampin (5 μg/ml) to cultures in certain experiments was done as noted below.

E. coli JM83 (38) and V850 (20) were grown on LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter). LB supplemented with either ampicillin (75 μg/ml) (LBA) or erythromycin (10 μg/ml) was used when selecting for transformants. LBA-agar plates containing 2 mg of starch azure (Sigma Chemical Co., St. Louis, Mo.) per ml were used for screening *E. coli* transformants for the cloned amylase activity. *B. subtilis* BR151 *trpC2 lys-3 metB5 amyE* (referred to below as *B. subtilis* BR151 *amyE*), a spontaneous amylase-negative mutant, was a gift from Glenn Chambliss, University of Wisconsin. *B. subtilis* BR151 *amyE* was grown on LB or LB supplemented with chloramphenicol (10 μg/ml) for selection of transformants containing pBS42 (2) or derivatives of this plasmid. Amylase-producing *E. coli* and *B. subtilis* clones were grown in LB containing the appropriate antibiotics to mid-log phase, centrifuged at 10,000 × *g* at 4°C for 10 min, and washed with 50 mM sodium phosphate (pH 6.8)–1 mM dithiothreitol. Cells were suspended to 5% of the original culture volume in the same buffer and broken with a French pressure cell at 12,000 lb/in^2. The broken cell suspension was centrifuged at 30,000 × *g* for 20 min at 4°C. The supernatant fluid (crude extract) was recovered and used for enzyme assays and protein analyses. The growth of all broth cultures was monitored spectrophotometrically (Spectronic 70; Bausch & Lomb, Inc., Rochester, N.Y.) by determining the optical densities of cultures at 660 nm. The glucose concentrations in culture fluids were determined enzymatically with a glucose oxidase and peroxidase diagnostic kit (Sigma Chemical Co.).

Protein and enzyme assays. The amylase activity of samples was determined by monitoring the increase in reducing sugar formation from starch by using the dinitrosalicylic acid reagent (6, 23). Reaction mixtures contained 0.5 ml of soluble potato starch (1.0% in 100 mM PIPES buffer, pH 6.8, containing 0.06% $CaCl_2 \cdot 2H_2O$) and 0.5 ml of an appropriately diluted enzyme sample. Mixtures were incubated at 39°C for 10 min, enzyme activity was stopped by the addition of 1.5 ml of dinitrosalicylic reagent, and resultant solutions were heated at 100°C for 15 min. The amount of reducing sugars formed was estimated by comparison to a glucose standard. One enzyme unit equaled 1 μmol of glucose equivalent formed per min. Cell protein was determined by the method of Lowry et al. (17) after hydrolysis of cells in base (0.1 N NaOH) at 70°C for 30 min. Protein concentrations of cell extracts were estimated by the dye-binding assay of Bradford (4) with the commercial Bio-Rad Laboratories (Richmond, Calif.) reagent. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (15). Proteins were stained with Coomassie brilliant blue R-250. Renaturation and detection of amylase activity following SDS-PAGE were carried out essentially as described previously (14). Amylase activities in supernatant fluids from glucose-grown *S. bovis* cultures were concentrated by using Millipore centrifugal filtration units with 30,000 NMWL ultrafiltration membranes (Millipore Corp., Bedford, Mass.) according to the manufacturer's instructions.

Amylose and amylopectin hydrolysis. The products of amylolytic attack were determined by measuring the release of maltooligosaccharides from amylose and amylopectin by a thin-layer chromatography method (7). Amylase-containing samples were combined with 20 ml of an amylose or amylopectin solution (1% final concentration in 100 mM PIPES buffer, pH 6.8, containing 0.06% $CaCl_2 \cdot 2H_2O$) and incubated at 39°C, and samples were removed at various times. Samples (10 μl) were applied to Whatman 150A K5 silica gel plates and developed three successive times in a solvent of nitroethane, ethanol, and water (1:3:1) (7). The plates were air dried, and spots were visualized by spraying the plates with *N*-(1-naphthyl)ethylenediamine hydrochloride (200 mg in 97 ml of methanol with 3 ml of concentrated sulfuric acid added) (3) and heating the plates to 100°C for 5 to 10 min. Oligosaccharides were identified by comparison to the migration of authentic standards.

DNA and cloning protocols. Genomic DNA from *S. bovis* JB1 and other *S. bovis* strains was isolated essentially as described by Saito and Miura (34). Plasmid pUC18 (38) was used for cloning, with *E. coli* JM83 (38) as the host. The genomic DNA was partially digested with *Pst*I, and 3- to 10-kb fragments were recovered from low-melting-point agarose by using the Elutip-d column (Schleicher & Schuell, Keene, N.H.) according to the manufacturer's instructions. The DNA was ligated into *Pst*I-digested and dephosphorylated pUC18 and transformed in *E. coli* JM83. Expression of amylase activity was screened by plating transformed cells onto LBA containing 2 mg of starch azure per ml and observing clearing zones around the colonies. Plasmid DNA from *B. subtilis* and *E. coli* was isolated by miniprep procedures or large-scale procedures using the Circleprep Kit (Bio101, La Jolla, Calif.). The *E. coli-B. subtilis* shuttle vector pBS42 (2) was used for subcloning and transformation in *B. subtilis* BR151 *amyE*.

DNA was analyzed by digestion with restriction endonucleases according to the manufacturer's instructions. Digested DNA was electrophoresed through agarose gels in 89 mM Tris–68 mM phosphoric acid–2 mM EDTA and stained with ethidium bromide. Southern hybridization analyses were carried out by standard protocols (21) with biotinylated probes. Biotinylated probes were prepared with a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) and 11-biotin-labeled dUTP according to the manufacturer's instructions. Hybridizations and staining with strepavidin-biotin-alkaline phosphatase were carried out with a DNA detection kit (Bethesda Research Laboratories, Gaithersburg, Md.).

Electroporation of *S. bovis* JB1. The plasmid pVA838, an *E. coli-S. sanguis* shuttle vector (20), and pVA838 containing the cloned *S. bovis* amylase gene were introduced into *S.*

TABLE 1. Effect of growth substrate on the production of amylase by *S. bovis*

Substrate	Amylase activity (U/mg of cell protein)[a]	Growth (μg of cell protein/ml)	% Distribution	
			Cells	Supernatant
Maltose	22.9 ± 0.38	174	7	93
Starch	23.8 ± 2.26	203	85	15
Glucose	1.3 ± 0.40	201	16	84
Sucrose	1.6 ± 0.13	188	24	76
Lactose	1.2 ± 0.07	179	17	83
Melibiose	1.0 ± 0.36	161	23	77
Inulin	1.1 ± 0.77	202	32	68
Cellobiose	1.3 ± 0.31	177	16	84
Fructose	1.0 ± 0.54	147	32	68

[a] Values are the means for triplicate cultures.

bovis JB1 by electroporation. The amylase gene was isolated from pWC22 by using the *Sma*I and *Sph*I sites of the multiple cloning site and inserted into the *Pvu*II and *Sph*I sites of pVA838. This plasmid was designated pVWC1. *S. bovis* cells were prepared and electroporated as previously described (37), except a Bio-Rad gene pulser was used at 24 μF and 200 Ω, with a field strength of 1.5 kV cm^{-1}. Following electroporation, cells were transferred to an anaerobic glove box, diluted into 2 volumes of RGM-glucose, and incubated at 37°C for 1 h. Cells were plated onto RGM-glucose-agar plates containing 5 μg of erythromycin per ml for selection of transformants.

cAMP determination. Cell samples of *S. bovis* grown on maltose or glucose were collected, suspended in 5% of the original volume of 0.1 N HCl, and heated to 95°C for 10 min (12). The resulting solution was centrifuged to remove cellular debris. Cyclic AMP (cAMP) in these samples was determined by radioimmunoassay using a commercially available kit (Biomedical Technologies Inc., Stoughton, Mass.) according to the manufacturer's instructions. Assays were also performed with *E. coli* JM83 grown on glucose and lactose (positive response) and *Bacteroides thetaiotaomicron* grown on glucose and maltose (negative control) (12).

Chemicals. A preparation of soluble potato starch (catalog no. S-2630; Sigma Chemical Company) that contained no detectable glucose, maltose, or other oligosaccharides (as determined by high-pressure liquid chromatography and thin-layer chromatography) was used as both the growth and amylase assay substrates. Oligosaccharide standards, amylopectin, and amylose also were obtained from Sigma Chemical Co. All other reagents were used at the highest grade available.

RESULTS

Effect of energy source on the production of amylolytic activity. *S. bovis* was grown overnight on a variety of substrates, and the effect of substrate on the growth, production, and distribution of amylase was determined. All the substrates used provided for good growth, but growth on only maltose and starch was associated with the accumulation of a high level of amylase activity (Table 1). The amylolytic activity of maltose-grown cultures was predominantly extracellular (>85%), while the greatest portion of amylase sedimented with the cell pellet when starch was the growth substrate. The degree of sedimentation of amylase in starch-grown cultures varied with the preparation of the starch substrate. If the starch used as the growth substrate was freshly prepared and *S. bovis* was inoculated immediately upon addition of starch to RGM, a greater portion of amylolytic activity was found in the culture fluid. However, if the same starch was stored for a period of time and allowed to undergo retrogradation, the amylase increasingly became associated with sedimentable material. The low level of activity produced when other substrates were provided for growth was predominantly extracellular (>80%).

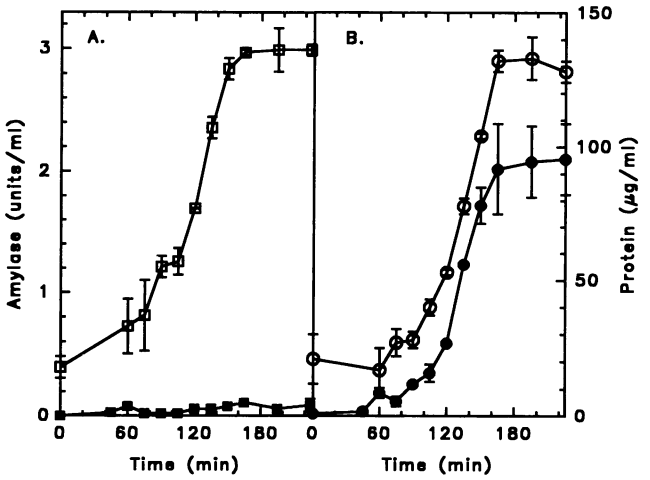

FIG. 1. Production of amylolytic activity by *S. bovis* growing on 0.2% glucose (A) or maltose (B). ■ and ●, amylase activity; □ and ○, cell protein.

Time-course experiments were conducted to study the pattern of amylase production by *S. bovis*. Growth on maltose resulted in an accumulation of amylase that generally paralleled the growth of the culture and accumulation ceased when growth ceased (Fig. 1). In contrast, the amylase activity of glucose-grown cultures remained low at all times, even after glucose was exhausted. These results suggested that the production of activity is regulated by the available carbohydrate source. To more clearly identify the factors influencing the production of amylase by *S. bovis*, a variety of experiments were conducted to evaluate the effects of growth substrates when provided in combinations. In these experiments, cultures were grown to the mid-logarithmic phase on one substrate, a second substrate was added, and amylase production was monitored until growth ceased. When glucose was added to cultures growing on maltose, the production of amylase activity ceased immediately but resumed again in approximately 45 min (Fig. 2). In the converse experiment, the addition of maltose to cultures growing on glucose had little effect until about 45 min after its addition when a rapid accumulation of amylolytic activity was noted. In both cases, the increase in amylase activity was coincident with the depletion of glucose in these cultures (i.e., when the glucose concentration dropped below 0.1 mM [data not shown]). In contrast to the glucose inhibition of amylase production, inulin exerted little effect (data not shown). Addition of inulin to maltose cultures did not decrease the accumulation of amylase, and the addition of maltose to inulin cultures resulted in an almost immediate increase in the amylolytic activity of these cultures (between 5 and 10 min). Cultures grown on inulin alone produced little amylase. A variety of other combinations of carbohydrates were tested for their effects when provided in combination with maltose. Both fructose and cellobiose, liked glucose,

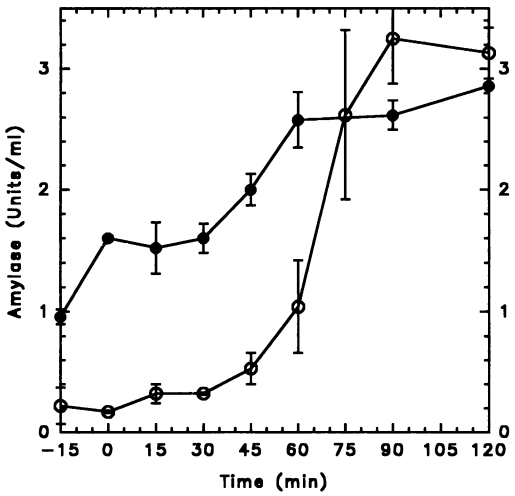

FIG. 2. Effect of the addition of glucose or maltose to growing cultures of *S. bovis* on subsequent production of amylase. Glucose (0.2%) was added to cultures growing on maltose (0.2%) (●) at zero time, while maltose (0.2%) was added to cultures growing on glucose (0.2%) (○).

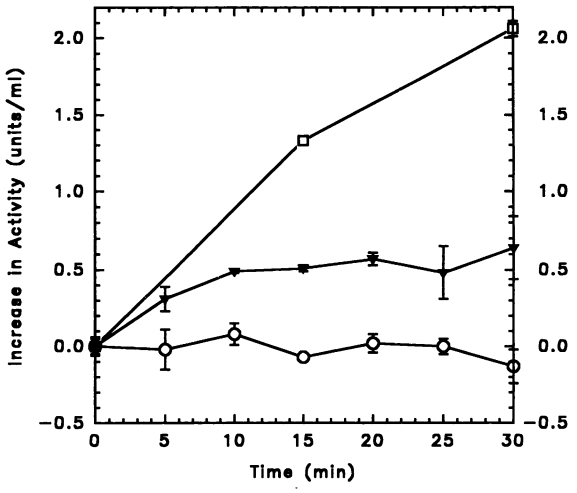

FIG. 3. Effect of the addition of antibiotics on the induction of amylase by maltose. Maltose (0.2%) was added to cultures growing on inulin (0.2%) 15 min prior to the addition of the antibiotics (zero time). The increase in amylase activity after the addition of rifampin (▼) or tetracycline (○) or with no added antibiotic (□) was monitored over time.

inhibited amylase synthesis. Lactose, like inulin, was found to have no effect.

Since *S. bovis* exhibited such a marked response in the production of amylase when glucose was added to maltose cultures, the possibility that catabolite repression involving cAMP might play a role in the control of amylase synthesis in this organism was considered. The levels of cAMP were measured in cells grown on glucose or maltose and compared with cAMP concentrations in *E. coli* and *B. thetaiotaomicron*. *S. bovis* cells contained similar levels of cAMP regardless of the growth substrate (0.095 nmol/ml). These levels were much lower than those observed for *E. coli* (2 to 6 nmol/ml) and were similar in concentration to those for *B. thetaiotaomicron* (0.054 nmol/ml).

Effect of antibiotics on the production of amylolytic activity. The antibiotics rifampin and tetracycline were tested for the ability to inhibit the maltose induction of amylase in inulin-grown cultures at the transcriptional and translational levels, respectively. Experiments were conducted in the same manner as were the induction-repression experiments described above except that antibiotics were added 15 min after the addition of maltose. The addition of either antibiotic to these cultures inhibited the accumulation of amylolytic activity (Fig. 3). In rifampin-supplemented cultures, a modest increase occurred during the first 10 min following the addition of the antibiotic, but little amylase accumulated after this time. The effect of tetracycline on amylase synthesis was even more pronounced. The addition of tetracycline resulted in cessation of amylase synthesis in less than 5 min. The effect of both antibiotics on the growth of these cultures was identical (data not shown). There was little effect for the first 10 min after addition, followed by a gradual decline in the growth rate of cultures.

Cloning of the amylase gene. A genomic library of *S. bovis* JB1 DNA was prepared in *E. coli* JM83 by using pUC18. The bacteria were screened on LBA-starch azure plates, and initially 7 clones from over 9,000 screened had clear zones around the colonies. Upon being restreaked, five retained the clearing zones. Plasmid DNA was isolated from these clones, and all were found to contain an identical *Pst*I insert of approximately 5 kb as determined by restriction endonuclease digestion analyses. Partial restriction maps for two of these clones, pWC2 and pWC7, are shown in Fig. 4. The insert in pWC7 was found to be in the opposite orientation from pWC2 (Fig. 4). Since both orientations expressed the amylase activity, this suggests that *E. coli* JM83 was recognizing an endogenous promoter.

DNA and enzyme analyses. Results of restriction digests and subcloning indicated that the amylase gene resided within the 2.3-kb *Pst*I-*Sst*I fragment (pWC30 in Fig. 4) of pWC2. This 2.3-kb fragment could also encode amylase activity when subcloned into the plasmid pBR322 (pBWC1). Interestingly, amylase activity in crude extracts from JM83/pWC30 was much greater than that observed for JM83/pWC2 (Table 2). Although the amylase is secreted by *S. bovis*, the activity in the *E. coli* clones remains primarily cell associated (Table 2). Osmotic shock experiments indicated that the amylase remained with the cell and was not present in the periplasm (data not shown). When the crude extract from pWC30 was subjected to SDS-PAGE and then renaturation, the enzyme had an apparent molecular weight of about 50,000 (Fig. 5, lane 4), which is well below the 70,000-molecular-weight enzyme produced by *S. bovis* grown on maltose (Fig. 5, lane 2). This suggests either that *E. coli* is processing the enzyme or that some misreading of the gene or message is occurring.

When the amylase gene was subcloned into the *E. coli-B. subtilis* shuttle vector pBS42 (pBSA5) and transformed into an amylase-negative strain of *B. subtilis* BR151, amylase activity was readily detected in both crude extracts and the culture supernatants (Table 2). These results indicated that *B. subtilis* could express the *S. bovis* amylase gene and secrete the amylase product. However, the insert was found to be unstable in *B. subtilis*, and further work with this construct was not attempted.

Southern hybridizations. Southern hybridization analysis of *Pst*I- or *Bam*HI-digested *S. bovis* JB1 genomic DNA with the cloned amylase gene revealed only one hybridizing band from each digest (data not shown), with the *Pst*I band having

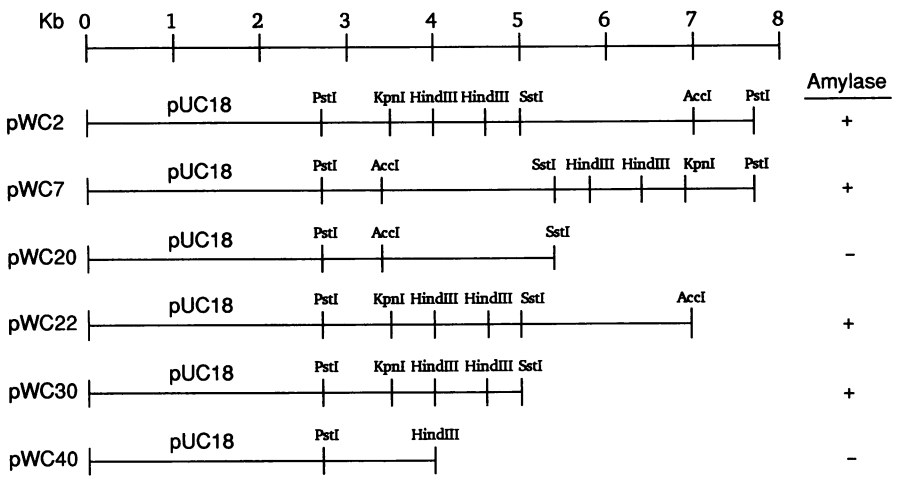

FIG. 4. Restriction endonuclease analysis of a 5.0-kb genomic insert for locating the site of the amylase gene. The presence (+) or absence (−) of amylase activity is indicated on the right.

the same size as the cloned *Pst*I fragment. These results indicate that only one gene copy is present in the chromosome and that no apparent rearrangement of the DNA occurred during cloning. When the cloned amylase gene was used as a probe against chromosomal DNA from *S. bovis* K27FF4, 21-09-6C, and SC101, a single hybridizing *Bam*HI fragment was detected under high stringency (data not shown). These results suggest that the amylase genes may be highly conserved among strains and may prove to be a potential probe for identification of *S. bovis* strains.

Comparison of amylase activities. Amylase activity from *S. bovis* JB1 was compared with activity from *E. coli* JM83/pWC22 and *S. bovis* JB1 containing pVWC1, the cloned amylase gene introduced back into JB1 on the plasmid pVA838. Culture fluid from *S. bovis* JB1 grown on maltose and crude extract from *E. coli* JM83/pWC22 were incubated with amylose and amylopectin, and digestion products were analyzed by thin-layer chromatography. The results are shown in Fig. 6. The extracellular amylase activity of *S. bovis* JB1 rapidly hydrolyzed amylopectin to a mixture of maltooligosaccharides (Fig. 6A, lanes 2 to 6). The major products of amylolytic digestion were maltose, maltotriose, and maltotetraose. Small amounts of maltopentaose and larger oligosaccharides were detected early during these incubations, but they had largely disappeared by 2 h. The amylase activity of *E. coli* JM83/pWC22 extracts also rapidly degraded amylopectin to oligosaccharides (Fig. 6A, lanes 8 to 12), but the pattern of products generated differed from those of *S. bovis* incubations. Maltohexaose and maltoheptaose were produced in addition to those products previously noted for *S. bovis* JB1. On the basis of the intensity of spots, the major products of amylopectin hydrolysis were maltotriose, maltopentaose, and maltohexaose. As with *S. bovis* incubations, there were disappearance of larger oligosaccharides over time and an increase in the intensity of spots corresponding to the major products. Similar incubations with unbranched amylose yielded slightly less-complex mixtures of products, but the same major products of

TABLE 2. Amylase activity in various *S. bovis* JB1, *E. coli* JM83, and *B. subtilis* BR151 *amyE* clones

Organism, strain, and plasmid (growth medium)	Amylase activity in:			
	Crude extract (U/mg of protein)	Culture (U/ml)		
		Supernatant	Cells	Supernatant (%)
S. bovis JB1				
No plasmid (maltose)	—[a]	5.0	0.4	93
pVA838 (glucose)	—	0.56	—	—
pVWC1 (glucose)	—	1.09	—	—
pVA838 (maltose)	—	5.11	—	—
pVWC1 (maltose)	—	5.55	—	—
B. subtilis BR151 *amyE*				
pBS42	<0.01	0	0	0
pBSA5	12.6	1.9	2.2	46
E. coli JM83				
pUC18	<0.01	0	0	0
pWC2	2.0	—	—	—
pWC30	73	3.2	18.0	15
pBWC1	160	0.2	55.9	1

[a] —, not determined.

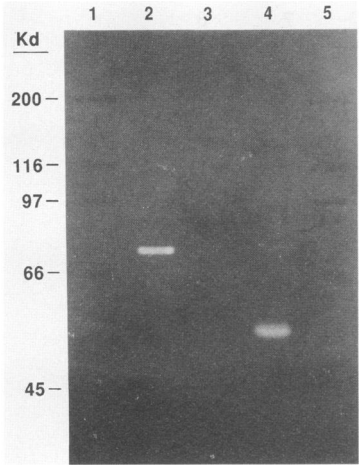

FIG. 5. Renaturation of amylase activity from *S. bovis* JB1 culture supernatant and *E. coli* JM83/pWC22 crude extract following SDS-PAGE. The gel was incubated in the presence of starch and stained with iodine as described in Materials and Methods. The light bands indicate the presence of amylase activity. Molecular size markers (in kilodaltons) are indicated on the left. Lanes: 1, molecular size markers; 2, JB1 grown on maltose; 3, JB1 grown on glucose; 4, JM83/pWC22 crude extract.

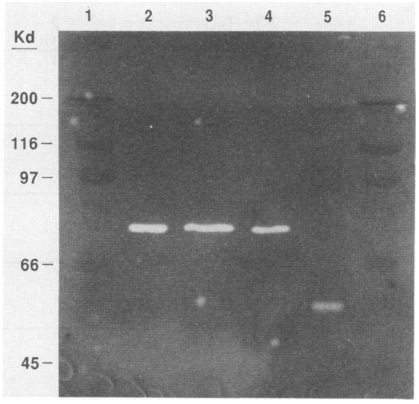

FIG. 7. Renaturation of amylase activity in culture supernatants from *S. bovis* JB1/pVA838, JB1/pVWC1 containing the cloned amylase gene, and *E. coli* JM83/pWC22 crude extract. Culture supernatants from glucose-grown cultures were concentrated as described in Materials and Methods. Lanes: 1 and 6, molecular size markers; 2, JB1/pVA838 grown on maltose; 3, JB1/pVA838 grown on glucose; 4, JB1/pVWC1 grown on glucose; 5, JM83/pWC22 crude extract. Molecular size markers (in kilodaltons) are indicated on the left.

digestion were detected (Fig. 6B). Differences in the patterns of products upon prolonged incubation (i.e., 24 h) were not as pronounced with the final products of maltose and maltotriose for *S. bovis* JB1 and maltose, maltotriose, and maltotetraose for *E. coli* JM83/pWC22. Experiments combining *S. bovis* JB1 amylase with *E. coli* JM83 extracts (no amylase) generated product patterns identical to those of *S. bovis* JB1 alone.

S. bovis JB1 containing pVA838 or pVWC1 was grown on glucose and maltose, and amylase activities in the culture fluids were compared. When grown on glucose, there was a reproducible twofold-higher increase in amylase activity in the pVWC1 culture than in the pVA838 culture (Table 2). Interestingly, following growth on maltose, the pVWC1 culture had only slightly higher amylase activity. When the amylase activity from the glucose-grown cultures was concentrated and subjected to SDS-PAGE and renaturation analysis, only one band of activity (Fig. 7, lane 4), which comigrated with the amylase from JB1/pVA838 (Fig. 7, lanes 2 and 3), was observed. This indicates that the entire amylase structural gene resides on the insert from pWC22.

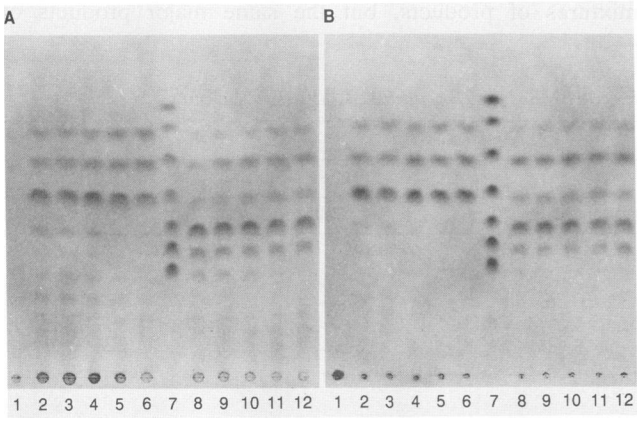

FIG. 6. Products of amylopectin and amylose hydrolysis by the amylase activities of *S. bovis* JB1 and *E. coli* JM83/pWC22. Reaction mixtures contained either 1% amylopectin (A) or 1% amylose (B) and 4.6 U of *S. bovis* amylase activity (lanes 2 to 6) or 4.8 U of *E. coli* JM83/pWC22 amylase activity (lanes 8 to 12). Mixtures were incubated and sampled at 0.25 h (lanes 2 and 8), 0.50 h (lanes 3 and 9), 1.0 h (lanes 4 and 10), 2.0 h (lanes 5 and 11), and 3.0 h (lanes 6 and 12). Amylopectin and amylose blanks (no enzyme added) are shown in lanes 1. Maltooligosaccharide standards (glucose-maltoheptaose) are shown in lane 7.

DISCUSSION

The current study represents a closer examination of the production and molecular characteristics of the extracellular amylase of *S. bovis* JB1 than has been previously conducted (6, 7). Amylolytic activity was produced under all growth conditions and, on the basis of renaturation of activities in polyacrylamide gels, was due to the action of a single 70,000-molecular-weight enzyme. This activity was located in the extracellular fluid, except when starch was the growth substrate and amylase sedimented with cells upon centrifugation. Differences in the sedimentation of amylase in starch-grown cultures raised the possibility that an additional, cell-associated amylolytic enzyme may be produced by *S. bovis* when grown on starch. Alternatively, the apparent association of amylase with cells may be due to binding of the enzyme to undegradated starch, which sediments upon centrifugation as previously suggested (6) and is known to occur with other amylases (13, 33). This appears to be correct since the distribution of activity between extracellular fluid and cell pellet fractions could be altered by the physical state of the starch substrate. Furthermore, renaturation gels of the extracellular fluid from maltose-grown cultures showing starch activity and protein extracted (SDS-PAGE sample buffer, 100°C, 5 min) from cell pellets of starch-grown cultures yielded bands with identical molecular weights. Therefore, the activities of these two cultures are likely the same and not representative of an additional cell-bound amylase. Previously, Walker had isolated a cell-

bound α-amylase from another strain of *S. bovis* (36). This activity was indistinguishable from the extracellular amylase produced by the same strain on the basis of the biochemical characteristics described. Furthermore, release of activity from cells required cell disruption, and the relative contribution of this activity to the total activity of the culture could not be estimated. This cell-bound activity might actually represent an intracellular precursor form of the extracellular amylase. Other investigations have noted that amylase is primarily extracellular even under conditions of growth on starch (9).

Although amylase was always produced by *S. bovis* cells, the level of activity varied considerably with the different growth substrates, indicating that production was regulated. Experiments examining the effects of different growth substrates provided singly and in various combinations, demonstrated that growth on maltose or starch was required for maximum production. The effect of the antibiotics rifampin and tetracycline on the maltose stimulation of amylase production indicated that the effect of maltose on synthesis was likely to be at the level of transcription of the gene. Amylase accumulation was inhibited by a number of simple carbohydrates. *S. bovis* JB1 has previously been shown to exhibit catabolic repression-like response to growth substrate (27). In those experiments, glucose repressed the utilization of maltose in a marked fashion. We found that glucose affected the production of amylase in an almost identical fashion. Because of the similarity to catabolic repression-like phenomena, the possible involvement of cAMP in the regulation of amylase was explored. *S. bovis* cells contained similar levels of cAMP regardless of the substrate provided for growth. These levels were much lower than those observed for *E. coli* in the classic, *lac* operon, negative-control response and were similar in concentration to those of *B. thetaiotaomicron*, an organism previously shown not to exhibit a cAMP response to the growth substrate (12). These results suggest that cAMP is not involved in the regulation of amylase production, and some other mechanism may be involved in the ability of *S. bovis* to respond rapidly to changes in the availability of carbohydrate sources.

Martin and Russell (22) showed that strain JB1 possessed phosphoenolpyruvate:sugar phosphotransferase system (PTS)-mediated transport mechanisms for the uptake of maltose, glucose, sucrose, and cellobiose. Furthermore, they were able to demonstrate that the glucose PTS function inhibited maltose PTS, but they did not note whether cellobiose exerted a similar inhibitory effect. Perhaps the repression of maltose-stimulated amylase production by glucose is mediated by its effect on maltose transport. Additionally, glucose may result in inducer expulsion, a phenomenon known to occur in other gram-positive bacteria (24). A combination of these mechanisms might be responsible for the very rapid inhibition of amylase synthesis upon the addition of glucose to maltose-growing cultures. The mechanism by which the nonpreferred substrate cellobiose exerts its inhibitory effect is unclear. The utilization of maltose by strain JB1 is not repressed by cellobiose (27); however, production of amylase was inhibited when cellobiose was added to maltose-growing cultures. It is interesting that the addition of cellobiose to maltose-grown cultures did not cause an immediate cessation of amylase synthesis that characterized the effect of added glucose. The inhibition of amylase by cellobiose is likely to be mediated through mechanisms other than that for glucose.

The amylase gene from JB1 was cloned and expressed in *E. coli* on a 5.0-kb *Pst*I genomic DNA fragment. The gene could be localized to a 2.3-kb *Pst*I-*Sst*I fragment. Since the gene was expressed in opposite orientations in pUC18, *E. coli* was apparently using an endogenous promoter for expression of the gene. However, when the gene was reintroduced into JB1 on pVA838, only a slight increase in activity over that typically noted following growth on maltose occurred. This would suggest that the region of DNA upstream of the amylase gene involved in maltose induction of expression is not present on the cloned DNA.

The amylase produced in *E. coli* was found to be of smaller molecular weight than that synthesized by JB1, suggesting that the gene is not expressed in its entirety or that the protein is processed by *E. coli*. This amylase also had catalytic characteristics different from those of JB1 amylase. The products of amylose and amylopectin digestion are consistent with the activity of an α-amylase, but the major products (G2 to G6) were different from those of the JB1 amylase (G2 to G4) in the culture fluid. These differences in products could be due to effects of the altered protein on the binding site and the catalytic activity of the enzyme produced by *E. coli*. However, when the gene was reintroduced into *S. bovis* JB1, only one form of amylase activity was detected, suggesting that the entire gene is present on this insert.

Recently, Clark et al. (5) reported the cloning and expression of an amylase gene from an *S. bovis* strain. However, the expressed activity in *E. coli* was drastically lower than that observed by us with the JB1 amylase gene (0.23 U/mg versus 73 U/mg), and no data on the products produced by the amylase were reported. We found that the JB1 amylase gene hybridized strongly to three other strains of *S. bovis* from the United States (strain SC101) and South Africa (strains K27FF4 and 21-09-6c). It would be interesting to determine whether the JB1 amylase gene hybridizes with the cloned amylase gene or genomic DNA from the strain used by Clark et al. (5), as well as other strains of *S. bovis*. If the gene is conserved among strains, then the JB1 amylase gene may serve as a genetic probe for detection and identification of *S. bovis* strains.

When the amylase gene was used as a probe to determine relative levels of mRNA for expression of the gene following growth on glucose and maltose, no detectable difference in mRNA levels was observed (data not shown). Such a small difference in mRNA levels may be involved in the almost immediate cessation of amylase synthesis following introduction of glucose to maltose-growing cells (Fig. 2). If the mRNA is turned over rapidly and gene expression is halted, then this could help to explain such a rapid event. The results of the addition of antibiotics to maltose-growing cultures (Fig. 3) also suggest the necessity of constant gene expression for an increase of amylase activity. Further analysis of the amylase expression system will be required to determine the regulatory elements involved. We are attempting to isolate amylase-negative mutants of *S. bovis* using Tn*916* mutagenesis as well as chemical agents. In addition, PTS mutants of *S. bovis* JB1 have been reported previously (26) and may prove useful for delineating the role of the PTS in induction and repression of the amylase gene in *S. bovis* JB1.

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