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Development of a DNA Probe for *Streptococcus bovis* by Using a Cloned Amylase Gene

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***Streptococcus bovis* is a normal inhabitant of the rumen but has been implicated as a causative agent for ruminal lactic acidosis and related problems. While rarely isolated from humans, *S. bovis* has been identified as a causative agent for endocarditis, meningitis, and septicemia. Recent reports have also suggested a correlation between human colonic carcinoma and increased levels of *S. bovis*. Identification of *S. bovis* strains of human origin has been problematic because of variations in results of biochemical tests compared with results for ruminal strains. We have tested a cloned amylase gene from the ruminal strain *S. bovis* JB1 as a potential DNA probe for rapid and accurate identification of *S. bovis* strains from all sources. DNAs from strains identified as *S. bovis*, of both human and ruminal origin, were found to hybridize with the probe under stringent conditions. The probe also hybridized with variants of *S. bovis* that did not grow on starch. The probe did not hybridize with DNA isolated from other bacteria of human colonic and ruminal origin, including *Bacteroides thetaiotaomicron*, *Bacteroides ruminicola*, *Butyrivibrio fibrisolvens*, and *Enterococcus faecalis* but did demonstrate hybridization with *Streptococcus salivarius*.**

Streptococcus bovis is a normal inhabitant of the rumen of ruminant animals such as cattle and sheep, existing at a level of less than 0.1% of the total ruminal microflora population (10). However, *S. bovis* has been identified as a probable causative agent for ruminal lactic acidosis and related conditions (11). These conditions arise as a result of alterations in feeding regimens which allow for rapid proliferation of *S. bovis* and increased production of lactic acid in the rumen.

While *S. bovis* is rarely isolated from humans, there are reports of human disease caused by strains of *S. bovis*. These disease states include meningitis, endocarditis, and septicemia (1, 8, 12, 20–23, 27, 30). More importantly, recent reports have suggested a potential relationship between increased fecal carrier levels of *S. bovis* and human gastrointestinal disease, primarily colonic cancer (1, 5, 8, 13, 14, 20, 22, 25, 30). However, problems in identification of *S. bovis* strains from human sources have been reported, compared with identification of strains of rumen origin (7, 15). Several biochemical characteristics, such as the ability to ferment mannitol and mellobiose, appear to vary among rumen and human *S. bovis* isolates (15). In addition, DNA-DNA hybridization studies indicate that human and ruminal isolates may be of different homology groups (6, 15).

While all strains of *S. bovis* exhibit the Lancefield group D surface antigen (as do enterococcal species), *S. bovis* strains are primarily identified by their ability to grow on starch and produce amylase activity (3, 28). However, several variants of *S. bovis* that do not hydrolyze starch have been isolated (15). In order to identify bacterial strains associated with disease, as well as potentially screen for colon cancer by monitoring levels of *S. bovis*, a more accurate diagnostic tool, such as a DNA probe, would be useful. We therefore tested a cloned amylase gene from the ruminal strain *S. bovis* JB1 (4) as a probe against DNA isolated from strains of *S. bovis* of human and ruminal origins. All strains identified as *S. bovis* were found to hybridize with the probe under stringent conditions, indicating that this gene or oligonucleotide probes derived from the DNA sequence may be used as a rapid and accurate means of identifying bacterial isolates as *S. bovis*.

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MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. Strains were from our culture collection (NCAUR) or obtained from the American Type Culture Collection (ATCC); J. R. Russell, Cornell University; Francis Macrina, Medical College of Virginia, Virginia Commonwealth University; N. O. van Gylswyk, Uppsala, Sweden; D. Odelson, Central Michigan University; and K. Ruoff, Massachusetts General Hospital. *S. bovis* V1477, V1387, and V1388 were isolated from endocarditis patients. All strains were grown anaerobically in routine growth medium (9), a complex Trypticase-yeast extract medium, with 0.2% carbon source. For studies of amylase production, strains were grown on routine growth medium containing glucose, maltose, or starch and growth was monitored with a Spectronic 21 spectrophotometer (Milton Roy Co., Rochester, N.Y.) at 660 nm. *S. bovis* biotypes were determined by using the RapidSTREP system (Vitek Systems, Hazelwood, Mo.).

Isolation of DNA and hybridization analyses. Chromosomal DNA from bacterial strains was isolated as described by Saito and Miura (29), except mutanolysin (Sigma Chemical Co., St. Louis, Mo.) was substituted for lysozyme for *Streptococcus* and *Enterococcus* species. The amylase gene from *S. bovis* JB1 was previously cloned into pUC18 on a 5.0-kb *Pst*I fragment and subsequently localized to a 2.3-kb *Pst*I-*Sst*I fragment (4). This 2.3-kb fragment was labeled by using the Genius Nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) and digoxigen-11-dUTP according to the manufacturer's instructions. Genomic DNA samples were digested with *Pst*I and electrophoresed through agarose gels. Gels were subjected to 0.25 HCl acid

TABLE 1. Sources and characteristics of bacterial strains

Species and strain	Source	Biotype (RapidSTREP)	Origin	Hybridization	Growth on starch[a]	Amylase activity[b]
S. bovis						
26	Russell	II	Rumen	+	+	40.8
21-09-6c	Russell	I	Rumen	+	+	29.1
JB1	Russell	I or II/1	Rumen	+	+	25.5
45s1	Russell	I or II/1	Rumen	+	+	22.7
K27FF4	van Gylswyk	II/1	Rumen	+	+	17.4
ATCC 15351	ATCC	II/1	Rumen	+	+	16.6
58laxy2	Russell	I or II/1	Rumen	+	+	11.1
SC101	Odelson	I	Rumen	+	+	1.6
7H4**	Russell	*S. milleri*	Rumen	−	−	ND
ATCC 33317	ATCC	II/1	Cow feces	+	+	9.4
ATCC 27960	ATCC		Cow udder	+	+	21.8
ATCC 43143	ATCC		Human blood	+	+	17.8
ATCC 43144	ATCC		Human blood	+	−	ND
ATCC 49147	ATCC		Clinical	+	+	19.8
1314	Ruoff	I	Clinical	+	+	7.8
2703	Ruoff	I	Clinical	+	+	9.8
9410	Ruoff	I	Clinical	+	+	17.1
6448	Ruoff	II/1	Clinical	+	−	ND
6	Ruoff	II/2	Clinical	+	−	ND
1499	Ruoff	II/2	Clinical	+	−	ND
V1477	Macrina	I	Clinical	+	+	10.5
V1388	Macrina		Clinical	+	+	2.9
V1387**	Macrina	*S. salivarius*	Clinical	+	−	ND
ATCC 49133	ATCC		Clinical	+	−	ND
ATCC 9809	ATCC		Unknown	+	+	23.9
S. salivarius						
ATCC 7073	ATCC		Human blood	+	−	ND
568	Ruoff		Clinical	+	−	ND
S. equinus ATCC 9812	ATCC			+	−	ND
Streptococcus sanguis V288	Macrina			−	−	ND
S. suis ATCC 43765	ATCC			−	+	<0.5
S. uberis ATCC 19436	ATCC			−	−	ND
Streptococcus agalactiae V372	Macrina			−	−	ND
E. faecalis JH2-2	NCAUR			−	−	ND
B. fibrisolvens H17c	NCAUR		Rumen	−	+	ND
B. thetaiotaomicron 5482	NCAUR		Human colon	−	+	ND

[a] +, growth on 0.2% soluble starch; −, less than 0.100 optical density unit.
[b] Units per milligram of cell protein. ND, not determined.

depurination for 10 min, then base denatured, and neutralized as described previously (19). The DNA was then transferred to a nylon membrane (Boehringer Mannheim) with a Bio-Rad (Richmond, Calif.) vacuum apparatus and 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M Na citrate, pH 7.0) as the buffer. The DNA was then cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene, La Jolla, Calif.), and hybridizations were carried out with standard high-stringency protocols (67°C, 16 h [19]) in hybridization buffer suggested by Boehringer Mannheim (5× SSC, 10% Boehringer Mannheim blocking reagent, 0.1% sarkosine, 0.05% sodium dodecyl sulfate [SDS]). Chemiluminescence detection of hybridizing DNA was carried out by using the Genius 1 kit (Boehringer Mannheim) according to the manufacturer's instructions.

Amylase and protein analyses. The amylase activity in starch-grown cultures was determined by monitoring the increase in reducing sugar formation from starch by using the dinitrosalicylic acid reagent as previously described (3, 24). Cultures were harvested by centrifugation (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. Amylase activities reported are the sum of cell-associated and extracellular activities. One enzyme unit equaled 1 μmol of glucose equivalent formed per min. Cell protein concentrations were estimated by the method of Lowry et al. (18) following hydrolysis of cells in base (0.1 N NaOH at 70°C for 30 min). Proteins in culture supernatants were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (17). Renaturation and detection of amylase activity following SDS-PAGE were carried out as previously described (4, 16). Briefly, SDS-PAGE gels were soaked in sodium phosphate buffer, followed by a starch solution. The gel was then stained with an iodine solution, and clear zones in the gel indicated locations of renatured amylase activity.

Materials. Soluble potato starch (catalog no. S-2630; Sigma Chemical Co.) containing no detectable glucose, maltose, or oligosaccharides was used for both growth and amylase assays. Digoxigenin-labeled lambda-*Hin*dIII DNA molecular size standards were purchased from Boehringer Mannheim. All other reagents were of the highest grade available.

RESULTS AND DISCUSSION

Chromosomal DNAs from 19 ruminal and human clinical isolates of *S. bovis*, as well as several known ruminal and human colonic bacteria, were probed with the amylase gene cloned from *S. bovis* JB1. The results are shown in Table 1.

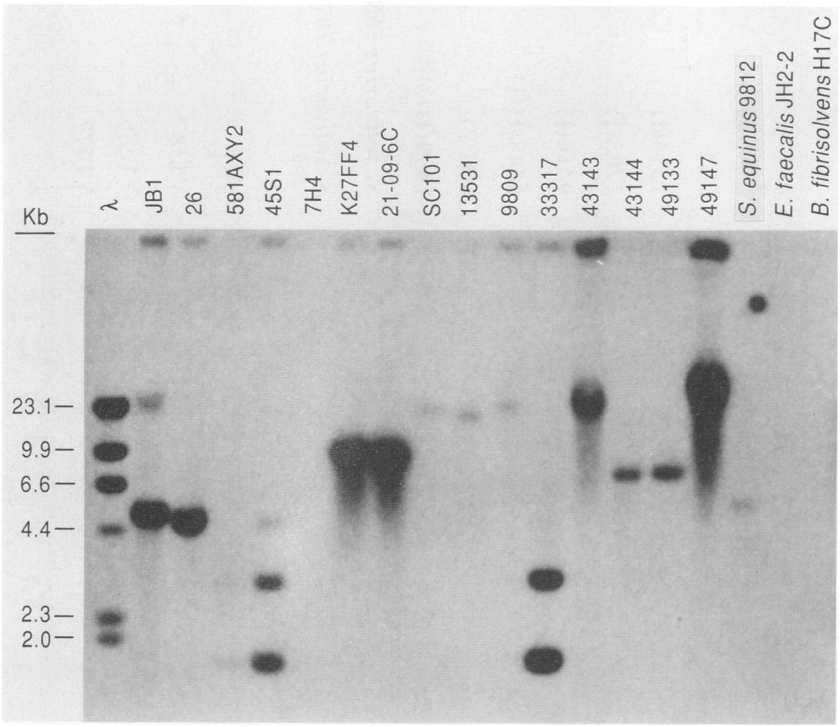

FIG. 1. Chemiluminescence detection of hybridization between *Pst*I digests of chromosomal DNA and the amylase gene from *S. bovis* JB1. *S. bovis* strain numbers and additional bacterial species are shown above the lanes. Molecular size markers (in kilobase pairs) are indicated on the left.

At least one hybridizing band was observed in all strains identified as *S. bovis*, and a representative Southern hybridization is shown in Fig. 1. The only bacteria other than *S. bovis* that demonstrated hybridization with the amylase gene were *Streptococcus equinus* 9812, the type strain of the species (Fig. 1), and *Streptococcus salivarius* (Fig. 2). Farrow et al. (6) previously reported that *S. equinus* ATCC 9812 and *S. bovis* ATCC 33317, the type strain, had greater than 70% DNA-DNA homology and therefore represented a single species. rRNA sequence comparison also indicates genetic relatedness (2). The DNA hybridization in Fig. 1 appears to support the view that these are the same species. The only other strain initially believed to be *S. bovis* is strain 7H4, which did not hybridize with the amylase probe (Fig. 1). However, Russell and Robinson (28) found that strain 7H4 was atypical in many biochemical and physiological characteristics compared with other *S. bovis* strains. They concluded that the classification of 7H4 as *S. bovis* was doubtful. The RapidSTREP system identified 7H4 as a strain of *Streptococcus milleri*. Again, our results appear to confirm this hypothesis that strain 7H4 is not an *S. bovis* strain. While the degree of hybridization varies between strains, it is evident that the amylase gene probe recognizes DNA from *S. bovis* of both ruminal and human clinical sources. An important note to make is that the probe was able to identify *S. bovis* strains from homology group I (ATCC 33317), group II (ATCC 43143 = F-1867), and group II-variant (ATCC 43144 = CDC 1723-81) as proposed by Knight and Shlaes (15). In addition, the probe did not hybridize with DNA from other common ruminal and human colonic bacterial species, such as *Streptococcus suis*, *Streptococcus uberis*, *Bacteroides thetaiotaomicron*, *Bacteroides ruminicola*, *Butyrivibrio fibrisolvens*, and *Enterococcus faecalis* (Table 1 and Fig. 1).

Unexpectedly, the amylase gene probe did hybridize with DNA from the two strains of *S. salivarius* tested (Fig. 2). We find this result very interesting in that the strains did not grow on starch and did not produce amylase activity, even though *S. salivarius* has been reported to ferment starch. An internal 0.6-kb fragment of the amylase gene containing only coding DNA also hybridized with the *S. salivarius* DNA, as well as the DNA of the *S. bovis* strains (data not shown). We are now testing specific oligonucleotides derived from the DNA sequenced thus far to determine whether these will hybridize only with *S. bovis*, *S. salivarius*, or both. Sequence comparisons of the rRNA sequences of *S. bovis* and *S. salivarius* suggest an ancestral association between these organisms (2), and it is possible that *S. salivarius* strains contain an unexpressed amylase gene similar to that found in *S. bovis*. An amylase gene that can hybridize with both *S. bovis* and *S. salivarius* may prove useful as a screen for colon cancer, as at least one report has demonstrated an association between colorectal cancer and endocarditis caused by *S. salivarius* (26).

A second aspect of the hybridization data in Fig. 1 and 2 is the potential use of restriction fragment length polymorphisms to categorize similar strains of *S. bovis*. Although the results are from single restriction digests, certain strains appear to have similar restriction patterns. For example, strains 581AXY2, 45S1, and ATCC 33317 have similar patterns, as do K27FF4 and 21-09-6C. Such restriction fragment length polymorphism patterns may be useful for identifying similar strains of *S. bovis* from clinical sources.

S. bovis is generally characterized by the ability to grow

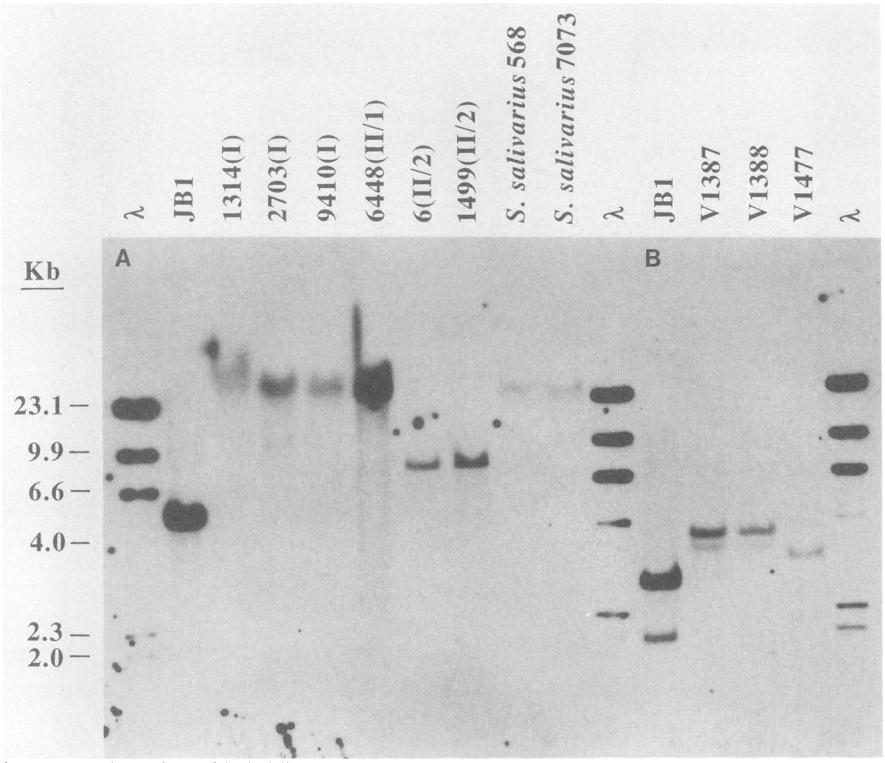

FIG. 2. Chemiluminescence detection of hybridization between restriction digests of chromosomal DNA and the amylase gene from *S. bovis* JB1. Strain numbers are shown above the lanes. (A) *Pst*I-digested DNA; (B) *Hin*dIII-digested DNA.

on starch and produce amylolytic activity (3, 28). However, variants of *S. bovis* that do not hydrolyze starch have been reported. We have previously characterized the amylase production in strain JB1 (4) and were therefore interested in comparing the abilities of the various *S. bovis* strains to grow on starch and produce amylase. The results are shown in Table 1. Of the strains tested, only V1387, ATCC 43144, ATCC 49133, and 7H4 were unable to grow on starch and produce amylase. Knight and Schlaes (15) also previously reported that strain ATCC 43144 did not hydrolyze starch when grown on starch-containing agar medium. *S. equinus* ATCC 9812 also did not grow on starch, although this strain and strains V1387, ATCC 43144, and ATCC 49133 did hybridize with the amylase gene probe (Fig. 1). The data suggest that these strains may be amylase-negative mutants of *S. bovis* which still retain the amylase gene sequence in the chromosome. The data also indicate that the DNA probe can identify amylase-negative variants of *S. bovis*.

The amylase activities of the *S. bovis* strains were further characterized by SDS-PAGE and renaturation of amylase activity as described in Materials and Methods. The results (data not shown) demonstrate the presence of at least one band of amylase activity from each strain, except for strains V1387, ATCC 43144, and ATCC 49133, which were previously demonstrated to lack amylase activity (Table 1). A major band of activity was noted at approximately 70,000 molecular weight for all the amylolytic strains, similar to that observed with *S. bovis* JB1 (4), which is the product of the cloned amylase gene.

The data presented in this report demonstrate that the cloned amylase gene from *S. bovis* JB1 will hybridize with analogous regions of DNA from *S. bovis* strains isolated from ruminal and human clinical sources. The gene did hybridize with *S. salivarius* but not with DNA from other common bacterial isolates from ruminal and colonic sources. The use of such a probe should allow investigators to more accurately identify bacterial strains isolated from both clinical and ruminal sources as *S. bovis*. We are currently determining the DNA sequence of the amylase gene, which should allow for production of specific oligonucleotide probes which may be used in place of the entire amylase gene for diagnostic identification of *S. bovis*. Such a diagnostic tool may also prove useful as a screen for colon cancer, considering the plethora of reports on the correlation of *S. bovis* fecal carrier levels and colon cancer (1, 5, 8, 13, 14, 20, 22, 25, 30).

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