JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2002, p. 2913–2918 Vol. 40, No. 8
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.8.2913–2918.2002

Identification of *Streptococcus bovis* Biotype I Strains among *S. bovis* Clinical Isolates by PCR

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Received 31 January 2002/Returned for modification 10 April 2002/Accepted 24 April 2002

Streptococcus bovis **causes 24% of all streptococcal infective endocarditis cases. There are many reports linking both *S. bovis* bacteremia and endocarditis with various forms of gastrointestinal disease (primarily colonic cancers). *S. bovis* is divided into two biotypes: I and II. The biotype I strain is much more frequently isolated from patients with endocarditis, gastrointestinal disease, or both. We describe here the isolation of biotype I-specific DNA sequences and the development of a PCR test which can identify *S. bovis* biotype I strains among *S. bovis* clinical isolates.**

Streptococcus bovis causes 11 to 14% of infective endocarditis cases and accounts for 24% of endocarditis episodes due to streptococci (3, 11, 26). *S. bovis* endocarditis is more common in people over 60 years old (41), causing a high mortality rate (45%), compared to that for non-*S. bovis* endocarditis (25%) (26). There is a well-documented high incidence of gastrointestinal (GI) cancers in patients with *S. bovis* bacteremia and/or endocarditis (3, 19, 24, 26, 29, 45). In some cases, the presence of bacteremia is the only indication of GI lesions (29). In fact, colonic tumors have been found up to 2 years after the initial infection. This possible connection between *S. bovis* and colonic tumors warrants closer evaluation (3). The high incidence of *S. bovis* endocarditis in people older than 60 years and the increase in the aging population itself (11, 41) means that *S. bovis* has become an increasingly important pathogen.

S. bovis carries the Lancefield group D antigen (38), shared by members of the enterococci. However, in terms of its physiological characteristics, it more closely resembles the viridans streptococci (15). There are two biotypes of *S. bovis*. The majority of *S. bovis* biotype I strains (the classical *S. bovis* strains) produce extracellular glucan from sucrose, ferment mannitol, and hydrolyze starch, whereas *S. bovis* biotype II strains (the variant *S. bovis* strains) are generally negative for these traits (15, 33).

Although *S. bovis* can be divided into two biotypes, there have been few studies correlating disease with biotype (15, 33). A study looking at the association between underlying gastrointestinal cancer and bacteremia caused by *S. bovis* biotypes I and II showed that 71% of patients with *S. bovis* biotype I bacteremia also had malignant or premalignant colonic lesions and, of those cases undergoing thorough colonic examination, this figure rose to 100% (37). In comparison, *S. bovis* biotype II was linked to colonic lesions in only 17% of cases, with this figure rising to 25% after thorough colonic examination. Similarly, the linkage between bacteremia and the presence of endocarditis was 94% with *S. bovis* biotype I and only 18% with *S. bovis* biotype II (37). The association between biotype I and both GI neoplasia and endocarditis suggests a possible biotype-specific adherence mechanism.

Methods identifying *S. bovis* infections have included two-dimensional crossed immunoelectrophoresis against a common antigen, designated c (22); immunoblotting against *S. bovis* protein bands of 66, 58, 52, and 4 kDa (6); and Southern blotting with a cloned amylase gene from the ruminal *S. bovis* JB1 (43). None have distinguished the more clinically significant *S. bovis* biotype I from biotype II. Previously, *S. bovis* biotypes were differentiated by commercially available rapid identification systems (16, 36). This report describes a PCR-based assay that can quickly and accurately identify *S. bovis* biotype I.

MATERIALS AND METHODS

Bacterial strains. *S. bovis* strains were grown overnight at 37°C in 5% CO_2, either standing in Todd-Hewitt broth (Oxoid, Inc.) or plated onto Columbia blood agar plates containing 5% sheep blood (Hemostat Labs). *Escherichia coli* strains were grown at 37°C either shaking in Luria-Bertani (LB) broth containing 100 μg of ampicillin/ml or plated onto LB broth-agar-ampicillin plates.

S. bovis genomic subtraction strains SS1189 (a biotype II strain) and DS1909-79 (a biotype I strain) were part of a collection kept at the Centers for Disease Control (CDC). *S. bovis* F1867 (a biotype I strain) is from the American Type Culture Collection. *S. bovis* strains of biotypes unknown to the authors were received from the CDC (19 strains) and E. J. Baron at Stanford Hospital (7 strains). K. L. Ruoff provided the 20 *S. bovis* strains tested in the clinical PCR (37).

Genomic DNA isolation. Overnight *S. bovis* cultures were spun at 5,000 rpm for 20 min, resuspended in 10 ml of 20 mM Tris (pH 8.0) containing 1 mg of lysozyme (Sigma)/ml, and incubated at 37°C for 5 h. Next, 48 μg of proteinase K (Sigma)/ml was added, and the mixture incubated at 37°C for 2 h. Full cell lysis was achieved by the addition of 50 mM EDTA (pH 8.0) and five to six repeated freeze-thaw cycles with dry ice-ethanol and 60°C baths, respectively. Unlysed cells and debris were removed by centrifugation at 5,000 rpm for 20 min. Streptococcal DNA in the supernatant was purified as recommended for the genomic DNA columns (100/G; Qiagen). DNA purity and concentration was monitored

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TABLE 1. Primer sequences for both the biotyping and clinical PCRs

PCR type	Sequence	
	Forward primer	Reverse primer
23S RNA	CTACCTTAGGACCGTTATAGTTAC	GAAGGAACTAGGCAAAATGGTGCC
Clone 40	CGTCTATTATCCCAAGCGCG	AGGCAGCATAATTTTCGAGG
Clone 51	GTCCTGCATTTGCTGAGCG	GCCTATTAAATAGGGCTTGGT
Clone DS14	GTTTATGGAACAAGTGCGG	GTTCCTTCTGTGCCTCGG

by measuring the optical density at 260 and 280 nm and by running 10-μl samples on 0.8% TAE agarose gels.

Genomic subtraction. PCR-Select Bacterial Genome Subtraction analysis was performed twice according to the manufacturer's instructions (Clontech). In the first experiment, *S. bovis* biotype I F1867 provided the tester DNA and biotype II SS1189 provided the driver DNA (14, 20). In the second experiment, biotype I DS1909-79 supplied the tester DNA, and SS1189 supplied the driver DNA. Briefly, tester and driver DNA were digested, the tester fragments were labeled with PCR primers, and tester and driver fragments were hybridized in the presence of excess driver DNA. Tester-specific fragments were then amplified from the mixture by PCR.

Tester-specific PCR products were cloned into pTAdv vectors as described in the AdvanTAge PCR Cloning method (Clontech). Clones were tested for insert DNA by PCR as follows: half-colonies were lysed in 5 μl of sterile distilled water, heated at 98°C for 10 min, and immediately placed on ice for 5 min. Master mix (20 μl), containing 2.5 mM of magnesium chloride ($MgCl_2$), 1× PCR buffer, 200 μM concentrations of each of the deoxynucleoside triphosphates, 0.25 μM concentrations of each of M13 reverse and M13 sequencing primers, and 1 U of *Taq* DNA polymerase (Gibco-BRL) was added to lysed bacteria. The following PCR conditions were used: 1 cycle of 94°C for 2 min, 63°C for 1 min, and 72°C for 1.5 min; followed by 14 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1.5 min; followed by a final cycle of 72°C for 2 min.

Individual *E. coli* colonies were subcultured and stored in 35% glycerol at −80°C.

DNA sequence analysis. Both DNA sequencing and the production of oligonucleotide primers were performed by the Protein and Nucleic Acid Facility, Beckman Center, Stanford University Medical Center, Palo Alto, Calif. Comparison of the three largest pTAdv clones with the GenBank database was performed on SeqWeb with BLASTP (2).

Southern blot analysis. Genomic DNA was digested with *Hin*dIII and separated on a 1% TAE agarose gel at 35 V overnight at 4°C. The reactions were denatured by incubating the gel in 0.5 M sodium hydroxide for 35 min and then transferred to a nylon membrane in 0.5 M sodium hydroxide for 6 h or overnight (Nytran [0.2 mm]; Schleicher & Schuell). The blot was prehybridized at 42°C for 2 h, hybridized with denatured probe overnight at 42°C, washed, and developed according to the manufacturer's directions supplied with the ECL Random Prime Labeling and Detection System (Amersham).

Probes were prepared by digesting insert DNA from the three largest pTAdv clones with *Eco*RI and labeling these inserts with fluorescein-labeled dUTP, as indicated by the ECL system manufacturer (Amersham).

Biotyping PCR. Genomic DNA from each unknown *S. bovis* strain was tested against four sets of primers in the biotyping PCR (Table 1). All PCRs were performed in a Robocycler 96 machine (Stratagene) and contained, in a 50-μl total volume, 250 ng of *S. bovis* genomic DNA, 2.5 mM $MgCl_2$, 1× PCR buffer, a 200 μM concentration of each of the deoxynucleoside triphosphates, a 0.25 μM concentration of each of the forward and reverse primers, and 1 U of *Taq* DNA polymerase (Gibco-BRL). The PCR conditions for the clone 40 and 51 biotyping PCRs were as follows: 1 cycle of 94°C for 2 min, 60°C for 1 min, and 72°C for 1 min; followed by 24 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; followed by a final cycle of 72°C for 2 min. The PCR conditions for the clone DS14 biotyping PCR were as follows: 1 cycle of 94°C for 2 min, 58°C for 1 min, and 72°C for 1 min; followed by 24 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; followed by a final extension cycle of 72°C for 2 min. The 23S rRNA PCR was included as a positive control that could be used under all PCR conditions.

PCR products (20 μl) were separated on a 2% TAE-agarose gel at 100 V for 1 h, next to either PCR markers or ϕX174/*Hae*III markers (Promega). Both DNA isolation and PCR experiments were performed twice for each strain.

Clinical PCR. For the clinical PCR, no. 3 MacFarland suspensions were prepared in 0.85% saline from *S. bovis* colonies, and 1.5-ml portions of these suspensions were then pelleted by centrifugation at 13,000 rpm for 2 min. The cell pellets were lysed by incubation at 37°C for 10 min in 180 μl of lysis buffer containing 10 mM Tris, 1 mM EDTA, 0.6% Triton, and 4 mg of lysostaphin (Sigma). Further purification involved proteinase K treatment and DNA purification on spin columns, as recommended by the Qiamp manufacturer (Qiagen). Clone 40/51 and clone DS14 PCRs were performed as described in the biotyping PCR.

RESULTS

Identification of specific biotype I gene fragments. Genomic subtraction allowed identification of specific gene fragments that were present in a biotype I strain but absent from a biotype II strain. This method produced two libraries, both from biotype I strains. One library contained DS1909-79-specific clones and the other contained F1867-specific clones. The three largest clones identified by PCR, clones DS14, 51, and 40, were selected for further study. Clone DS14 was obtained from the DS1909-79 library, and clones 40 and 51 were obtained from the F1867 library. Southern blot analysis confirmed that these clones did not contain sequences homologous to the biotype II SS1189 driver DNA. Clone inserts were digested from the pTAdv vector by using *Eco*RI and used to probe *Hin*dIII digests of either (i) SS1189 and DS1909-79 genomic DNA or (ii) SS1189 and F1867 genomic DNA. Figure 1 shows that for each of these three clones, the probe only reacted with the biotype I tester DNA used in the genomic subtraction method and not the biotype II SS1189 driver DNA. Therefore, these clones contain only sequences homologous to the parental biotype I genome.

Analysis of gene fragments. DNA sequencing of clones DS14, 51, and 40 gave 1.01-, 1.22-, and 0.41-kb sequences, respectively. Determination of the deduced amino acid sequences gave ca. 335, 407, and 135 amino acid sequences for clones DS14, 51, and 40, respectively. Only clone 51 contained an entire protein sequence with both start and stop codons.

Comparison of the deduced clone DS14 peptide sequence to the GenBank database by using BLASTP (2) gave a low level of homology with two overlapping fragments from a hypothetical protein 2 upstream of the SR protein in *S. mutans* OMZ 175 (31). The percent identity for each fragment was 36% for the 85-amino-acid fragment and 44% for the 45-amino-acid fragment, with E values of 2e-08 and 8e-04, respectively. Lack of homology over the entire region makes conclusions drawn from these results very tentative. The clone 51 deduced protein sequence carried significant homology, 49% identity, and 69% similarity to the Rgg protein from *S. gordonii* Challis (40), with an E value of 7.0e-111. The clone 40 peptide possessed significant homology to protein components from the lactose-specific family of phosphotransferase system (PTS) proteins, with E values of 4.2e-21, 1.8e-11, and 1.7e-09 to the *Bacillus subtilis* cellobiose-specific permease IIC (18), the *Staphylococcus au-*

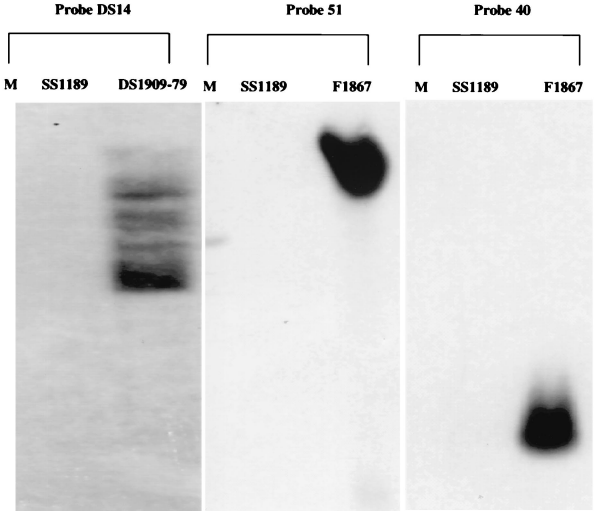

FIG. 1. Southern blot analysis of *Hin*dIII-digested SS1189 genomic DNA (lanes 2, 5, and 8), DS1909-79 genomic DNA (lane 3), and F1867 genomic DNA (lanes 6 and 9). Lanes 1, 2, and 3 were probed with fluorescein-labeled clone DS14 DNA. Lanes 4, 5, and 6 were probed with clone 51 DNA, and lanes 7, 8, and 9 were probed with clone 40 DNA. No homologous sequences were observed in the SS1189 DNA (lanes 2, 5, and 8). Lanes 1, 4, and 7 contain λ*Hin*dIII-digested DNA markers.

reus lactose-specific permease IIBC (4), and the *S. mutans* lactose-specific permease IIBC components (35).

Biotype specificity of gene fragments. A blind trial involving 26 *S. bovis* strains of unknown biotype was undertaken to test whether clones DS14, 51, and 40 were found in other *S. bovis* strains. PCR assays were designed based on the internal primers from the clone DS14, 51, and 40 DNA sequences (Table 1). Figure 2 shows a sample of the results obtained for these experiments. The DS14 PCR gave three PCR bands; the 880-bp band is the expected band, although all three bands were present in all biotype I strains tested. This is not surprising, since the Southern blot analysis results in Fig. 1 show that the DS14 probe reacts with more than one DS1909-79 genomic DNA band, suggesting that either more than one copy of this gene fragment exists or that sequences homologous to clone DS14 exist in multiple places in the genome. The clone 51 PCR gave a single 1.1-kb band with all biotype I strains, and the clone 40 PCR gave a single 240-bp band with only some biotype I strains. The 23S rRNA positive control reaction was included in each experiment.

In these blind trials, PCR-based identification was confirmed by the CDC after the experiments were completed. Table 2 gives a summary of the results obtained for all 26 *S. bovis* strains. Clone DS14 and 51 PCRs produced the clone-specific 880-bp and 1.1-kb bands, respectively, with all 10 of the *S. bovis* biotype I strains. The clone 40 PCR, on the other hand, reacted only with a subset of the biotype I strains, yielding the clone-specific 240-bp band with 7 of the 10 biotype I strains. The 16 *S. bovis* biotype II strains tested did not react with any of the clone-specific PCRs and only produced bands in the 23S rRNA positive control PCR. We conclude that clones 51 and DS14 are biotype I specific and that clone 40 is specific to some subset of the biotype I strains.

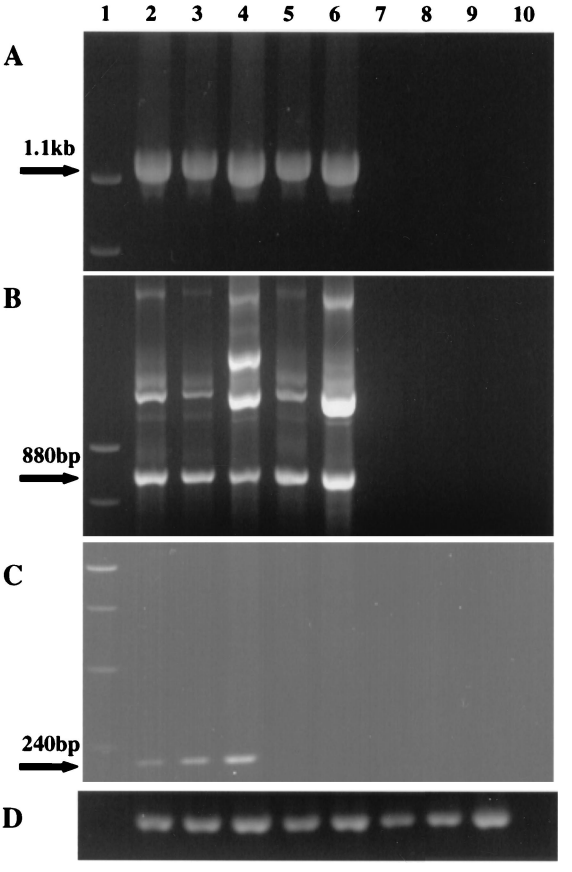

FIG. 2. Sample PCR results. Lanes 2 to 6 show the PCRs with DNA from five *S. bovis* biotype I strains obtained by using clone 51 primers (A), clone DS14 primers (B), clone 40 primers (C), and the 23S RNA positive control primers (D). Lanes 7 to 9 show the reactions of these primers with DNA from three biotype II strains. Lane 1 contains ϕX174/*Hae*III DNA markers, and lane 10 contains the distilled water negative PCR control.

Biotyping PCR assay distinguishes between biotypes in a clinical setting. To determine whether the clone DS14 and 51 PCRs could be used in a clinical setting, a second blind trial was undertaken. Table 3 lists the 20 *S. bovis* strains tested, the PCR results obtained, and the biotype and disease correlations revealed after the biotype PCR assays were performed. All *S. bovis* biotype I strains were again PCR positive in the clone DS14 and 51 reactions, thus confirming our earlier results showing that these two clones are specific for biotype I strains. All biotype I strains were PCR positive, regardless of the presence of either endocarditis or GI disease; the exception being one patient who had neither endocarditis nor GI disease. Neither the clone DS14 PCR nor the clone 51 PCR reacted with either the II/1 or II/2 subtypes of biotype II, regardless of the presence of disease.

We also tested whether clone 40 results correlated with disease. Approximately half of the biotype I strains isolated from patients with both endocarditis and GI disease were PCR positive for clone 40. However, all biotype I strains from patients with only one or neither of these conditions gave the clone 40-specific PCR band. While clone 40 reacts with a subset of biotype I strains, this does not seem to be linked to

TABLE 2. Association between PCR results and CDC identification

S. bovis strain	CDC biotype	PCR result[a] with:			
		Clone DS14	Clone 51	Clone 40	23S RNA
1892	I	+	+	+	+
1275	I	+	+	+	+
336	I	+	+	+	+
888	I	+	+	+	+
139	I	+	+	+	+
2262	I	+	+	+	+
1872	I	+	+	+	+
752	I	+	+	−	+
955	I	+	+	−	+
2487	I	+	+	−	+
6992	II	−	−	−	+
4265	II	−	−	−	+
5112	II	−	−	−	+
458	II	−	−	−	+
1313	II	−	−	−	+
1433	II	−	−	−	+
7332	II	−	−	−	+
1815	II	−	−	−	+
25	II	−	−	−	+
329	II	−	−	−	+
2468	II	−	−	−	+
87	II	−	−	−	+
2127	II	−	−	−	+
2469	II	−	−	−	+
663	II	−	−	−	+
2822	II	−	−	−	+

[a] +, PCR band observed; −, no PCR band observed.

disease manifestation. None of the biotype II strains reacted in the clone 40 PCR test.

DISCUSSION

In the present study, we identified two gene fragments, based on clones DS14 and 51, which correctly distinguished between *S. bovis* biotypes I and II strains in 100% of 46 isolates (26 biotype I strains and 20 biotype II strains). No correlation existed between disease and the presence of either clone DS14 or 51 PCR bands, implying that these PCRs will react only with *S. bovis* biotype I strains, regardless of the disease presentation. Since this biotype is the predominant biotype in 94% of endocarditis cases and 71 to 100% of the GI disease cases, these PCR tests could prove clinically useful (37).

A third gene fragment, clone 40, reacted with 69.2% of 26 biotype I strains and did not react with any biotype II strains tested. Approximately 50% of the 11 biotype I strains acquired from patients with both endocarditis and GI disease possessed the clone 40 PCR band. Further investigation into the presence of this PCR band with the presence of a specific type of GI disease for the 20 clinical strains demonstrated no strong correlation between specific GI disease and PCR (results not shown). This suggests that clone 40 might represent allelic variation in a subset of biotype I strains. However, that subset is not uniquely linked to a clinical presentation of GI malignancy. Further investigation is required to study the long-term medical outcome in patients harboring clone 40 PCR-positive strains.

Comparison between clone DS14 and proteins in the public databases yielded 36 and 44% identities, respectively, for two overlapping fragments from the same hypothetical protein upstream of the *sr* gene in *S. mutans* OMZ 175 (31). This low level of homology means that conclusions from the sequence comparisons are tentative. However, the SR protein from *S. mutans* OMZ 175 has 88% homology at the amino acid level to the *S. mutans* PAc protein (32) that belongs to the family of oral streptococcal antigen I/II surface adhesins (12, 17, 23, 27). These proteins are responsible for a variety of host-bacterium interactions, including binding to human salivary glycoproteins and components of the oral microflora (5, 10, 21, 30, 44). It is possible that the DS14 clone encodes some regulatory protein that controls expression of a downstream protein homologous to the antigen I/II adhesin family.

DNA homology studies between clone 51 and known genes showed a 49% identity and a 69% similarity to the *S. gordonii rgg* gene (40). The *S. gordonii* Rgg protein is a positive transcriptional regulator of the downstream glucosyltransferase gene, *gtfG* (39). The GtfG enzyme produces glucans with both α1,3 and α1,6 glucosidic links from sucrose (37, 40, 42). Increased levels of GtfG expression produces the Spp^+ phenotype (sucrose-promoted phenotype) and a hard colony morphology (40). In our hands, when *S. bovis* biotype I strains were plated onto Todd-Hewitt broth plates containing 3% sucrose, 70% produced vast quantities of glucans (unpublished observations), confirming the observation by Coykendall (9). No biotype II strains produced this phenotype, and of the 30% of biotype I strains with the Spp^- phenotype it is hypothesized that, like the *S. gordonii* GtfG, specific changes in the carboxyl terminus of GtfG affected glucan production (42). Glucosyltransferases have also been recognized as virulence determi-

TABLE 3. Comparison between PCR results and both biotype and disease

S. bovis strain	Biotype	PCR result[a] with:				Disease status[b]	
		Clone DS14	Clone 51	Clone 40	23S RNA	Endocarditis	GI disease
3358	I	+	+	+	+	+	+
5912	I	+	+	+	+	+	+
5927	I	+	+	+	+	+	+
6066	I	+	+	+	+	+	+
7786	I	+	+	+	+	+	+
1314	I	+	+	+	+	+	+
365	I	+	+w	−	+	+	+
22195	I	+	+	−	+	+	+
9925	I	+	+	−	+	+	+
726	I	+	+w	−	+	+	+
4203	I	+	+w	−	+	+	+
8298	I	+	+	+	+	−	−
5897	I	+	+	+	+	+	?
9410	I	+	+	+	+	+	−
2703	I	+	+	+	+	+	−
7422	I	+	+	+	+	+	−
8250	II/1	−	−	−	+	−	−
6448	II/1	−	−	−	+	+	+
1053	II/2	−	−	−	+	+	+
4499	II/2	−	−	−	+	−	−

[a] +, PCR band observed; −, no PCR band observed or no disease observed; +w, weak but distinctive PCR band observed.

[b] +, Condition observed; −, condition not observed; ?, condition not known at present.

nants in *S. mutans* dental caries, and mutations in *S. mutans* genes involved in glucan and fructan production caused a 46% reduction in the incidence of endocarditis in rats (28). Similarly, the *S. pyogenes rgg* sequence which carries 22% identity and 34% similarity to the *S. gordonii rgg* gene was shown to contribute to virulence by controlling the production of streptococcal erythrogenic toxin B (SPE B) at the transcriptional level (7). SPE B has been shown to contribute to the virulence of *S. pyogenes* in mice (25). Given these published reports linking *rgg* to dental caries and endocarditis, where a change in colony morphology could affect binding of the organism in the first stages of either of these diseases, it is not surprising to find that the clone 51 PCR identified *S. bovis* biotype I strains that are the more prevalent strain in endocarditis.

Clone 40 has homology to many gram-positive carbohydrate PTS proteins belonging to the lactose-specific family. These proteins are involved in both the uptake and the phosphorylation of specific carbohydrates (34). The highest level of homology to clone 40 was observed in the *B. subtilis* cellobiose-specific enzyme IIC (18). Together with enzyme IID, these proteins interact to provide the domain responsible for both sugar binding and transmembrane channel formation (34). Interestingly, the level of homology between clone 40 and the *S. aureus* lactose-specific IIBC component (4) was lower than that for the *B. subtilis* component, although this protein, together with the *Lactobacillus casei*, *S. mutans*, and *Lactococcus lactis* proteins, belongs to the same lactose class of PTSs (1, 13, 35). Exactly why some biotype I strains of *S. bovis* possess this gene fragment and others do not is unclear. At least 20 *S. bovis* human strains have been found to possess a PTS for lactose transport with significant homology to an *S. bovis* ruminal PTS protein (8). It could be that the biotype I strains which are clone 40 PCR positive harbor some residual genes leftover from cellobiose metabolism of an ancestral *S. bovis* ruminal strain.

The biotype I-specific gene fragments identified here are predicted to be involved in the regulation of known streptococcal adhesion mechanisms. Whether this association is directly related to disease pathogenesis remains to be tested. Although the present study does not directly link *S. bovis* with either endocarditis or GI cancer, the biotype PCRs with clones DS14 and 51 may prove to be useful markers in further investigations of the potential link between *S. bovis* and the development of GI cancer. Meanwhile, the biotype PCRs with clones DS14 and 51 provide a fast and economical test that can be used to identify *S. bovis* biotype I strains.

ACKNOWLEDGMENTS

We are grateful to Ellen J. Baron and Susan Munro for providing clinical *S. bovis* isolates. We thank Meg Vickerman for helpful discussions of the Spp phenotype and how it relates to glucosidic linkages. We also thank Luda Diatchenko, Clontech, Inc., for help with the genomic subtraction method. We are indebted to Jacqueline Eversley and Sheila Masci for excellent technical assistance. We thank Anthea K. Lee, Corrella S. Detweiler, and Denise Monack for critical review of the manuscript.

This work was supported by a contract from Protein Design Labs, Inc.

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