**Molecular cloning and characterization of the spaB gene of Streptococcus sobrinus**

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A gene of *Streptococcus sobrinus* 6715 (serotype g) designated spaB and encoding a surface protein antigen was isolated from a cosmid gene bank. A 5.4 kb *HindIII/AvaI* DNA fragment containing the gene was inserted into plasmid pBR322 to yield plasmid pXI404. Analysis of plasmid-encoded gene products showed that the 5.4 kb fragment of pXI404 encoded a 195 kDa protein. Southern blot experiments revealed that the 5.4 kb chromosomal insert DNA had sequence similarity with genomic DNA of *S. sobrinus* 6715, *S. sobrinus* B13 (serotype d) and *Streptococcus cricetus* HS6 (serotype a). The recombinant SpaB protein (rSpaB) was purified and monospecific antiserum was prepared. With immunological techniques and the anti-rSpaB serum, we have shown: (1) that the rSpaB protein has physico-chemical and antigenic identity with the *S. sobrinus* SpaB protein, (2) the presence of cross-reactive proteins in the extracellular protein of serotypes a and d of the mutans group of streptococci and (3) that the SpaB protein is expressed on the surface of mutans streptococcal serotypes a, d and g.

**Introduction**

In recent times, the use of recombinant DNA methodology to study genetic determinants of the mutans group of streptococci has become quite common. The majority of these studies has involved the isolation and characterization of genes that function in the metabolism of sucrose (Robeson et al., 1983; Gilpin et al., 1985; Hayakawa et al., 1986; Sato & Kuramitsu, 1986; Pucci et al., 1987). Other studies have been on DNA sequences encoding (i) a glucan-binding protein (Russell et al., 1983), (ii) biological determinants not directly associated with virulence (Jagusztyn-Krynicka et al., 1982), (iii) dextranase (Barrett et al., 1987) and (iv) surface protein antigens (Holt et al., 1982; Lee et al., 1988; Ogundipe & Holt, 1989). In addition to providing basic information on the structure and function of mutans streptococcal genes, recombinant DNA technology also provides the means to identify novel antigens and to produce sufficient quantities of these antigens free of contaminants for assessment of the efficacy of various candidate antigens in the development of a safe anti-caries vaccine.

Presently, surface proteins and peptide derivatives of surface proteins are candidate antigens for the production of an protective immune response and subsequent development of the anti-caries vaccine. Furthermore, several studies have implicated streptococcal surface proteins as virulence determinants in the onset of the caries process (Curtiss et al., 1983, 1986). Several surface protein antigens have been evaluated in animals for their ability to protect against caries. One group of proteins, designated as antigen B (Russell, 1979), antigen I/II (Russell et al., 1980), antigen IF (Hughes et al., 1980), and antigen P1 (Forester et al., 1983) has been extensively characterized by different laboratories. Although these proteins have different names, they are immunologically identical proteins of about 185 kDa in size. Lehner et al. (1981) and also Russell et al. (1983) have shown that antigen I/II and antigen B, respectively, can confer protection against caries. However, these antigens have been reported to possess immunological cross-reactivity with heart tissue (Hughes et al., 1980; Forester et al., 1983). On the other hand, other researchers, either using monoclonal antibodies to detect cross-reactivity between antigen I/II and heart tissue (Smith et al., 1984; Ayakawa et al., 1987) or testing for anti-human heart antibodies in animals immunized with purified antigen I/II (Bergmeier & Lehner, 1983), could not confirm these reports. Because of the issue of heart cross-reactivity, work on better methods for the production of desired antigens and also on the identification of other potential immunogens need to be pursued. Recombinant
DNA technology offers significant promise in the pursuit of both these goals. We are particularly interested in surface proteins of the mutans group of streptococci that have the potential to function as immunogens for the prevention of dental caries. We report here the cloning of a gene, designated spaB, that encodes a protein antigen, SpaB, whose prevalence appears to be restricted to Streptococcus sobrinus and Streptococcus cricetus.

Methods

Bacteria and growth conditions. S. sobrinus 6715 (serotype g) and the minicell-producing Escherichia coli X1274 were obtained from R. Curtiss, III, Washington University, St Louis, Missouri, USA. Representative strains of serotypes a, b, c, e, and f of the mutans group of streptococci were kindly provided by F. Macrina, Virginia Commonwealth University, Richmond, Virginia, USA. S. sobrinus B13 (serotype d) was provided by R. Linzer, State University of New York at Buffalo, USA. The genotypes and sources of E. coli strains HBlOl, HB2688 and HB2690 have been described elsewhere (Ogundipe & Holt, 1989). E. coli strains were routinely cultured in Luria medium and streptococcal cells were grown in either brain heart infusion or FMC-NZ medium (Ogundipe & Holt, 1989). When appropriate, ampicillin (50 μg ml\(^{-1}\)) or tetracycline (12.5 μg ml\(^{-1}\)) was added to media.

Preparation of DNA. Streptococcal DNA was isolated from mutansynol-digested cells as described by Robeson et al. (1983). Cosmid and plasmid DNA were purified by a combination of alkaline lysis and cesium chloride gradient centrifugation as described in Maniatis et al. (1982).

Construction of cosmid library and identification of immunoreactive recombinant E. coli. A cosmid library of S. sobrinus DNA was prepared using the procedures described previously (Ogundipe & Holt, 1989). Briefly, S. sobrinus chromosomal DNA was partially restricted with EcoRI, and DNA fragments greater than 20 kb were ligated into EcoRI-cleaved and phosphatase-treated cosmid vector pJC74. The ligated DNA was packaged into phage heads using extracts of E. coli lysogens HB2688 and HB2690 by the method of Collins et al. (1978) and then the packaged DNA was used to infect E. coli HB101. Ampicillin-resistant colonies were screened for production of material reactive with antiserum against S. sobrinus extracellular protein or SpaA protein by the immunoassay procedure of Hohn & Holt, 1989). The 5.4 kb HindIII fragment of S. sobrinus rSpaB, SpaA or extracellular protein fractions of streptococcal strains were prepared as previously described (Ogundipe & Holt, 1989). Polyethylene glycol (PEG) and lyophilized material were mixed in the ratio of 1:5 (w/v) agarose prepared in 0.06 M-barbital buffer, pH 8.6. For immunoelectrophoresis, plates were incubated in a humidified chamber at room temperature for 16 h and washed exhaustively with saline solution. For visualization of reactions, the agarose on the plates was dried and precipitin reactions were stained with Coomassie brilliant blue.

Immunological procedures. Immunological procedures were carried out in 1.5% (w/v) agarose prepared in 0.06 M-barbital buffer, pH 8.6. For immunoelectrophoresis, plates were incubated in a humidified chamber at room temperature for 16 h and washed exhaustively with saline solution. For visualization of reactions, the agarose on the plates was dried and precipitin reactions were stained with Coomassie brilliant blue.

Crossed and tandem crossed immunoelectrophoresis were performed using barbital running buffer and the following procedure. Protein fractions applied into wells were allowed to diffuse for 15 min prior to electrophoresis at 15°C for 1 h at 8 V cm\(^{-1}\) (first dimension). After this electrophoresis, a portion of the gel above the path of electrophoresis of the protein was removed and replaced with agarose.
Characterization of \(\text{spaB}\) gene

Results

Cloning of the \(\text{spaB}\) gene

The cosmig gene library constructed in \(p\text{JC74}\) was screened by colony immunoblotting for recombinant \(E.\) \(\text{coli}\) reactive with antiserum against the extracellular protein fraction of \(S.\) \(_{\text{sobrinus}}\) 6715. The recombinant \(E.\) \(\text{coli}\) exhibiting positive reactions were then screened using antiserum to the highly immunogenic surface protein antigen \(A\) (SpaA) of \(S.\) \(_{\text{sobrinus}}\) which has been described previously (Holt et al., 1982). Of approximately 500 colonies screened, nineteen clones were identified that failed to react with SpaA antibodies. One of these clones was designated \(\text{HB101 (pXI400)}\) and chosen for further characterization. \(\text{HB101 (pXI400)}\) was found by immunoblot using antiserum to \(S.\) \(_{\text{sobrinus}}\) extracellular protein to express a protein of 195 kDa which was also produced by \(S.\) \(_{\text{sobrinus}}\) 6715 cells (data not shown).

To determine the coding region for the 195 kDa protein, we performed a number of subcloning experiments using the plasmid vector \(p\text{BR322}\). Fig. 1 shows partial restriction maps of \(p\text{XI400}\) and \(p\text{BR322}\) derivatives constructed for this study. During subcloning, we found that plasmids \(p\text{XI402}\) and \(p\text{XI403}\) both encoded the 195 kDa protein, which suggested that the promoter sequences for the gene had also been cloned along with the sequences encoding the protein. Ultimately, the entire coding region of the 195 kDa protein was isolated on recombinant plasmid \(p\text{XI404}\), which contained a 5-4 kb \(\text{HindIII}/\text{AvaI}\) fragment of \(S.\) \(_{\text{sobrinus}}\) DNA. We designated the \(S.\) \(_{\text{sobrinus}}\) gene encoded by \(p\text{XI404}\) as \(\text{spaB}\) (see Discussion) and the product of the cloned gene as recombinant surface protein antigen \(B\) (rSpaB).

Analysis of plasmid-encoded products

Plasmids \(p\text{BR322, pXI404 and pXI405}\) were used to transform \(E.\) \(\text{coli x1274}\) and newly synthesized proteins of minicells prepared from these strains were examined. \(p\text{XI405}\) contains the 3-1 kb \(\text{HindIII}/\text{BamHI}\) end of the 5-4 kb insert of \(p\text{XI404}\) inserted into \(p\text{BR322}\) (Fig. 1). Strains harbouring \(p\text{XI405}\) were found by immunoblotting to produce a truncated polypeptide (data not shown). A major 195 kDa protein was observed in minicells containing \(p\text{XI404}\) (Fig. 2, lane 3). A truncated protein of 140 kDa was observed in minicells containing \(p\text{XI405}\) (Fig. 2, lane 4). These results indicate that \(p\text{XI404}\) encodes the 195 kDa rSpaB protein and that transcription of the gene is initiated from the \(\text{HindIII}\) side of the 5-4 kb insert of \(p\text{XI404}\).
Fig. 2. Analysis of plasmid-encoded products synthesized by E. coli minicells. The newly synthesized polypeptides were labelled with [\textsuperscript{35}S]methionine and electrophoresed through a 7% SDS-polyacrylamide gel. The lanes contain proteins synthesized by minicells isolated from X1274 (1), X1274(pBR322) (2), X1274(pXI404) (3) and X1274(pXI405) (4). The numbers to the left are the sizes, in kDa, of the protein standards run along with the samples.

**Purification and characterization of rSpaB protein**

Using SDS-PAGE and immunoblot analyses, we obtained results suggesting that rSpaB was predominantly translocated to the periplasmic space of E. coli cells. Therefore, the protein was purified from this fraction as described in Methods. The purified rSpaB protein exhibited a single polypeptide band of 195 kDa. Polyclonal antibodies were prepared against the purified rSpaB protein. In immunodiffusion analysis, precipitin reactions of complete identity were observed between the S. sobrinus SpaB protein and the purified rSpaB protein (Fig. 3a).

CROSSED IMMUNOELECTROPHORESIS OF EXTRACELLULAR PROTEIN OF S. sobrinus 6715 revealed several peaks of antigen-antibody reactivity when the protein was run into an agarose layer containing antiserum against total extracellular protein of S. sobrinus 6715. By using the same serum, and tandem crossed immunoelectrophoresis of S. sobrinus extracellular protein and a lysate of E. coli HB101(pXI404), we determined which of the precipitin peaks corresponded to the SpaB protein of S. sobrinus (Fig. 3b, arrow).

Fig. 3. (a) Immunodiffusion analysis using anti-rSpaB protein serum. Shown are precipitin reactions between antigens of (1) S. sobrinus extracellular protein fraction, (2) purified rSpaB protein, (3) cellular lysate of E. coli HB101(pBR322) and (4 and 5) anti-rSpaB protein serum. (b) Crossed immunoelectrophoretic analysis of an S. sobrinus extracellular protein fraction with anti-S. sobrinus extracellular protein serum. The arrow indicates the peak corresponding to the SpaB protein.

**Distribution of the spaB gene and SpaB protein among the mutans group of streptococci**

Southern hybridization experiments using the 5.4 kb HindIII/AvaI fragment of pXI404 as probe and high stringency conditions showed that sequences similar to that of the spaB gene were present on the chromosomes of other species of the mutans group of streptococci. Positive hybridization signals were detected to 5.4 kb fragments of S. sobrinus strains and to 4.8 kb and 2.7 kb fragments of S. cricetus. DNA of the S. mutans strains and the S. rattus strain did not hybridize with the probe (data not shown).
To determine the distribution among the mutans group of streptococci of antigens that cross-react with antibodies to the rSpaB protein, extracellular protein fractions were prepared from representative strains of the mutans group of streptococci and probed by immunoblotting with anti-rSpaB serum. This analysis showed that cross-reactive antigens were produced by the S. sobrinus strains and the S. cricetus strain. The antiserum did not react with the protein of the S. mutans strains nor with that of the S. rattus strain (data not shown).

Immunofluorescence studies were performed on washed cells of representative species of the mutans group of streptococci to determine if the SpaB protein could also be found on the surface of cells. With indirect immunofluorescent staining, the intensity of fluorescence varied among the strains examined. The S. sobrinus strains exhibited very intense fluorescence while reactivity with the S. cricetus strain was moderate. The S. mutans and S. rattus strains displayed no reactivity (data not shown). On S. sobrinus cells, more fluorescence was observed to occur at the junction between neighbouring cells in a chain (Fig. 4).

**Discussion**

We have characterized the spaB gene of S. sobrinus, which encodes a protein that is both associated with the surface of S. sobrinus cells and excreted extracellularly. Isolation of the spaB gene was achieved by differential immunoscreening of a library of recombinant E. coli containing S. sobrinus DNA fragments. Initially, the library was screened for recombinants that expressed S. sobrinus extracellular proteins. The resulting clones were then screened for the production of the very immunogenic SpaA protein (Holt et al., 1982), which left only clones producing proteins other than the SpaA protein. The product of the spaB gene exhibited no sucrose or dextran hydrolysis activity, which indicated that the gene did not encode a glucosyltransferase or dextranase enzyme. Immunofluorescence studies with washed streptococcal cells, using binding of antibodies raised against the purified recombinant DNA derived product, indicated that the product of the spaB gene was tightly associated with the cell surface of S. sobrinus and S. cricetus cells. Furthermore, the fluorescent label appeared as a series of 'Xs' between the individual cells of a chain, indicating that the SpaB protein is preferentially localized to the region of the cell wall where new material is being formed. These results justify designation of the SpaB protein as a surface protein antigen.

Immunodiffusion and tandem crossed immunoelectrophoretic analyses indicated that the cloned product was immunologically identical to the product made by S. sobrinus cells. The latter technique also revealed that the SpaB protein was the second most immunoreactive protein in the extracellular protein fraction of S. sobrinus. Only the SpaA protein was more reactive. Therefore, it appears that the SpaB protein is a major antigen in this fraction and probably is also a major constituent of the cell surface of S. sobrinus cells. The molecular mass of rSpaB was also identical to that of the S. sobrinus product as demonstrated by gel electrophoresis of the purified cloned product and immunoblot analysis. Moreover, analysis of plasmid-encoded gene products confirmed the results of the immunoblot studies.

It has been demonstrated here that antigens immunologically cross-reactive with the SpaB protein are produced by S. sobrinus and S. cricetus but not by S. mutans or S. rattus. Although a serotype h strain was not examined, it is very likely that this species also expresses a similar protein. Reports have shown that this serotype is related to serotypes d and g (Beighton et al., 1981; Okahashi et al., 1984). Considering the species distribution of the SpaB protein, it is reasonable to think that the SpaB protein could be used in the development of a vaccine against caries with specificity against serotypes a, d, g and h of the mutans group of streptococci.

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References


