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-Krynica *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 produc-

tion 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

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DNA technology offers significant promise in the pursuit of both of 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* χ 1274 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 HB101, BHB2688 and BHB2690 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 μ g ml⁻¹) or tetracycline (12.5 μ g ml⁻¹) was added to media.

Preparation of DNA. Streptococcal DNA was isolated from mutanolysin-digested cells as described by Robeson *et al.* (1983). Cosmid and plasmid DNA were purified by a combination of alkaline lysis and caesium 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 kbp were ligated into *EcoRI*-cleaved and phosphatase-treated cosmid vector pJC74. The ligated DNA was packaged into λ phage heads using extracts of *E. coli* lysogens BHB2688 and BHB2690 by the method of Collins & Hohn (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 Helfman *et al.* (1983) using reagents and protocols supplied in a commercially available immunoblot assay kit (Bio-Rad).

Subcloning, transformation and minicell analysis. For each subcloning experiment, 2.0 μ g of recombinant plasmid DNA and 1.0 μ g of plasmid pBR322 DNA were separately digested with restriction enzyme(s). The recombinant plasmids and the enzymes used for their construction were as follows: pXI401 (*EcoRI*), pXI402 (*HindIII*), pXI403 (*HindIII*), pXI404 (*HindIII/AvaI*) and pXI405 (*HindIII/BamHI*). Following digestion with restriction enzyme, the restricted recombinant plasmid DNA and the digested pBR322 DNA were ligated using a 3:1 mass ratio of insert to vector. In instances where a number of fragments of different sizes were to be ligated, the size of the largest fragment generated was used in the mass ratio determinations. Ligated DNA was transformed into *E. coli* HB101 by the calcium chloride procedure described in Maniatis *et al.* (1982). Transformants were screened for the expression of immunoreactive determinants by the immunoassay and the size of the product was determined by immunoblot analysis (Towbin *et al.*, 1979).

Minicell analysis was performed as described by Ogundipe & Holt (1989). Plasmids pBR322, pXI404 and pXI405 were transformed into *E. coli* χ 1274 and minicells were isolated from the resulting strains. Plasmid-encoded products were radiolabelled with [³⁵S]methionine and resolved on a 7% (w/v) SDS-polyacrylamide gel; autoradiography of the dried gel was performed with Kodak X-Omat AR film.

Immunofluorescence staining. Cells from 100 μ l of a culture were pelleted in a microfuge and washed twice with 1.5 M-NaCl. The cells were then suspended in 100 μ l anti-rSpaB serum diluted 1:20 in phosphate-buffered saline (PBS) and incubated for 1 h with agitation. After two washes with PBS, the cells were suspended in 100 μ l fluorescein-conjugated goat anti-rabbit IgG diluted 1:10 in PBS. The suspension was incubated in the dark for 30 min without agitation followed by two washes of the cells with PBS. Finally, the cell pellet was suspended in 75 μ l PBS and samples were examined using a Zeiss model IM-35 fluorescence microscope.

Southern hybridization. Chromosomal DNA of mutant group streptococci and DNA of plasmid pXI404 were digested with *AvaI* and *HindIII*, fractionated by agarose gel electrophoresis and transferred by the method of Southern (1975) to nitrocellulose membranes as described previously (Ogundipe & Holt, 1989). The 5.4 kb *AvaI/HindIII* fragment of pXI404 was gel purified, radiolabelled by nick-translation with [³²P]dCTP and used to probe, using high stringency conditions, the membranes as described previously (Ogundipe & Holt, 1989).

Protein isolation and antibody production. Recombinant SpaB protein was purified from concentrated periplasmic protein fractions of *E. coli* HB101(pXI404) prepared as described by Hazelbauer & Harayama (1979). Concentrates were subjected to gel filtration chromatography using a Pharmacia fast protein, peptide, polynucleotide liquid chromatography (FPLC) system. A sample containing 3 mg protein in 200 μ l elution buffer [20 mM-Tris/HCl (pH 8.0), 150 mM-NaCl] was injected onto a Superose 12 column (10 mm $\times$ 30 mm) that had been equilibrated with two column volumes of elution buffer. Protein was eluted at a flow rate of 0.5 ml min⁻¹ and 1 ml fractions were collected. Fractions were analysed for the presence of rSpaB protein by immunodiffusion analysis using anti-*S. sobrinus* extracellular protein serum. Fractions that were positive were then analysed for homogeneity by SDS-PAGE (Laemmli, 1970).

Cellular lysates of recombinant *E. coli* derivatives and extracellular protein fractions of streptococcal strains were prepared as previously described (Ogundipe & Holt, 1989).

Polyclonal antisera against *S. sobrinus* rSpaB, SpaA or extracellular proteins were raised in female New Zealand White rabbits by subcutaneous injection, into several sites on the backs of the animals, of 100 μ g, 200 μ g or 1 mg of the respective proteins emulsified in Freund's complete adjuvant. Booster injections using incomplete adjuvant were given two weeks after the initial injections. Animals were bled two or three weeks after the booster injections.

Immunological procedures. Immunological procedures were carried out in 1% (w/v) agarose prepared in 0.06 M-barbital buffer, pH 8.6. For immunodiffusion analyses, 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

Fig. 1. Restriction endonuclease cleavage site map of pXI400 and pBR322 subclones. The heavy and light lines indicate pJC74 and *S. sobrinus* chromosomal DNA, respectively. The size, in kb, of the chromosomal DNA insert is given beneath the recombinant cosmid or plasmid designations. The letters indicate the restriction sites for *Ava*I (A), *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hind*III (H), *Pst*I (P) and *Sal*I (S). The arrow indicates the approximate coding region for the *spaB* gene and the direction of transcription.

containing antiserum (10%, v/v) against the extracellular protein of *S. sobrinus*. In the second dimension, samples were electrophoresed into the antibody-containing layer at 15 °C for 18 h at 2 V cm⁻¹. In both dimensions, the samples were electrophoresed from cathode to anode. Precipitin peaks were visualized after staining of gels as described above.

Results

Cloning of the *spaB* gene

The cosmid gene library constructed in pJC74 was screened by colony immunoblotting for recombinant *E. coli* reactive with antiserum against the extracellular protein fraction of *S. sobrinus* 6715. The recombinant *E. coli* exhibiting positive reactions were then screened using antiserum to the highly immunogenic surface protein antigen A (SpaA) of *S. 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 HB101(pXI400) and chosen for further characterization. HB101(pXI400) was found by immunoblot using antiserum to *S. sobrinus* extracellular protein to express a protein of 195 kDa which was also produced by *S. 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 pBR322. Fig. 1 shows

partial restriction maps of pXI400 and pBR322 derivatives constructed for this study. During subcloning, we found that plasmids pXI402 and pXI403 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 pXI404, which contained a 5.4 kb *Hind*III/*Ava*I fragment of *S. sobrinus* DNA. We designated the *S. sobrinus* gene encoded by pXI404 as *spaB* (see Discussion) and the product of the cloned gene as recombinant surface protein antigen B (rSpaB).

Analysis of plasmid-encoded products

Plasmids pBR322, pXI404 and pXI405 were used to transform *E. coli* χ 1274 and newly synthesized proteins of minicells prepared from these strains were examined. pXI405 contains the 3.1 kb *Hind*III/*Bam*HI end of the 5.4 kb insert of pXI404 inserted into pBR322 (Fig. 1). Strains harbouring pXI405 were found by immunoblotting to produce a truncated polypeptide (data not shown). A major 195 kDa protein was observed in minicells containing pXI404 (Fig. 2, lane 3). A truncated protein of 140 kDa was observed in minicells containing pXI405 (Fig. 2, lane 4). These results indicate that pXI404 encodes the 195 kDa rSpaB protein and that transcription of the gene is initiated from the *Hind*III side of the 5.4 kb insert of pXI404.

Fig. 2. Analysis of plasmid-encoded products synthesized by *E. coli* minicells. The newly synthesized polypeptides were labelled with [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 *Hind*III/*Ava*I 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).

Fig. 4. Indirect immunofluorescence analysis of *S. sobrinus* 6715 SpaB protein. *S. sobrinus* 6715 cells were stained and spread for microscopy as described in Methods. Magnification $\times 1000$.

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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