Cloning and sequencing the endocarditis immunodominant antigen of *Streptococcus sobrinus* strain MUCOB 263

W. BROOKS and J. P. BURNIE*

Department of Medical Microbiology, University of Manchester Medical School, Oxford Road, Manchester M13 9PT

Summary. Immunoblotting sera from cases of *Streptococcus mutans* or *S. sobrinus* endocarditis against an extract from *S. sobrinus* strain MUCOB 263 had identified three immunodominant antigenic bands at 190, 200 and 220 kDa. A lambda ZAPII DNA library was produced from the sheared genomic DNA of *S. sobrinus* MUCOB 263 and six identical positive clones were identified when this library was screened with serum from a patient with endocarditis caused by a bacterium from the mutans group of streptococci. On subcloning and sequencing, a protein containing 1548 amino acids was identified with a 99·2 % homology to the SpaA antigen of *S. sobrinus* and 68·4 % homology to the PAc antigen of *S. mutans*.

Introduction

Infective endocarditis remains an important disease in patients with underlying rheumatic or congenital heart valve lesions. Previous work has shown that antibody to streptococcal antigens is raised in streptococcal endocarditis and produces a pattern on immunoblot which is species specific.¹ In the case of *Streptococcus sobrinus*, patients with active endocarditis produced antibody to bands at 220, 200 and 190 kDa. These antigens have not been identified, but an antigen alternatively called antigen B,² I/II,³ IF,⁴ MSL-1,⁵ P1⁶ or PAc,⁷ has been implicated in the development of dental caries. This antigen has an apparent mol. wt of 185 kDa.²

The purpose of this work was to clone the immunodominant antigens of *S. sobrinus* strain MUCOB 263 and thereby ascertain their identity. This was done by preparing a library in the expression vector lambda ZAPII and screening the library with serum from a patient with active endocarditis. The aim was to find out whether one of the endocarditis-specific antigens of *S. sobrinus* is related to antigen I/II. This is important, as antigen I/II has been used successfully as an anti-caries vaccine in Rhesus monkeys⁸ and antibody against it has been shown to cross-react with heart tissue.⁴

This approach was preferred to the identification of an antigen from a gel by direct amino-acid sequencing, as several proteins may run on the same place on the gel and this may be misleading. Antibody selection of the antigen in an expression system avoids this

problem and also preferentially detects linear epitopes. These might be synthesised subsequently and form the basis of a test to detect endocarditis-specific antibody without the need for immunoblotting.

Materials and methods

DNA isolation and lambda ZAPII library preparation

S. sobrinus strain Manchester University Collection of Bacteria No. 263 (MUCOB 263) was used both here and in the previous immunoblotting paper.¹ Initially, strain MUCOB 263 was identified as *S. mutans*, a fact reflected in the aforementioned paper. However, more detailed testing of this strain revealed that MUCOB 263 is biochemically more similar to *S. sobrinus* (Professor D. Bratthall, personal communication). To avoid future confusion, the organism has now been renamed *S. sobrinus* MUCOB 263. DNA was isolated essentially by the method described by Chassy.⁹ Bacterial cells were grown overnight at 37°C with shaking in brain-heart infusion broth containing glucose 0·2 % and 40 mM D, L-threonine (Sigma). The bacteria were harvested by centrifugation at 5000 rpm for 10 min before being washed and resuspended in 12·5 ml of 0·02 M Tris, pH 8·2; 25 ml of 20 M polyethylene glycol (PEG) 24 % in distilled water were then added and mixed. Lysozyme was added at 34·6 mg/ml, equivalent to 100 µg/unit obtained by measuring the OD₆₀₀ of a 10-fold dilution of bacterial cultures. After incubation for 1 h at 37°C, the spheroplasts formed were centrifuged at 5000 rpm for 10 min and thoroughly resuspended in 50 ml of 10 mM Tris-Cl, pH 8·0, 1 mM EDTA (TE). The spheroplasts were lysed with 5·5 ml of sodium dodecyl sulphate (SDS)

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* Correspondence should be sent to Professor J. P. Burnie.

10%, at 60°C for 15 min. The DNA was purified by standard procedures,¹⁰ treated with RNAase A (Sigma) (after pre-boiling the RNAase at 100°C for 15 min), treated with proteinase K, extracted with phenol:chloroform:isoamyl-alcohol (25:24:1) and precipitated with ethanol. Plasmid DNA was removed by ethanol precipitation in the presence of 0.3 M ammonium acetate, pH 5.2. Further purification was achieved by dialysis against TE over 36 hr at 4°C.

The lambda ZAPII library with an insert size range of 2–7 kb was prepared by Clontech Laboratories Inc., Cambridge Biosciences. The DNA was sheared mechanically, *EcoRI* linkers were added, the DNA was fractionated, and inserts were ligated into the lambda ZAPII vector arms.

Antibody screening

Serum was taken from the patient with *S. mutans* endocarditis described previously¹ and used for antibody screening. *Escherichia coli* XL1-Blue cells were infected with the lambda ZAPII phage on L broth agar (bacto-tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, maltose 2 g/L, bacto-agar 15 g/L) at c. 3000 pfu/85-mm plate. This was incubated at 42°C for 3 h. Plaques were transferred to nitrocellulose filters (0.45 µm pore size; Sartorius AG, Goettingen, Germany), impregnated with 10 mM isopropyl β-D-thiogalactopyranoside (IPTG), at 37°C for 2 h. These filters were blocked overnight at 4°C with bovine serum albumin (BSA; Sigma) 3% in buffered saline (150 mM NaCl, 10 mM Tris). Serum, diluted 100-fold in BSA 3%, was added to the filters and incubated at room temperature for 2 h. The filters were then washed for 30 min in washing solution (150 mM NaCl, Tween 20 0.05%), before the second antibody, anti-human IgG conjugated to alkaline phosphatase (Sigma) diluted 1000-fold in BSA 3%, was added. After 1 h at room temperature, the filters were again washed and stained with equal volumes of naphthol ASMX phosphate (0.4 mg/ml in distilled water; Sigma) and Fast Red TR salt (6 mg/ml in 0.2 M Tris, pH 8.2; Sigma) (the Fast Red stain). Positive plaques were transferred to 1.5-ml tubes containing 200 µl of SM (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM MgSO₄, gelatin 0.0001%), and two-to-three drops of chloroform. Plaque purification was performed by the above method.

Fusion protein and immunoblot preparation

E. coli XL1-Blue cells were infected with purified positive phage clones on L broth agar to give 5000 pfu/85-mm plate, and incubated at 42°C for 4 h. Fusion protein was induced by adding 1 ml of 10 mM IPTG/plate and incubation at 37°C for 3.5 h. The crude lysates were recovered by adding 0.5 ml of lysis buffer (β-mercaptoethanol 25%, SDS 10%, 6 M urea, 300 mM Tris-HCl, pH 6.8) and gently shaking the sealed plates at 37°C for 10–15 min. The buffers,

containing the fusion proteins, were centrifuged at 4000 rpm for 10 min to remove debris and stored at –20°C.

Protein gel electrophoresis and immunoblotting were performed. Fusion protein samples (75 µl) and Rainbow Protein Molecular Weight Markers (25 µl; Amersham International plc) were made up to a total volume of 100 µl with cracking buffer (SDS 2.6%, 2-β mercaptoethanol 1.3%, glycerol 6%, bromophenol blue 0.2%, 0.05 M Tris-HCl, pH 6.8), and heated at 100°C for 3 min. The samples were then loaded on to an acrylamide 10% slab gel, and run in a discontinuous buffer system (Protean II xi Slab Cell; BioRad Laboratories Ltd, Herts) at 80 mA for 3 h. Proteins were transblotted on to nitrocellulose paper at 500 mA for 30 min (Trans-Blot Cell Model; BioRad). The nitrocellulose was blocked overnight at 4°C in BSA 3%. Immunoblotting was performed as for the antibody screening method described above, with a 1000-fold dilution of anti-β-galactosidase mouse monoclonal antibody (Promega) in BSA 3% as the primary antibody, and a 4000-fold dilution of anti-mouse polyvalent immunoglobulin-alkaline phosphatase conjugate in BSA 3% as the secondary antibody. The alkaline phosphatase stain—660 µl of nitroblue tetrazolium (NBT) 0.05 g/ml in N,N-dimethyl formamide (DMF) 70% added to 100 ml of 100 mM NaCl, 52.6 mM magnesium chloride, 100 mM Tris-Cl, pH 9.5, buffer, before 330 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in DMF 70%, was added—was substituted for the Fast Red stain.

Antigen-directed antibody selection

Two 100-µl volumes of resuspended *E. coli* XL1-Blue cells were infected with a dilution of a high titre stock of purified positive phage on L broth agar to give 5000 pfu/85-mm plate. Phage adsorption proceeded at 37°C for 30 min. The adsorption mixes were added to 2.5-ml volumes of L broth agarose 0.8%, mixed, and poured on to L broth agar plates. These plates were incubated at 42°C for 2.5 h, before nitrocellulose filters (0.45 µm pore size; Sartorius), pre-soaked in 10 mM IPTG and dried at room temperature for at least 1 h, were added and incubated at 37°C overnight. Each filter was then washed three times over a 30-min period in Tris-buffered saline (150 mM NaCl, 10 mM Tris-HCl, pH 7.2), and blocked overnight at 4°C in BSA 3%. A 10-fold dilution of serum in BSA 3% was added to the filters and incubated with shaking at room temperature for 3 h. This serum was removed, defined as “the depleted serum” and stored at 4°C. The filters were washed five times over a 100-min period with Tween 20-Tris-buffered saline (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, Tween 20 0.05%), and once with salt-Tween 20 solution (150 mM NaCl, Tween 20 0.05%). The bound antibody was eluted by adding 5 ml of glycine saline buffer (150 mM NaCl, 200 mM glycine-Cl, pH 2.8), and shaking at room temperature for 30 min. The buffer was aspirated into

a 7-ml glass bottle, containing Tris 0.04 g, mixed, defined as "eluted antibody" and stored at 4°C. A Western blot of *S. sobrinus* MUCOB 263 was performed by the immunoblot method described above with the original serum, depleted serum and eluted antibody as the primary antibodies. The antigen was the supernate of the strain after crushing in an X-press. The secondary antibody was a 1000-fold dilution of anti-human IgM and IgG alkaline phosphatase conjugates in BSA 3%, and the alkaline phosphatase stain was used.

In-vivo excision of DNA insert

A 10-ml overnight culture of *E. coli* XL1-Blue cells grown at 37°C in L broth containing tetracycline 125 µg/µl, was centrifuged at 2000 rpm for 10 min, resuspended in 4 ml of 10 mM MgSO₄ and stored at 4°C. In a 50-ml plastic conical tube, 200 µl of the resuspended *E. coli* XL1-Blue cells, 180 µl of high titre antibody-positive phage, (c 1.78×10^8 /85-mm plate), and 1 µl of R408 helper phage were combined and incubated at 37°C for 15 min (helper phage and *E. coli* XL1-Blue cells were from Clontech). Then, 5 ml of 2 × YT media (NaCl 5 g/L, yeast extract 10 g/L, bacto-tryptone 16 g/L, pH 7.0) were added to the dual-infected bacteria and incubated with shaking at 37°C for 3 h. The cells were killed by heating at 70°C for 20 min and ruptured by vortex mixing for 5 min. The cells were centrifuged at 4000 rpm for 5 min, and the supernate containing pBluescript SK(−) phagemid was decanted and stored at 4°C. The phagemid was propagated by adding 10 µl of the phagemid solution to 200 µl of resuspended *E. coli* XL1-Blue cells, incubating the culture at 37°C for 15 min, and plating 1-, 25-, 75- and 100-µl volumes on to L broth agar plates containing ampicillin 50 µg/ml.

Plasmid DNA preparation and denaturation

The pBluescript SK(−) plasmid DNA was purified from the bacterial colonies with the Magic Minipreps DNA Purification System (Promega) and alkaline denatured by the method of Maniatis *et al.*¹⁰ Hence, 8 µl of solution containing DNA 1.5–2.0 µg, was added to 2 µl of 2 M NaOH, briefly vortex mixed and centrifuged, and left at room temperature for 10 min. Three µl of 3 M sodium acetate (pH 4.8), 7 µl of distilled water and 60 µl of absolute ethanol at −20°C were then added and the DNA was precipitated at −70°C for 30 min. The precipitate was pelleted by centrifugation at 13000 rpm for 1 min, washed with ethanol 70% at −20°C and vacuum-dried. The dried pellets were stored at −20°C.

DNA sequencing

DNA sequencing was performed by the two-step chain-termination method, with Sequenase Version 2.0 (Cambridge Bioscience). The annealing step was

carried out at 66°C for 2 min, the labelling at 22°C for 4 min and the termination at 38°C for 5 min. Areas containing secondary structure were resolved by the substitution of dITP for dGTP. Sequencing reactions were run on acrylamide 6% gels, for 11, 8, 5 and 2 h at 46°–51°C.

Subcloning with the TA cloning[®] system

The phagemid clone did not carry the 5' end of the cloned gene, therefore it was decided to subclone the 5' end by polymerase chain reaction (PCR), followed by TA Cloning. The following PCR reagents were added to each of three tubes containing 0, 0.7 and 1.8 µg of *S. sobrinus* MUCOB 263 genomic DNA, with distilled water added to give a total volume of 64.3 µl: 5.3 µl of Primer 1 (CAGTCTCCGTCCCAACGACTGCG), 0.1 µg/µl, 4.4 µl of Primer 2 (GCTCCTCTTGAC-ATGGTC), 0.1 µg/µl, 10 µl of 10 × Taq buffer (Northumbria Biologicals Ltd, Northumberland; nbl); and 16 µl of dNTP (12.5 µl each of dATP, dGTP, dCTP, dTTP; Promega, added to 950 µl of de-ionised water). Then, 60 µl of mineral oil was carefully layered on top of the PCR reactions, the DNA was denatured at 95°C for 10 min, and 2.5 Weiss units of Taq (nbl) were added. The PCR conditions were: 94°C for 1.5 min, 50°C for 1.5 min and 72°C for 3 min for 30 cycles, before a final long extension of 72°C for 10 min. The PCR products (30 µl) were run on a TBE 0.8% gel at 50 V for 2 h, alongside 4 µl of *Eco*RI-*Hind* III cut lambda DNA markers (nbl).

Insert DNA was subcloned with the TA Cloning System Version 1.3 (InVitrogen Corporation, British Biotechnologies Ltd, Oxon) as specified in the manufacturer's protocol. Overnight, 12°C ligation reactions containing 3.0 µl of a four-fold dilution, in distilled water, of the 0.7-µg PCR reaction, 4.7 µl of a doubling dilution of the 0.7-µg PCR reaction and a negative control were performed. In the transformation, white recombinant colonies were picked after overnight incubation at 37°C and twice replated on kanamycin (50 µg/ml)/5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; 25 µl of 40 mg/ml solution/plate) to achieve pure and stable subclones.

Plasmid DNA was obtained, purified, denatured and sequenced as described above.

Southern blotting

Five restriction enzyme digests (*Pst*I, *Sac*I, *Sau*3AI, *Eco*RI, and *Bam*HI) of *S. sobrinus* genomic DNA were probed with [α -³²P] dCTP-labelled, *Eco*RI-digested plasmid subclone DNA. Briefly, genomic DNA (5 µg) was digested overnight at 37°C and the digest fragments were separated on a TBE agarose 1% gel, run at 50 V for 2.5 h. The DNA was nicked by UV irradiation for 5 min, denatured in 0.5 M NaOH for 35 min (changing the solution once), and transferred to a nylon membrane (Hybond-N Nylon, 0.45-µm, hybridisation transfer membrane; Amersham) in

10×SSC (1.5 M NaCl, 0.15 M tri-sodium citrate, pH 7.0) by means of Model 785 Vacuum Blotter (BioRad) operated for 2 h. The DNA was fixed to the membrane by UV irradiation for 5 min. Pre-hybridisation was performed at 68°C for 1.5 h in a hybridisation oven (Appligene, Chester-le-Street, Durham). The pre-hybridisation solution was 5× Denhardt's solution, 6×SSC and SDS 0.5% in distilled water, and contained 50 µl of salmon testes DNA (Sigma) 10 mg/ml, which had been sonicated, denatured and pre-boiled at 100°C for 3 min before use.

The subcloned DNA was digested with *EcoRI* at 37°C for 2 h. The insert DNA was separated from the TA vector arms on a TAE low melting point 0.8% gel at 20 V overnight and excised from the gel. This probe DNA was prepared with the Prime-a-Gene Labelling System (Promega), and incubated at room temperature for 1.5 h. The reaction was terminated by heating at 100°C for 2 min. The probe was purified by passing it through a 1-ml Sephadex G-50 DNA Grade (Fine) column (Pharmacia Ltd, Pharmacia LKB, Biotechnology Division, Milton Keynes) before being added to the pre-hybridisation solution. Hybridisation was performed overnight at 68°C.

The following washes were performed: three 2-min washes at room temperature with SDS 0.1%, 2×SSC solution; two 1-h washes with SDS 0.1%, 2×SSC solution at 68°C; and two 1-h washes with SDS 0.1%, 0.5×SSC solution at 68°C. The autoradiograph was developed after incubation overnight at -70°C.

DNA homology study

Comparison of the *S. sobrinus* DNA sequence with the EMBL database was performed on Seqnet with the ISIS package SWEEP,¹¹ and the alignment was achieved with the GCG program PILEUP.¹²

Results

Antibody screening and fusion protein preparation

Six antibody-positive clones were isolated and purified from the 60 000 pfu initially screened with serum from a patient with culture-positive endocarditis (CPE). All six clones produced a fusion protein of c. 185 kDa apparent mol. wt. Fusion protein production was induced in the presence of IPTG and not without it (fig. 1).

Antigen-directed antibody selection showed that the cloned, expressed sequence contained epitopes shared by *S. sobrinus*, as the eluted antibody bound to the 190-kDa antigen (fig. 1).

Southern blotting

One phage clone was chosen and the insert was subcloned by the in-vivo excision method (Clontech). Southern blotting of this plasmid subclone with five

Fig. 1. Tracks 1 and 2, fusion protein production in the absence and presence of IPTG, respectively; 3, an immunoblot of *S. sobrinus* MUCOB 263 with serum from a patient with endocarditis; 4, eluted antibody.

Fig. 2. Tracks 1–5 show *S. sobrinus* genomic DNA digested with *Pst*I, *Sac*I, *Sau*3A1, *Eco*RI and *Bam*HI, respectively. The blot was probed with the 4.7-kb insert encoded by the pBluescript SK(–) phagemid.

restriction endonuclease digests of *S. sobrinus* MUCOB 263 genomic DNA produced the patterns shown in fig. 2. This blot confirmed that the clone contained streptococcal DNA originating from *S. sobrinus* MUCOB 263.

DNA sequencing

The sequence of the phagemid clone is shown in fig. 3. This was highly homologous to the gene encoding the *S. sobrinus* SpaA antigen, with the first 275 bp from the 5' end of the gene missing. This finding agreed with the size of the fusion protein and the cross-reaction of the eluted antibody with the 190-kDa antigen of *S. sobrinus*. To obtain the 5' end of the gene, two primers were made—an *S. sobrinus* MUCOB 263 internal primer and a primer from the conserved 5' end of the *S. sobrinus* *spaA* gene. In PCR, these primers produced one band of c. 0.3 kb which was subcloned into the TA vector. After DNA sequencing, the 0.3-kb

sequence was added to that obtained from the phagemid clone.

DNA homology study

The cloned gene showed 99.2% homology to the *spaA* gene of *S. sobrinus*, 91.4% homology to the gene encoding *S. sobrinus* antigen I, 68.4% homology to the *pac* gene of *S. mutans*, 67.6% homology to the *spaP* gene of *S. mutans*, and 40.0% homology to the *S. sanguis* agglutinin receptor gene.¹³ This degree of homology implies that the protein is a member of the surface protein family first identified in studies of dental caries. Fig. 3 shows that the protein contains the

Fig. 3. See p. 335 for legend.

2210	2230	2250	2270	2290
GCCTCAGCCTATACGGGTCAAAACGAGAAGTACCTCTTATCAAGAAATTCACCTTCTACGATGAAGACGGTAATCCCATCGACTTTGATA				
A S A Y T G Q N E K D T S I F I K N E F T F Y D E D G N P I D F D				
2310	2330	2350	2370	2390
ATGCCCTCTTGTTCAGTTGCTCCCTTAACAGGGAACACAAATTCAGATGGCCAGGACTACAGCGGTACCTTCGTTAAGATTCTGGCTCATCCAT				
N A L L S V A S L N R E H N S I E M A K D Y S G T F V K I S G S S I				
2410	2430	2450	2470	2490
TGGTGAAGAAATGGCATGATCTATGCGACCGACACCTCAACTTTAAAGGGTGAAGGCGGTTCCTTCACACCATGTACACGAGCAAGTGAAGCCT				
G E K N G M I Y A T D T L N F K K G E G G S L H T M Y T R A S E P				
2510	2530	2550	2570	2590
GGTTCAGTTTGGGACTCTGCTGATGCTCCTAATCTTGGTATGGTGGTGGTGTGTCAGAATGTCCGGCCCCAACTACATCACTTTGGGGGCAACCT				
G S G W D S A D A P N S W Y G A G A V R M S G P N N Y I T L G A T				
2610	2630	2650	2670	2690
CAGCGCAATGTCTCAGCCTAGCTGAAATGCCAGGTACCTGGTAAAGATAATACTGCTGGTAAAAACCAATATCTGGTATTCCTTAATGGTAA				
S A T N V L S L A E M P Q V P G K D N T A G K K P N I W Y S L N G K				
2710	2730	2750	2770	2790
GATTCGGGACGTAATGTCCCTAAAGTGACCAAGGAAAAACCAACCCACAGTTGAGCCAAACGAGACGAGCGAGTCTATGAAGTTGAGAAGGAA				
I R A V N V P K V T K E K P T P P V E P T K P D E P V {Y E V E K E				
2810	2830	2850	2870	2890
TTGGTAGATCTGCCAGTTGAACCAAGCTACGAAAGGAACCAACCCACCAAGACTCCAGACCAAAATATCCAGACCAACAGTAGAGCTACTT				
L V D L P V E P S Y E K E P T P P S K T P D Q N I P D K P V E P T}				
2910	2930	2950	2970	2990
ATGAGTTGAAAAGGAGCTGGAACCGGCACCAAGTTGAGCCAAAGCTACGAAAGGAACCAACCCACCAAGCAAGACTCCGGATCAAGCGATTCCAGACAA				
{Y E V E K E L E P A P V E P S Y E K E P T P P S K T P D Q A I P D K				
3010	3030	3050	3070	3090
ACCGTAGAGCCAACTATGAGTTGAAAAGGAGTTGGAACCAAGTACCTGTAGAAACAACTACGAAAGGAACCAACCCCGCTCAGTCAACCCAGAC				
P V E P T} {Y E V E K E L E P V P V E T N Y E K E P T P P Q S T P D				
3110	3130	3150	3170	3190
CAAGAAGAGCCCAACCAACCGGTGGAACCAAGTACCAAGCTTGCCAAACCCAGTGGCAGCGACTTATGAAAAGGTTCTGGTCTGTGCTGTGTC				
Q E E P T K P V E P S} Y Q S L P T P P V A P T Y E K V P G P V S V				
3210	3230	3250	3270	3290
CAACGGTTCGGTACCCTACTATAAAGTACCGGTCCAACCCCGGTCCACCAAGAAATCAAAACCCAGGATGACCTGGATATTGACAAGACCCCTGGTGGC				
P T V R Y H Y Y K L A V Q P G V T K K I K N Q D D L D I D K T L V A				
3310	3330	3350	3370	3390
TAAGCAGTCGACGGTTAAGTTCAATTTGAAGACGACGCTGCCAGCGGTGCTCCAGAAACGACCTCTTGTCTTGTATGGATCTCTGCAAGCGGT				
K Q S T V K F Q L K T A D L P A G R P E T T S F V L M D P L P S G				
3410	3430	3450	3470	3490
TACCAACTTAATCTGGAAGCTACCAAGGTGCCAGCCAGCGTTTGAAGCTAGCTATGATGCCATGACCCATACGGTAACCTTCATCGCAACCGCTGAGA				
Y Q L N L E A T K V A S P G F E A S Y D A M T H T V T F I A T A E				
3510	3530	3550	3570	3590
CCTTGGCGGCTCAACAGGATCTGACCAAGCGGTGGCGACTATCTACCCACAGTTGTGGGACAAGTCTCAACAGTGGCGCTACCTACCAATAAA				
T L A A L N Q D L T K A V A T I Y P T V V G Q V L N D G A T Y T N N				
3610	3630	3650	3670	3690
CTTACCCCTGATGGTCAATGATGCTTACGCTATTAATCCAATATCGTTCGGTGGTACCACAGGGAACCTAACGACCCAGACACCAAGCAACAAC				
F T L M V N D A Y G I K S N I V R V T T P G K P N D P D N P S N N				
3710	3730	3750	3770	3790
TACATCACCCCGCACAGGTCAACAAGATGAAAACGGTGTGGTATGATGGTAAAGTCCGCTAGCTGGTACCACCACTACTATGAATGACTTGGG				
Y I T P H K V N K N E N G V V I D G K S V L A G T T N Y Y E L T W				
3810	3830	3850	3870	3890
ACCTGGACCAATACAGGGCGATAAATCGCCAGGAGACCTCCAAAAGGCTTCTTCTATGGATGACTCTCTGAAGAAGCGCTGGACTTGGCGAC				
D L D Q Y K G D K S A K E T I Q K G F F Y V D D Y P E E A L D L R T				
3910	3930	3950	3970	3990
CGACCTGATTAAAGCTGACCGATGCCAAGCGGTGCTGGTGTGAGCGTGGCTGACTACGCCAGTCTGGAGGCGGACCCAGCAGCTGTTCAAGAC				
D L I K L T D A N G K A V T G V S V A D Y A S L E A A P A A V Q D				
4010	4030	4050	4070	4090
ATGCTCAAGAGGCCAATACCCCTAAGGGAGCCTTCAAGTCTTTACCGCTGACGATCTCTCAGGCTTCTACGATGCTATGTTGGTTACCGGAACTG				
M L K K A N I T P K G A F Q V F T A D D P Q A F Y D A Y V V T G T				
4110	4130	4150	4170	4190
ACCTGACCATCGTCACTCCAATGACGGTCAAGGCTGAGATGGGCAAGATCGGTGGTATGATGAAAACAAGGCTTACCAGATTGACTTTGGTAAATGGCTA				
D L T I V T P M T V K A E M G K I G G S Y E N K A Y Q I D F G N G Y				
4210	4230	4250	4270	4290
TGAATCTAATATTGTGATTAACAATGTGCGCAATCAATCTGAAAAGGATGTGACCTTGACCATGGATCCAGCGGATAGTACCAATGTGGATGGACAG				
E S N I V I N N V P Q I N P E K D V T L T M D P A D S T N V D G Q				
4310	4330	4350	4370	4390
ACCATCGCCCTCAATCAGTCTTTAATACCGTCTCATCGGTGATCATTCCAGCGGACCATGCCAAGAGCTCTTGTAGTACAGCTTTAGCGATGACT				
T I A L N Q V F N Y R L I G G I I P A D H A E E L F E Y S F S D D				
4410	4430	4450	4470	4490
ATGACCAAACTGGAGACGATACAGGGCCAAATACAAGGCTTGGCAAGGTTGACCTGACCCCTCAAGGATGGTACAATCATCAAGCGGCTACTGACTT				
Y D Q T G D Q Y T G Q Y K A F A K V D L T L K D G T I I K A G T D L				
4510	4530	4550	4570	4590
GACTTCATATACAGAAGCGCAAGTTGATGAAGCTAATGGCCAAATTTGCTGACCTCAAGGAAGATTCTTGGCGTCTGTCTGTAGACTCGGCCTTC				
T S Y T E A Q V D E A N G Q I V V T F K E D F L R S V S V D S A F				
4610	4630	4650	4670	4690
CAAGCGGAAGTCTACCTACAGATGAAGCGGATAGCGGTGCGGACCTTTGCCAATACCTATGTCAATACCGTCAATGGAATTACCTATAGCTCTAATACGG				
Q A E V Y L Q M K R I A V G T F A N T Y V N T V N G I T Y S S N T				
4710	4730	4750	4770	4790
TAAGACCCAGCACACGAGCGAAGCAGCAAGTCCAGTGGTACCTAAGACCACTACTACGGTAGTCTTCCAGCCTCGTCAGGGTCAAGCTTATCAGCC				
V R T S T P E P K Q P S P V V P K T T T T V V F Q P R Q G Q A Y Q P				
4810	4830	4850	4870	4890
AGCGCCGACGAGGAGCTCAATGCCAGCCACAGGGGATAGTAGCAATGCTTACCTGCCACTTTTAGGCCTCGTAAGCCTGACTGTGGCTTTAGCCTG				
A P P A G A Q L P A T G D S S N A Y L P L L G L V S L T A G F S L				
4910	4930	4950	4970	4990
TTAGGACTGCGCGGAAGCAGGACTAAAGAATCCACAAGAAAAATGGGAAGTTTGGCTTTCTCATTTTATATATCCAGCTAGCTGAGTAGTCAAG				
L G L R R K Q D *				
5010	5030	5050	5070	
AAGTACTCTTAGAAAACCCCTAGAGAACATTAGCTAACTTTTCCAAACCGATAGACGTTTATTTTAGTCTAAGTATGG				

Fig. 3. *S. sobrinus* MUCOB 263 DNA sequence and the deduced amino-acid sequence. The LPXTG signal sequence for the postulated membrane anchor attachment site is indicated by (---), and the A- and P-regions are bracketed ({}).

3-tandem repeated alanine and proline regions described by Brady *et al.*,¹⁴ and the LPXTG signal sequence, postulated by Kelly *et al.*¹⁵ to be the glycoprotein membrane anchor attachment site. The wall-spanning and leader regions, described by La-Polla *et al.*¹⁶ are also present in fig. 3, but not highlighted.

Discussion

This study demonstrated that the 190-kDa immunodominant endocarditis-specific antigen Esa of *S. sobrinus* MUCOB 263 was 99.2% homologous to the SpaA antigen of *S. sobrinus*.¹⁶ This finding cast doubt on the classification of the strain previously published as *S. mutans* MUCOB 263. Re-testing of the organism by Professor D. Bratthall identified the strain as *S. sobrinus*, on the basis of glucose and mannitol fermentation and failure to ferment acetyl-glucose-amine and arbutine. This strain is now referred to as *S. sobrinus* MUCOB 263. The sequence also showed 68.4% homology to *S. mutans* antigen I/II.³ This antigen has been linked to the initial sucrose-independent attachment of *S. mutans* to tooth surfaces and is thought to function as an adhesin.¹⁷ Local passive immunisation with monoclonal antibodies against *S. mutans* antigen I/II prevented colonisation with *S. mutans* in man.¹⁸

Re-classification of the *S. sobrinus* MUCOB 263 isolate raised questions concerning the identity of the streptococcal isolate cultured from the patient with CPE whose serum was used for antibody screening of the lambda ZAPII library. Unfortunately, the original organism was no longer available for further identification. However, both *S. mutans* and *S. sobrinus* belong to the mutans group of streptococci, and are the most closely related bacteria within this group. Moreover, this paper illustrates that the Esa protein, together with the *S. sobrinus* SpaA and *S. mutans* PAC proteins, are so closely related that an antibody response raised against one organism would probably cross-react with the other. Further work is required to study cross-reactions between sera from patients with CPE with different viridans streptococci.

The findings presented in this paper are consistent with the immunodominance of the related *S. mutans* antigen I/II in endocarditis. Elevated levels of antibody against biochemically purified *S. mutans* antigen I/II has been shown by ELISA in five patients with *S. mutans* endocarditis.¹⁹ This approach has problems, in that it is labour intensive to prepare the protein and every preparation is subject to batch-to-batch variation. The present cloning strategy will tend to detect linear epitopes that could be chemically synthesised. The size of the cloned protein was too large to do this but the positive clone will be re-cloned, following restriction endonuclease digestion, to ascertain the position and sequence of the key epitopes. The antigen could also be used as an expressed fusion protein in an indirect ELISA. This has a drawback, in that antibody against the β -galactosidase part of the fusion protein will also be detected, but this approach has worked for another immunodominant antigen specific to *Enterococcus faecalis* endocarditis.²⁰ A possible link^{4, 6, 21, 22} between the *S. mutans* antigen I/II and the production of human heart-specific antibodies has now been discounted and shown to be associated with a 62-kDa membrane protein containing myosin-like epitopes.²³

The cloned protein also showed 40.0% homology to the agglutinin receptor gene from *S. sanguis*.¹³ A probe from the 3' region of the gene which encoded residues 857–1207 of *S. mutans* antigen I/II has been shown previously to hybridise with DNA from a range of α -haemolytic streptococci including *S. sanguis*, *S. intermedius*, *S. anginosus*, *S. gordonii* and *S. oralis*.¹⁷

These cross-reactivities may mean that the presence of a high level of antibody against the cloned antigen does not give a specific diagnosis of endocarditis due to either *S. mutans* or *S. sobrinus*. It may act as a wider test for endocarditis due to a range of α -haemolytic streptococci. This is of little clinical importance, as all of these bacteria are sensitive to standard therapy with benzylpenicillin and gentamicin.

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