Regions of the *Streptococcus sobrinus spaA* Gene Encoding Major Determinants of Antigen I

RAÚL MAX GOLDSCHMIDT,† MARILYN THOREN-GORDON, AND ROY CURTISS III*

Department of Biology, Washington University, St. Louis, Missouri 63130

Received 10 October 1989/Accepted 28 March 1990

Surface protein antigen A (SpaA), also called antigen B, antigen I/II, or antigen P1, is an abundant cell envelope protein that is the major antigenic determinant of *Streptococcus sobrinus* and other members of the *Streptococcus mutans* group of cariogenic bacteria. This laboratory has previously reported the cloning and expression in *Escherichia coli* of a *Bam*HI restriction fragment of *S. sobrinus* DNA containing most of the *spaA* gene (pYA726) and encoding antigen I. Regions of *spaA* encoding immunodeterminants of antigen I were analyzed by either deletion mapping or expressing selected restriction fragments from the *trc* promoter. SpaA proteins produced by mutants harboring nested deletions, constructed by BAL 31 exonuclease treatment at a unique *Sst*I site located towards the 3' end of the gene, were examined by Western immunoblot with rabbit serum against SpaA from *S. sobrinus*. Only SpaA polypeptides larger than 56 kilodaltons reacted with anti-SpaA serum. Various restriction fragments of the region of *spaA* encoding the antigenic determinants were cloned into an expression vector. The immunoreactive properties of the polypeptides encoded by those fragments indicated that expression of the immunodominant determinant required topographically assembled residues specified by noncontiguous regions located within 0.48-kilobase *Pvu*II-to-*Sst*I and 1.2-kilobase *Sst*I-to-*Hind*III fragments which were adjacent on the *spaA* map.

The *Streptococcus mutans* group of bacteria constitutes a major etiologic agent in the development of dental caries (31). Differences in DNA base composition and sequence have led to the distinguishing of seven species in the *S. mutans* group which are correlated with eight serotypes independently defined through differences in the structures of cell wall-associated polysaccharides: *Streptococcus cricetus* (serotype a), *S. rattus* (serotype b), *S. mutans* (serotypes c, e, and f), *S. ferrus* (serotype c), *S. macacae* (serotype c), *S. sobrinus* (serotypes d and g), and *S. downei* (serotype h) (4, 67; for a review, see reference 31). Though the various species forming this group are related and compete for the same ecological niche, they differ significantly in physiological properties as well as in DNA composition (31).

The SpaA protein (34), also known as antigen I/II (57), antigen B (59), or antigen P1 (25), is an abundant cell surface protein of *S. mutans* which has been defined through its antigenic properties. SpaA is highly immunogenic in rabbits, mice, and monkeys (46, 56). Found among serotypes a, c, d, e, f, g, and h of *S. mutans* but not in b (34, 51, 57, 60), its widespread occurrence suggests that SpaA may serve an important function for bacteria in the *S. mutans* group. However, the function of SpaA is still not known. Inhibition of *S. mutans* adherence to saliva-coated hydroxylapatite by antisera against SpaA (23) and genetic evidence (17) suggest that SpaA may be a virulence determinant involved in one of the steps leading to the adherence of *S. mutans* to tooth surfaces.

From a practical perspective, the high immunogenicity of SpaA and its ubiquity among the *S. mutans* group of bacteria make this protein an attractive candidate for the development of vaccines against *S. mutans*-induced dental caries (19, 20, 46). SpaA can induce protective immunity against dental caries in monkeys (46).

Biochemical characterization by proteolytic digestion of

the antigenic determinants in SpaA protein of serotype c of *S. mutans* has defined two antigens, I and II, within polypeptides of 150 and 69 kilodaltons (kDa) (56–58). However, technical difficulties resulting from the extensive in vivo and in vitro degradation suffered by SpaA and other *S. mutans* surface proteins (59) have prevented any further characterization of the two antigens into smaller polypeptides. Recombinant DNA technology has offered an alternative approach to the characterization of SpaA. An 8.3-kilobase (kb) DNA fragment from *S. sobrinus* 6715, encoding a protein that cross-reacted with antiserum prepared against purified SpaA protein, was cloned into a plasmid vector and introduced into *Escherichia coli* K-12 (34). This recombinant plasmid was called pYA726. In *E. coli*, the cloned *spaA* gene is expressed from its own promoter (34). The SpaA protein synthesized by recombinant *E. coli* is smaller than the 200-kDa protein synthesized by *S. sobrinus* and contains the determinants of the stronger antigen (antigen I) but lacks the determinants of antigen II (34). Antiserum prepared against the SpaA protein specified by *E. coli* shows cross-reactivity with SpaA from *S. sobrinus* and is able to recognize cell surface proteins on cells from various *S. mutans* serotypes (c, d, e, f, g, and a, but not b) (34).

The present work characterizes regions of *spaA* encoding the immunodeterminants of antigen I and, in particular, those determining the immunodominant determinant(s). It shows that two distinct and noncontiguous fragments of the *spaA* gene are jointly required to express the immunodominant determinant(s) of antigen I. Elsewhere (29) we show that these regions of the *spaA* from *S. sobrinus* also determine the polypeptide domains responsible for the major cross-reactivity with the SpaA proteins from other serotypes.

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids are shown in Table 1. Plasmids constructed during this work are described

* Corresponding author.

† Present address: 7539 Oxford Dr., St. Louis, MO 63105.

TABLE 1. Strains or *E. coli* and plasmids

Strain or plasmid	Genotype	Source or reference
HB101	F ⁻ <i>ara-14 leuB6 proA2 lacY1 glnV44 galK2 recA13 rpsL20 xyl-5 mtl-1 thi-1 hsdS20</i>	Boyer and Rouland-Dussoix (9)
χ925	F ⁻ <i>thr-1 ara-13 leuB6 azi-8 fhuA lacY1 minA1 glnV44 gal-6 λ⁻ minB2 rpsL135 malA1 xyl-7 mtl-2 thi-1</i>	Frazer and Curtiss (26)
χ2813	F ⁻ <i>lacY1 glnV44 galK2 galT22 recA56 ΔthyA57 metB1 hsdR2</i>	Jacobs et al. (38)
CC118	F ⁻ <i>araD139 Δ(ara-leu)7697 ΔlacX74 ΔphoA20 galE galK recA1 rpsE argE(Am) rpoB thi</i>	Manoil and Beckwith (49)
χ6060	CC118[F ⁺ (<i>traD36 proA⁺ proB⁺ lacI^a ΔlacZM15</i>);::Tn5]	R. Goldschmidt, this work
pACYC184	<i>cat tet</i>	Chang and Cohen (13)
pYA770 ^a	<i>cat spaA</i> (see Fig. 1)	This work
pKK233-2	<i>bla</i> (see Fig. 6)	Amann and Brosius (1)

^a Described within the text.

below. Strain stocks were prepared in 1% peptone–5% glycerol and stored at –70°C; working stocks in 1% peptone–50% glycerol were kept at –20°C.

Antisera. Rabbit (New Zealand White) antiserum prepared against SpaA protein from *S. sobrinus* purified by antibody affinity chromatography was a gift from John Barrett. SpaA protein used as an antigen was purified in the absence of detergents by a modification from a previously described method (34) and injected into rabbits without the addition of adjuvants. Two batches of antisera, obtained from two separate rabbits, were preadsorbed with *E. coli* K-12 strain χ2813 containing plasmid vector pACYC184, first against whole cells and then against Sepharose 4B-coupled cell extracts. When used for assaying SpaA polypeptides produced in *E. coli* backgrounds other than χ2813 (Table 1), dilutions of those sera were further purified by additional preadsorption against the new strain with the appropriate vector, first against whole cells and next against nitrocellulose filter-bound cell extracts. All serum preparations derived from one of the rabbits were used at final dilutions of 1:1,000; those derived from the second rabbit were used at 1:500. All assays described in this work were performed with antisera from both rabbits. Though differing in titer, both antisera were indistinguishable with regard to specificities, as assayed by Western immunoblots and immunodot blots. For consistency purposes, the assays described in the various figure legends were all performed with antiserum from the same rabbit and at 1:1,000 dilutions. An alkaline phosphatase conjugate of goat anti-rabbit immunoglobulin G (Sigma Chemical Co.) was used as a secondary antibody in Western and colony immunoblots at a 1:2,000 dilution.

Growth conditions. Complex media for routine cultivation of bacteria were L broth (47) and Penassay base agar (antibiotic medium 2; Difco Laboratories) containing, per ml, 40 μg of thymidine, 25 μg of ampicillin (Sigma), 12.5 μg of chloramphenicol (Sigma), and 12.5 μg of tetracycline (Sigma), as necessary. Rich synthetic medium based on morpholinopropanesulfonate (MOPS)-buffered medium (52) was used for radioactive labeling of bacterial proteins, omitting the labeling amino acid from the supplements.

Enzymes. Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, and the large fragment (Klenow) of *E. coli* DNA polymerase I were purchased from Bethesda Research Laboratories, Inc., International Biotechnologies, Inc., New England Biolabs, Inc., and Promega Biotec, whereas BAL 31 F nuclease was obtained from International Biotechnologies. Unless specified otherwise, reactions were carried out in buffers recom-

mended by the suppliers and under conditions described by Maniatis et al. (48).

Ligation of two or more blunt-ended DNA fragments was carried out at 23°C for 14 h, in the presence of 1 mM hexamine cobalt chloride (55), to enhance intermolecular ligation events, followed by a further 6 h of incubation in the presence of 40 mM KCl and additional ligase, to allow plasmid circularization.

Plasmid analysis, restriction mapping, gel electrophoresis, and transformation. Recombinant clones were screened for plasmid DNA by a modification (62) of the Birnboim minisatellite technique (7), starting from 1.5- or 5-ml cultures. These DNA preparations were used for transformation, restriction endonuclease mapping, and gel electrophoresis. *Hind*III digests of bacteriophage λ and *Hae*III digests of φX174 DNAs served as molecular weight markers (Bethesda Research Laboratories). Plasmids were introduced by transformation into the various backgrounds, as required. *E. coli* cells were transformed by the method of Dagert and Ehrlich (21), and the transformation mix was plated on appropriate selective plates, following 40 to 60 min of incubation to allow phenotypic expression of drug resistance traits.

Plasmid and restriction fragment purification. Plasmid purification from 25-ml to 1-liter bacterial cultures was done by the preparative method of Birnboim (7), modified in its last two steps. After the two ethanol precipitations that followed treatment with 5 M LiCl–0.05 M MOPS (pH 8.0), the pellet containing plasmid DNA was suspended in 10 mM Tris (pH 7.8)–5 mM EDTA–0.5% sodium dodecyl sulfate (SDS) and treated with 200 μg of proteinase K per ml for 2 to 3 h at 65°C. Following a phenol-chloroform extraction, two chloroform extractions, precipitation with isopropanol, and two washes with 70% ethanol, the pellets were suspended in 10 mM Tris (pH 8)–1 mM EDTA (TE buffer). Generally, this plasmid preparation was used to isolate and purify particular restriction fragments. Whenever necessary, plasmids were further purified by CsCl-ethidium bromide buoyant density gradient centrifugation. Restriction fragments generated by cleavage of a plasmid with a particular restriction enzyme were separated by electrophoresis in SeaKem GTG or NuSieve GTG agarose (FMC Corp.) gels containing 0.5 mg of ethidium bromide per ml. Restriction fragment bands of interest were transferred electrophoretically onto strips of DE 81 DEAE cellulose paper (Whatman, Inc.) by the procedure of Dretzen et al. (24). The paper was macerated, and the DNA was eluted in high-salt buffer.

Construction of plasmids. pYA770 was derived from pYA726 (34) via two steps. The 4.8-kb *Hind*III fragment of

S. sobrinus DNA in pYA726 was first inserted into the *Hind*III site of pACYC184 (13), yielding pYA759. This subcloning removed 0.24 kb from the 3' end of the *spaA* gene in pYA726 (besides 3.2 kb of unessential *S. sobrinus* DNA distal to the 5' end of the gene): the *spaA* genes in pYA759 and pYA726 were transcribed from their own promoters and had similar orientations relative to the split *tet* gene of the vector. In a second step, the 276 nucleotides between the *Bam*HI and *Sal*I sites of the *tet* gene of the vector were removed from pYA759 by successive digestions with the two restriction endonucleases, and the free ends of the largest fragment were ligated together, after filling in protruding 5' ends by treatment with the Klenow fragment of DNA polymerase. Deletion of the two *Kpn*I sites of pYA759, separated by 272 base pairs, allowed the construction of plasmids pYA761 and pYA762, differing in the reading frame of the region encoding the C terminus of their SpaA proteins. pYA761 was constructed by ligating pYA759 cleaved with *Kpn*I restriction endonuclease, after making the 3' protruding ends blunt ended with T4 DNA polymerase. pYA762 was constructed by ligating pYA759 cleaved with *Kpn*I. pYA101 to pYA144 are derivatives of pYA770 containing a series of nested deletions of the *spaA* gene, generated by nuclease BAL 31 digestion of plasmid DNA cleaved at the *Sst*I site, and bridging the gap ends with a *Bam*HI linker (see below).

pYA99 is an expression vector, derived from pKK233-2 (Pharmacia LKB; 1) digested with *Pst*I, treated with T4 DNA polymerase to make the protruding 3' ends blunt ended, and followed by ligation in the presence of a 2,000-fold molar excess of an octamer *Sst*I linker (New England BioLabs). pYA170 was constructed by inserting into expression vector pYA99 the 1.2-kb *Sst*I-to-*Hind*III fragment of pYA770, maintaining the reading frame of the *spaA* gene. pYA171 contains the 2.8-kb *Hinc*II *spaA* fragment of pYA770 inserted into the *Hinc*II site of the multiple cloning site of pUC18 (68), oriented against the transcription direction from the *tac* promoter of the vector. pYA175 contains the 0.85-kb *Pvu*II fragment of pYA171 (including the 0.64-kb *Pvu*II-to-*Hinc*II *spaA* fragment) inserted at the *Sma*I site of the multiple cloning site of pUC18 and oriented in parallel to the transcription direction imposed by *Plac*. This construction results in (i) destruction of the *Pvu*II and *Sma*I sites and (ii) positioning of *Kpn*I and *Sst*I sites immediately next to the destroyed *Pvu*II site. The 486-nucleotide *Sst*I fragment of this plasmid contains the *Pvu*II-to-*Sst*I fragment of pYA770 in the correct reading frame to provide expression when inserted into the *Sst*I site of either pYA99 or pYA170. The reading frame of the fragment is such that multimers of it also provide the correct reading frame. Insertions of this 486-nucleotide fragment into pYA99 and pYA170 yielded plasmids pYA176 and pYA177, respectively, whereas pYA178, pYA179, pYA180, and pYA181 contain dimeric, trimeric, tetrameric, and pentameric forms of the *Sst*I fragment inserted into the *Sst*I site of pYA170. pYA190 contains the dimeric form of the *Sst*I fragment inserted into pYA99.

pYA182 resulted from ligating the 0.64-kb *Kpn*I fragment of pYA175 with the 4.9-kb *Kpn*I fragment of pYA190, conserving expression of the *spaA* segment from the *trc* promoter. pYA184 was derived from pYA182 by digestion with both *Sma*I and *Hind*III and ligation after filling in the 5' overhanging end with the Klenow fragment of DNA polymerase I. This construct expressed the *spaA Pvu*II-to-*Hinc*II region of pYA770 from *Ptc* in pYA99.

Generation of deletions through nuclease BAL 31 digestion. Plasmid pYA770 cleaved with *Sst*I and extracted with phenol-chloroform was suspended at a concentration of 20 ng/ml

in 300 μ l of BAL 31 buffer (20 mM Tris, pH 8.0, 600 mM NaCl, 12.5 mM $MgCl_2$, 12.5 mM $CaCl_2$). After 1 min of preincubation at 30°C, 2.5 U of nuclease BAL 31 "fast" form (International Biotechnologies) was added to the reaction mixture, and 30- μ l samples were withdrawn into 15 μ l of stop buffer [125 mM EDTA, 50 mM ethylene bis(oxyethylenitrilo)tetraacetic acid (EGTA)] at various times thereafter (1, 2, 4, 6, 8, 10, 12, 14, 20, and 30 min), as determined from a previous test reaction. DNA pooled from the samples and extracted with phenol-chloroform was treated with Klenow enzyme, and the blunted ends were ligated to previously phosphorylated decameric *Bam*HI linkers (New England BioLabs) whose purpose was to serve as a structural marker when characterizing the deletions. After elimination of free linkers and polylinkers by passage through a Sephacryl 200 column (Pharmacia), the DNA was digested with excess *Bam*HI enzyme and, following repeated ethanol precipitations, circularized by ligation under dilute conditions. DNA was digested once more with *Sst*I to eliminate the background of plasmids that might have escaped the initial cleavage and then was transformed into χ 2813. Transformants were screened for the size of their plasmid DNA. Plasmids smaller than pYA770 were cleaved with *Hind*III, alone and in conjunction with *Bam*HI, to determine the extent of the deletions by the sizes of the generated fragments.

Labeling. Exponentially growing cells were grown at 37°C to a density of 4×10^8 to 5×10^8 cells per ml in Neidhardt medium (52) lacking methionine. To pulse, 1 ml of cells was transferred to a Wasserman tube containing 10 μ Ci of [35 S]methionine per μ l (1,066 mCi/mmol; Amersham Corp.) and incubated at 37°C for 1 to 3 min on a vertical rotator. The pulse was stopped by adding a 400-fold molar excess of methionine. Immediately thereafter, two samples were placed in Eppendorf tubes containing 0.5 g of crushed ice kept at -20°C: (i) 400 μ l for immunoprecipitation and (ii) 100 μ l for total protein analysis. The remaining labeled cells were grown for one generation, and then the sampling was repeated. All samples were sedimented for 5 min at 4°C in an Eppendorf centrifuge and washed first in 10 mM Tris (pH 8.0)-1 mM methionine and then in 10 mM Tris, pH 8.0. Total protein samples were suspended in 50 μ l of dissociation buffer (62.5 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 5% β -mercaptoethanol), heated at 100°C for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis on slab gels (43), loading equal numbers of disintegrations per minute on each well. A 14 C-methylated protein mixture (Amersham) was used for molecular mass standards.

Immunoprecipitation. Samples were processed as described by Oliver and Beckwith (54), except that Pansorbin (Calbiochem-Behring) was used as a staphylococcal protein A-antibody adsorbent. Samples of equal volume were subjected to SDS-polyacrylamide gel electrophoresis.

Fluorography. Gels containing labeled proteins were processed by the fluorographic system described by Jen and Thach (39), dried at 80°C under vacuum, and exposed to prefogged (44) X-Omat AR X-ray film (Eastman Kodak Co.).

Minicells. Preparation of minicells from derivatives of χ 925 containing various plasmids and their labeling with [35 S]Met have been described previously (16), except that cells were grown and labeled in Neidhardt medium (52).

Western blot analysis. Cells lysed in dissociation buffer were subjected to SDS-polyacrylamide electrophoresis (43), loading approximately 2 μ g of protein per well, as determined by the bicinchoninic acid protein assay (63) on parallel cell samples lysed in boiling 1% SDS. A mixture of proteins

FIG. 1. Map for restriction sites and antigenic determinant-encoding regions of the *spaA* gene in pYA770. The *Hind*III fragment of *S. sobrinus* DNA is 4.7 kb. The rationale for boundary assignments is explained in the text. The immunodominant determinant is encoded by noncontiguous domains within the region represented by the densely striped area (see text). The heavy arrow indicates the direction of transcription and protein synthesis of *spaA*. The sequencing strategy for the DNA region encoding the immunodominant determinant is shown, with numbers referring to the distance in nucleotides from the promoter-distal *Hind*III site. Arrows indicate start and extent of DNA-primed synthesis in the sequence analysis. Symbols: ■, major *SpaA* antigenic determinant; ▨, minor *SpaA* antigenic determinant(s).

(Bio-Rad Laboratories) was used as molecular mass standards. The separated proteins were transferred onto a nitrocellulose sheet (Schleicher & Schuell, Inc.) in a Transphor electrophoresis unit (Hoefer Scientific Instruments) at 4°C in either 25 mM Tris–192 mM glycine–20% methanol (pH 8.3) for 12 h at 180 mA (66) or 20 mM phosphate buffer (pH 6.8) for 2 h at 1 A. The nitrocellulose sheet was trimmed, and the section containing the molecular mass standards was removed for later analysis. After a brief wash (100 mM Tris, pH 8.0, 0.05% sodium azide, 0.04% polyoxyethylene sorbitan mono-oleate [Tween 80; Sigma]), the nitrocellulose sheet was blocked for at least 1 h in blocking buffer (100 mM Tris, pH 8.0, 0.05% sodium azide, 0.25% gelatin [Bio-Rad], 0.25% bovine serum albumin [fraction V; Sigma], 0.04% Tween 80). After removal of buffer, fresh blocking buffer containing a 1:1,000 dilution of preadsorbed rabbit antiserum against purified *S. sobrinus* SpaA was added, and the sheet was incubated for 12 h. Following five 10-min washes, the nitrocellulose sheet was incubated for 2 h or more with blocking buffer containing a 1:3,000 dilution of an alkaline phosphatase conjugate to goat anti-rabbit immunoglobulin G (Sigma). The cycle of five washes was repeated, including 0.5% SDS in the first three (40), followed by a brief wash and exposure to 5-bromo-4-chloroindoxyl phosphate and Nitro Blue Tetrazolium (8). Optical densitometry tracings (Joyce-Loebel) of the bands developed on the nitrocellulose paper were used for quantitative comparisons. Molecular mass standards were visualized by washing the corresponding slice of nitrocellulose four times at 37°C in large volumes of 10 mM phosphate buffer (pH 7.2)–150 mM NaCl–0.3% Tween 20 and staining at room temperature in the same buffer with 1 µl of India ink per ml.

Colony immunoblot. Clones expressing *SpaA* antigenic determinants were often identified by direct immunological

screening (32) with the same buffers, antisera, and histochemical reagents as those described for Western blots.

DNA sequence analysis. The DNA sequence was determined by the dideoxynucleotide chain termination procedure (61) with an M13 cloning DNA sequencing system (New England BioLabs). As templates, particular DNA fragments obtained by single or double digestion with restriction enzymes were cloned into either M13mp18 or M13mp19 (68). Heptadecamers overlapping the M13 sequences next to the polylinker region were used as primers.

RESULTS

Mapping of antigenic determinants. In *E. coli*, the *spaA* genes of pYA726 (34) and pYA770 are transcribed from their own promoters (Goldschmidt, unpublished results). Translation of the genes starts at a site located approximately 0.1 kb leftwards from the *Hinc*II site located 0.9 kb from the *Hind*III boundary of the *S. sobrinus* insert in pYA770 (Fig. 1) and results in polypeptides having apparent molecular masses of 157 (pYA726) and 148 kDa (pYA770), as determined by SDS-polyacrylamide gel electrophoresis of the *in vivo* pulse-radiolabeled polypeptides (Goldschmidt, unpublished results). In preliminary experiments, a number of deletions and frameshift mutations were constructed at the 3' end of the gene by using various available restriction sites which were promoter distal to the *Sst*I site (Fig. 1). They resulted in the production of shorter *SpaA* polypeptides, whose immunoreactive properties were examined by either Western immunoblots or immunoprecipitation. Mutations which interrupted protein synthesis at restriction sites promoter distal to the first *Kpn*I site resulted in *SpaA* polypeptides that were immunoprecipitated (Fig. 2). However, mutations which interrupted protein synthesis at restriction

FIG. 2. Immunoprecipitation of SpA polypeptides produced by HB101 derivatives containing pYA770, pYA761, or pYA762. Exponentially growing cells were pulsed for 1 min with [35 S]methionine and chased with an 800-fold molar excess of cold methionine for one generation. P, Samples taken at the end of the pulse; C, samples taken at the end of the chase. The molecular masses of the SpA polypeptides produced by pYA770, pYA761, and pYA762 were determined to be 148, 138, and 119 kDa, respectively.

sites promoter proximal to the first *KpnI* site resulted in SpA polypeptides that were not immunoprecipitated (data not shown). Immunoprecipitation failed to detect any undergraded SpA polypeptides of expected molecular masses smaller than 110 kDa. In contrast, all of these initially examined mutants produced SpA polypeptides that were reactive in Western immunoblots (data not shown).

To map *spaA* regions specifying antigenic determinants located promoter proximal to the *SstI* site, we examined by Western blot the SpA polypeptides made by *E. coli* strains containing a series of nested deletions of the *spaA* gene generated by BAL 31 exonuclease. pYA770, a derivative of pYA726 with no *BamHI* sites, was used for this analysis. Though slightly different in size, the SpA polypeptides encoded by pYA770 and pYA726 showed almost indistinguishable immunoreactivities both by immunoprecipitation of in vivo pulse-radiolabeled polypeptides and by Western immunoblot analysis (Goldschmidt, unpublished results).

Of 420 analyzed BAL 31-deleted clones, 44 that contained plasmids of significantly different sized were selected for further study. Figure 3 shows the sizes of the BAL 31-generated deletions for a set of these plasmids as well as the predicted sizes of the corresponding encoded SpA polypeptides. The predicted molecular masses of the corresponding encoded SpA polypeptides were between 3.7 and 93 kDa.

Production of polypeptides encoded by the *spaA* genes carried in several of the previously described plasmids was also examined in [35 S]methionine-labeled minicells (Fig. 4; Fig. 3, "Molecular mass" column). Polypeptides encoded by the various deletions of *spaA* were identified as bands absent from minicells containing the vector without any insert. Minicells isolated from χ 925 derivatives containing plasmids pYA104, pYA108, pYA129, pYA131, pYA133, and pYA141 all produced polypeptides whose molecular weights were consistent with the sizes of the corresponding *spaA* gene fragments (Fig. 3 and 4). pYA134 produced a polypeptide of 77 kDa instead of the predicted 62.9 kDa (Fig. 3 and 4). DNA sequence analysis of the two regions of *spaA* DNA from pYA134 that were separated by the *BamHI* linker, used to close the gap generated by BAL 31 treatment, showed the formation of a fused protein. The nucleotide sequence indicated that the open reading frame of the promoter-proximal region of *spaA* extended for approximately 0.25 kb after the *BamHI* linker, even though the reading frame of this region had been altered (data not shown). Thus, only the first 63 kDa of the protein produced by pYA134 contains SpA-like amino acid sequences. DNA sequence analyses established that for pYA104, pYA108, pYA131, pYA137, and pYA141, the respective BAL 31-generated deletions had resulted in reading frame shifts that introduced one or more termination codons within the following 50 nucleotides (data not shown). The same is likely to have occurred also for pYA129 and pYA133, given the good agreement found between the predicted and observed molecular masses of the encoded SpA polypeptides (Fig. 3). In addition, the immunoreactive properties of the SpA polypeptides encoded by the plasmids described in Fig. 3 were not altered when the respective *spaA* regions promoter proximal to the *BamHI* linker were subcloned, in both orientations, into different sites of a cloning vector.

All SpA polypeptides whose predicted molecular masses were equal to or greater than 62.9 kDa showed reactivity with rabbit anti-SpA serum in Western blot analysis (Fig. 3 and 5). The observed failure of the protein products from the *spaA* genes of plasmids pYA104, pYA108, pYA111, pYA120, pYA123, and pYA141 to show reactivity with rabbit anti-SpA serum is not correlated with a defect in synthesis nor with an intrinsic insolubility or higher instability of the corresponding polypeptides, since they could be detected in minicells (Fig. 4). Thus, the first 56-kDa region of SpA either does not contain significant antigenic determinants or does not fold correctly to display them in the absence of carboxy-terminal regions.

Optical densitometric analyses of the bands developed in multiple Western immunoblots measuring the immunoreactivities of SpA polypeptides produced by recombinant clones indicated that, generally, immunoreactivity increased with predicted size for SpA polypeptides between 63 and 94 kDa (data not shown). This suggests that antigenic determinants are distributed throughout this protein region, rather than concentrated at a particular site. But, it should be noted that the immunoreactivities of all of these SpA polypeptides were significantly lower than those of the SpA polypeptides encoded by pYA770 and pYA726 (Fig. 5 and data not shown). Thus, the *spaA* region promoter proximal to the *SstI* site of pYA770 did not appear to specify immunodominant determinants.

It is apparent from Fig. 5 that extensive in vivo proteolytic degradation is suffered by most SpA polypeptides and that many of the smaller degradation products could be detected in Western immunoblots. Degradation products of SpA

FIG. 3. Properties of SpaA polypeptides produced by mutants containing BAL 31-generated deletions of *spaA*. Restriction maps of the various *spaA* genes show the extents of the deletions (▨). Predicted molecular masses were calculated from distances between protein initiation sites and the *Bam*HI sites of the linkers introduced to join the ends of the deletions, on the basis of experimentally determined restriction fragment sizes. The molecular masses of the encoded polypeptides (-, not done) were determined by analysis of the polypeptides produced by [³⁵S]methionine-labeled minicells (see Fig. 4). Reactivity with anti-SpaA serum (+, detected; -, not detected) was determined from Western blot analyses (see Fig. 5).

could not usually be detected by immunoprecipitation of in vivo pulse-radiolabeled polypeptides. Indeed, as mentioned earlier, immunoprecipitation of pulse-radiolabeled polypeptides even failed to detect any undegraded SpaA polypeptides of expected molecular masses smaller than 110 kDa. Thus, the in vivo pulse-radiolabeled SpaA polypeptides produced by plasmids containing BAL 31-generated deletions of *spaA* also failed to be immunoprecipitated (data not shown). Given that immunoprecipitation (of in vivo pulse-radiolabeled proteins) and Western immunoblot analysis offer roughly similar detection sensitivities for SpaA polypeptides, the apparent discrepancy between the results probably reflects differences in configuration of the same protein molecules under the different conditions of the two assays.

Expression of the immunodominant determinant(s). The previously described results suggested, by exclusion, that immunodominant determinants were probably encoded within the *spaA* region promoter distal to the *Sst*I site of pYA770. To test this hypothesis, we examined the immunoreactivities of SpaA polypeptides synthesized by expressing DNA restriction fragments of that *spaA* region and adjacent ones. Expression vector pYA99, which uses the *trc* promoter (1), was constructed to test the presence of encoded antigenic sites within the promoter-distal region to the *Sst*I

site of *spaA* and adjacent regions (Fig. 6). Thus, the following fragments of pYA770 (Fig. 1 and 6B) were introduced into pYA99: the 1.2-kb *Sst*I-to-*Hind*III, 0.48-kb *Pvu*II-to-*Sst*I, 1.7-kb *Pvu*II-to-*Hind*III, and 0.65-kb *Pvu*II-to-*Hinc*II fragments.

Plasmid pYA99 allowed the insertion of the 1.2-kb *Sst*I-to-*Hind*III fragment of pYA770 while maintaining the reading frame of the *spaA* gene (pYA170 in Fig. 6). To insert the 0.48-kb *Pvu*II-to-*Sst*I fragment of pYA770 into the *Sst*I sites of pYA99 and pYA170, it was first converted into a 486-nucleotide *Sst*I fragment. The CAGCTG sequence of the *Pvu*II site was substituted by the hexadecanucleotide GAGCTCGGTACCCCTG, designated *Pvu*II*. As a result, the Ala at the amino terminus of the encoded polypeptide was replaced by the pentapeptide Glu-Leu-Gly-Thr-Pro. When inserted in the proper orientation at the *Sst*I sites of either pYA99 or pYA170, the 486-nucleotide *Sst*I fragment was in the correct reading frame to provide expression of the encoded SpaA polypeptide. Because the length of the fragment in nucleotides was divisible by three, multimers in tandem of the fragment also provided the correct reading frame (see below). Insertion of the 486-nucleotide *Sst*I fragment into pYA99 yielded plasmids pYA176 (Fig. 6) and pYA190 (data not shown), containing monomeric and dimeric forms of the insert, respectively. Insertion of the

FIG. 4. Proteins synthesized by minicells from χ 925 derivatives containing the indicated plasmids. Minicells incubated in Neidhardt medium were pulsed for 10 min with 10 μ Ci of [35 S]methionine and allowed to incubate for 30 more min in the presence of a 300-fold molar excess of cold methionine. A similar number of counts per minute was laid into each well. MW, Molecular weight (10^3).

486-nucleotide fragment into pYA170 yielded plasmids pYA177, pYA178, pYA179, pYA180, and pYA181, which contained monomeric, dimeric, trimeric, tetrameric, and pentameric forms of the *Sst*I fragment, respectively. In χ 6060, biosynthesis of the SpaA polypeptides encoded by all these plasmids was inducible by isopropyl- β -D-thiogalactoside (data not shown). Thus, as expected, the *spaA* fragments were all expressed from the *P_{trc}* of the vector.

Comparisons among band intensities in Western immunoblot assays measuring the reactivities of anti-*S. sobrinus* SpaA serum with SpaA polypeptides encoded by these various plasmids suggested that synergistic contributions from polypeptide segments specified by noncontiguous regions of the *spaA* gene were required to generate the immunodominant determinant(s). When expressed from *P_{trc}*, the immunoreactivity of the SpaA polypeptide produced by the 1.68-kb *Pvu*II-to-*Hind*III fragment was very much stronger (Fig. 7, lane 6) than the combined contributions of the weak immunoreactivities of the polypeptides produced by the individual 0.48-kb *Pvu*II-to-*Sst*I and 1.2-kb *Sst*I-to-*Hind*III fragments (Fig. 7, lanes 2 and 5).

Furthermore, the immunoreactivities of polypeptides encoded by constructs containing two copies in tandem of the *Pvu*II-to-*Sst*I fragment (Fig. 7, lanes 3 and 7) were stronger than those encoded by the corresponding constructs with one copy of the fragment (Fig. 7, lanes 2 and 6), indicating that the *Pvu*II-to-*Sst*I fragment-encoded polypeptide (or parts thereof) appears to be directly involved in the major

FIG. 5. Immunoreactivity of polypeptides encoded by derivatives of pYA770 having BAL 31-generated deletions within *spaA*. All plasmids were in the χ 1813 background. Cells in early stationary phase were harvested and lysed, and their proteins were resolved by SDS-polyacrylamide gel electrophoresis. Approximately 0.4 μ g of cell extract protein was used for the strain containing pYA770, and 4 μ g was used for all others. Reactivity to rabbit antiserum prepared against purified SpaA from *S. sobrinus* was tested by Western blot assays.

antigenic determinant and not simple serving as a structural scaffolding for the latter. Thus, the *Pvu*II-to-*Sst*I fragment appears to be necessary, though not sufficient, to code for strong antigenic determinants.

Essentially similar results were obtained when the reactivities of anti-*S. sobrinus* SpaA serum with the SpaA polypeptides encoded by all of these plasmid constructs were compared under the nondenaturing conditions of immunodot blots. However, there was one important difference. In immunodot blots but not in Western blots, the immunoreactivities of SpaA polypeptides encoded by plasmid constructs containing more than two copies in tandem of the *Pvu*II-to-*Sst*I fragment increased in a strictly monotonic relationship to the number of fragment copies (data not shown).

The immunoreactivity of SpaA polypeptides encoded by constructs expressing the *Pvu*II-to-*Hind*III fragment (Fig. 7, lanes 6 and 7) was comparable to that shown by the SpaA polypeptide produced by pYA770 (Fig. 7, lane 8). This indicated that the immunodominant determinant(s) of the SpaA protein produced by pYA770 was encoded within the *Pvu*II-to-*Hind*III region. Thus, there were two obvious explanations for the failure of the *Pvu*II-to-*Sst*I and *Sst*I-to-*Hind*III regions to code for strong antigenic determinants. The *spaA* region encoding the immunodominant determinant(s) could be split by the *Sst*I site. Alternatively, the region could consist of noncontiguous DNA segments lo-

FIG. 6. *P_{trc}*-dependent expression of fragments from the 3' region of the pYA770 *spaA*. (A) Derivation of expression vector pYA99 and derivatives containing *spaA* fragments. Vector pKK233-2 (1) can express recombinant DNA inserted at the *Pst*I and *Hind*III sites when the reading frames of vector and insert are in phase. pYA99 was tailored to maintain the reading frame of *spaA* at the *Sst*I site by inserting an *Sst*I octameric linker to replace the *Pst*I site of the original vector. The vector was a pBR322 derivative. *P_{trc}*, Expression promoter; T1T2, *rrnBT1T2* (transcription termination signal for rRNA synthesis in *E. coli*); bp, base pairs; T4 DNA pol. exo., exonuclease activity of T4 DNA polymerase; fragm., fragment. The enlarged fragments show for the various plasmids the regions in which protein synthesis is initiated when driven from *P_{trc}*. (B) *spaA* fragments expressed from *P_{trc}*. *Pvu*II*, *Pvu*II site altered to provide an *Sst*I site in the correct reading frame to allow expression from pYA99 (see text). Inserts for pYA176 and pYA184 were inserted into the *Sst*I site of pYA99; those for pYA177 and pYA178 were inserted into the *Sst*I site of pYA170.

cated on different sides of the *Sst*I site. To distinguish between these possibilities, we examined the immunoreactivity of a SpaA polypeptide synthesized by expressing the 0.66-kb *Pvu*II-to-*Hinc*II region of *spaA*, which includes the *Pvu*II-to-*Sst*I fragment and 0.17 kb of the adjacent region (pYA184 in Fig. 6B).

The low reactivity of the SpaA polypeptide encoded by pYA184 (Fig. 7, lane 4) excludes the possibility that the *Sst*I site splits a region encoding strong antigenic determinants in either pYA170 or pYA176. Furthermore, expression of the *Pvu*II-to-*Eco*RI region from the *trc* promoter resulted in a polypeptide whose immunoreactive properties were also similar to those of the polypeptide expressed by pYA184 (unpublished results). Thus, the immunoreactivity of the immunodominant determinant of the antigen I component of the SpaA protein appears to result from strongly synergistic contributions by noncontiguous polypeptide regions that are encoded within the *Pvu*II-to-*Sst*I and *Sst*I-to-*Hind*III fragments of *spaA*. In other words, expression of the immunodominant determinant seems to require the topographical assembly of amino acid residues distantly located on the SpaA polypeptide chain.

Since denaturing conditions had been used to separate proteins during electrophoresis, it was somewhat surprising that the configurational nature of the immunodominant determinant could still be detected by Western immunoblots. It follows that the structure of the immunodominant determinant of antigen I is either stable under the employed denaturing conditions or renatured upon transfer of the proteins onto nitrocellulose paper.

To exclude *in vivo* proteolytic degradation as the major cause for the poor immunoreactivity shown by SpaA polypeptides produced by pYA170, pYA176, pYA184, and pYA190, the stability of these polypeptides was tested in pulse-chase experiments. Immunoprecipitation of the SpaA polypeptide encoded by pYA177 synthesized during a 1-min pulse, with and without a subsequent 1-h chase, revealed two polypeptides of 84 and 82.5 kDa (Fig. 8, lanes 9 and 10). No SpaA polypeptides encoded by pYA170, pYA176, pYA184, and pYA190 were immunoprecipitated under similar conditions (Fig. 8, lanes 1 through 8). Furthermore, all of these SpaA polypeptides were produced in approximately similar molar amounts in pulse-labeled minicells, though they varied somewhat in stability (data not shown). How-

FIG. 7. Immunoreactive properties of polypeptides encoded by various DNA fragments from the 3' end region of the pYA770 *spaA*. Cells from derivatives of CC118 containing the indicated plasmids were grown to late exponential phase in L broth. Proteins from cells lysed in dissociation buffer were separated by electrophoresis on an SDS-polyacrylamide gel. Approximately 1 μ g of cell extract protein was used for the pYA770-containing strain, and 4 μ g was used for all others. Reactivity to anti-*S. sobrinus* SpaA serum was tested by Western blot assays. MW, Molecular weight (10^3).

ever, the observed protein degradation was not high enough to account for the lower immunoreactivity shown by the polypeptides encoded by pYA176, pYA184, and pYA190. Rather, there is a qualitative difference between these polypeptides and that encoded by pYA177, which allows the latter, but not the former, to be immunoprecipitated and to react significantly more strongly in Western immunoblot and immunodot blot analyses.

The results of these experiments, together with those of the earlier described immunoprecipitations and deletion mapping, are summarized in Fig. 1. The right boundary for the antigenic determinant region has been located approximately 860 nucleotides promoter proximal from the *Sst*I site of pYA770, to agree with data in Fig. 3. In addition, the region assumed to code for the major antigenic determinant(s) has been bracketed between the *Pvu*II site and the first *Kpn*I site to conform with the results in Fig. 7 and 8 and with the previously described observation that amino terminus-containing SpaA polypeptides had to have a minimum molecular mass of 110 kDa to be immunoprecipitated with rabbit anti-SpaA serum.

DNA sequence. The sequence of 1,187 nucleotides that encodes the major antigenic determinant of SpaA and adjacent regions (Fig. 9) was generated by the chain-terminating method (61). The sequencing strategy, indicating primer extension, is shown in Fig. 1. Of the sequence described,

41% was generated from both strands. Multiple additional overlaps were created with the set of BAL 31-generated deletions. The only open reading frame permitted the uninterrupted synthesis of 395 amino acids and extended beyond to the carboxy terminus of the SpaA protein (data not shown). Codons GGC, GTC, GCC, AAT, ATT, TCA, CAA, CTC, CCT, and CCC, which have low rates of occurrence in *E. coli* (30, 41), were used frequently. Also, codons occurring rarely among a compilation of genes from *Staphylococcus aureus* and *Streptococcus* sp. (33), such as CTC, TCC, GCC, CAG, and CCC, were used frequently.

DISCUSSION

The model for the antigen I of *S. sobrinus* that emerges from this work has two main features (Fig. 1): first, that of epitopes distributed throughout the C-terminal two-thirds of the SpaA polypeptide specified by pYA726; second, that of an immunodominant determinant(s) generated by the topographical assembly of amino acid residues (5) widely separated in the protein sequence or by polypeptide folding. Deletion mapping of the *spaA* region encoding antigenic determinants in pYA770 defined a right boundary located approximately 860 nucleotides promoter proximal from the *Sst*I site (Fig. 1). Thus, the 56-kDa N-terminal region of SpaA does not appear to encode antigenic determinants, which are distributed throughout the remaining part of the molecule. Furthermore, this mapping also suggested that antigenic determinants mapping in the region between the right boundary and the *Sst*I site (Fig. 1) only weakly contributed to the overall antigenicity of the SpaA polypeptide encoded by pYA770. Somewhat surprisingly, contribution of the *Sst*I promoter-distal region of *spaA* to the antigenicity of the pYA770-encoded SpaA polypeptide was weak, too. Generation of the immunodominant antigenic determinant(s) required topographically assembled amino acid residues specified by noncontiguous regions of *spaA*.

Serum responses from individual rabbits were the same with regard to determinants recognized but not titers (data not shown). Elsewhere (29), we have shown that mice and rabbits responded similarly to the same antigenic and immunogenic determinants of the *S. sobrinus* SpaA protein. Thus, it appears that the immunodeterminants characterized in the present work correspond to topographical features of the SpaA protein that are recognized by different host species.

Because the *spaA* gene in pYA726 is truncated (35, 53), producing a SpaA polypeptide devoid of antigen II (34, 35, 53) and yet with an antigen I component indistinguishable by immunodiffusion from that in the *S. sobrinus* protein (34), it was considered ideal for the present study. Indeed, the immune response towards purified undenatured SpaA from *S. sobrinus* appears to be predominantly directed towards the immunodominant determinant(s) of antigen I (29). The contribution of antigen II to the overall antigenicity of SpaA is thus significantly weaker than that of antigen I. As shown elsewhere (29), rabbit antisera to either a recombinant polypeptide containing the immunodominant antigenic determinant defined in this work or purified undenatured SpaA from *S. sobrinus* displayed qualitatively and quantitatively similar immunoreactivities and cross-reactivities when tested against the multiple-size SpaA polypeptides found extracellularly in cultures from *S. mutans* of various serotypes. Thus, the *spaA* region of *S. sobrinus* missing in pYA726 probably does not contribute significantly to the immunogenicity of antigen I. Not unexpectedly, differences in immunoreactivity between the SpaA polypeptides specified by

FIG. 8. Immunoprecipitation of SpaA polypeptides produced by CC118 derivatives containing the indicated plasmids. Exponentially growing cells were pulsed (P) for 1 min with [35 S]methionine and chased (C) for one generation in the presence of a 400-fold molar excess of cold methionine. Similar numbers of counts per minute were processed for each sample. MW, Molecular weight (10^3).

pYA726 and pYA770 were negligible, though the *spaA* gene of the latter was shortened by 0.24 kb at its 3' end.

Our results for the antigenic determinant structure of the SpaA protein of *S. sobrinus* are not easily compared with previous biochemical characterizations. The latter, which refer to the SpaA protein of *S. mutans* serotype c, have assigned the determinants of antigens I and II to two possibly overlapping polypeptide fragments of 150 and 69 kDa, respectively (56–58). More recent biochemical characterizations describe the isolation of polypeptide fragments of various sizes, possessing antigenic properties, which were obtained either from culture supernatant fluids (27) or from purified SpaA protein cleaved with cyanogen bromide (6). However, the relationships among the various fragments, and also between each fragment and the whole protein, are not known (6). Homology between the *spaA* genes of *S. mutans* serotype c and *S. sobrinus* serotype g can only be shown under low-stringency hybridization conditions (18, 53, 65). The region of homology is small and maps toward the middle of both genes, showing 62% nucleotide matching and 66% homology between the deduced amino acid sequences of the two proteins (65). From the nucleotide sequences (Fig. 9; 64), it follows that the region of greatest homology between the two *spaA* genes extends from a site located at least 57 nucleotides 5' to the *HincII* site to a region between the two *KpnI* sites (Fig. 1); but significant homology exists almost up to the *HindIII* site (Fig. 1). Characterization of recently cloned *spaA* genes from *S. mutans* serotype c (45; R. G. Holt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D-99, p. 87) and possibly (64) should allow a more-detailed comparison between the antigenic structures of the

encoded proteins and that of the SpaA protein of *S. sobrinus*.

Studies using immunofluorescence (57, 59) and immunoelectron microscopy (2, 51) have shown the presence of the SpaA protein on the outer surface of *S. mutans*. Our mapping of the antigenic determinants of the SpaA protein shows that the amino-terminal one-fourth of the molecule displays no antigenicity, suggesting that this region of the SpaA protein may be concealed by the conformation of the purified protein or that it is intrinsically unable to elicit an antibody response. However, the region of the SpaA protein containing the antigenic determinants is located on the surface of the molecule. The hydropathic profile (42) of the 395-amino-acid SpaA polypeptide determined by the DNA sequence in Fig. 9 was calculated by using the Kyte and Doolittle algorithm and parameter assignments (42) and averaging over a window of 9 residues. It did not indicate any potential membrane-spanning sequence (data not shown). In addition, a computer program written by Modrow and Wolf (50) which superimposes local hydrophilicity values (36) onto the algorithm for secondary structure predictions of Chou and Fasman (14, 15) was used to generate a predicted secondary structure of the polypeptide. The highest local hydrophilicity peak, often a successful predictor of the position of an antigenic determinant (36, 50), occurred at amino acid residues 83 and 84 of the polypeptide in Fig. 9 (data not shown). This position coincided exactly with the first of 51 potential β -turns (data not shown). The His at position 83 formed part of a highly hydrophilic heptapeptide Glu-Lys-His-Lys-Asn-Glu-Asp, including six charged amino acid residues. The significance of this predicted antigenic

```

10      20      30      40      50      60      70      80      90      100     110     120
CAGCTGCTAAGCAAGAAATATGAGCAGAAAGTTCAAGAAAGCTAATGCTAAAAATCCGCAAAATTCAGGAAGCCAACTGCTATCCGTGAACGCAATGCCAAGGCCAAGACAGACTATGAAC
AlaAlaLysGlnGluTyrGluGlnLysValGlnLysAlaAsnAlaLysAsnAlaGluIleThrGluAlaAsnArgAlaIleArgGluArgAsnAlaLysAlaLysThrAspTyrGluL
BbvI
PvuIIEspI
130     140     150     160     170     180     190     200     210     220     230     240
TCAAATGCTCTAAGTACCAAGACAGCTTGTCTAGTACAAGAACGACCTAGCGCAATACCCAGCTAAATCTCAAGCCTATCAAGATGAACCAAGGCCAATCAAGCCAGCTCTGCCAGACT
eulysLeuSerLysTyrGlnGluGluLeuAlaGlnTyrLysLysAspLeuAlaGluTyrProAlaLysLeuGlnAlaTyrGlnAspGluGlnAlaAlaIleLysAlaAlaLeuAlaGluL
DraII      MspBII      BbvI
250     260     270     280     290     300     310     320     330     340     350     360
TGGAAAAACACAAGAACGAGACGGGAACCTCAGCGAGCCCTCAGCCCAAACTCTGCTCTATGATTTAGAGCCCAATGCTCAGGTTTCCCTAGTAACTGACGGGAAATGCTGAAAGCAT
euGluLysHisLysAsnGluAspGlyAsnLeuSerGluProSerAlaGlnSerLeuValTyrAspLeuGluProAsnAlaGlnValSerLeuValThrAspGlyLysLeuLeuLysAlaS
370     380     390     400     410     420     430     440     450     460     470     480
CAGCCCTTGACAGGCCCTTAGCCATGATGAGAAAAATTATAATAACCATCTCTCTACAACAGATAATCTGAATGTGACCTATCTGGAGCAGGCTGATGATGTGGCTCTCTCAGTAGAGC
erAlaLeuAspGluAlaPheSerHisAspGluLysAsnTyrAsnAsnHisLeuLeuGlnProAspAsnLeuAsnValThrTyrLeuGluGlnAlaAspAspValAlaSerSerValGluL
StuI      HgiA
490     500     510     520     530     540     550     560     570     580     590     600
TCTTTGGTAATTTGGTGATAAGGCTGGTGGACAAACCTGTCTCAGCAATGGTTTCAGAAAGTTAAGTTTGGCTCTCTCTCTCAAGCGTGGCCAGAGTGGCTACAGCCACCTTATACCAACC
euPheGlyAsnPheGlyAspLysAlaGlyTrpThrThrValSerAsnGlySerGluValLysPheAlaSerValLeuLeuLysArgGlyGlnSerAlaThrAlaThrTyrThrAsnL
I      BalI
610     620     630     640     650     660     670     680     690     700     710     720
TAAAAAATCTTACTACATAATGGTAAGAGATTCTTAAGGTGGTCTACAGTATACGGTTGACCCCTGACTCCAAAGTTCCAAAATCTACTGGTAAGCTTTGGCTGGGCTCTTTACCGACC
eulysAsnSerTyrTyrAsnGlyLysLysIleSerLysValValTyrLysTyrThrValAspProAspSerLysPheGlnAsnProThrGlyAsnValTrpLeuGlyIlePheThrAspP
HincII      MaeII
730     740     750     760     770     780     790     800     810     820     830     840
CAACCTAGGGGTCTTTGCTCAGCTTATACGGGTCAAAACGAGAGGATACCTTATCTTATCAAGAAATGAATTCACCTTCTACGATGAAGACGGTAATCCCATGCACTTTTGATAATG
roThrLeuGlyValPheAlaSerAlaTyrThrGlyGlnAsnGluLysAspThrSerIlePheIleLysAsnGluPheThrPheTyrAspGluAspGlyAsnProIleAspPheAspAsnA
AvrII      XmnIEcoRI
850     860     870     880     890     900     910     920     930     940     950     960
CCCTCTTATCAGTTCCTCCCTTAACAGGGAAACACAATTCCATTGAGATGGGCCAAGGACTACAGCGGTACCTTCGTTAAGATTTCTGGCTCATCCATTGGTGAAGAAATGGCATGATCT
laLeuLeuSerValAlaSerLeuAsnArgGluHisAsnSerIleGluMetAlaLysAspTyrSerGlyThrPheValLysIleSerGlySerSerIleGlyGluLysAsnGlyMetIleT
BstXI      BalI      BanI      KpnI      FokI
970     980     990     1000    1010    1020    1030    1040    1050    1060    1070    1080
ATCGACCGATACCTTCACTTAAAGAGGCTCAAGGCGCTTCCCTTCACACCATGTACACAGCAAGTCAGGCTGGTTCAGGTTGGGACTCTGCTGATGCTCTTAATCTTGGTATG
yrAlaThrAspThrLeuAsnPheLysLysGlyGluGlyGlySerLeuHisThrMetTyrThrArgAlaSerGluProGlySerGlyTrpAspSerAlaAspAlaProAsnSerTrpTyrG
DraI
1090    1100    1110    1120    1130    1140    1150    1160    1170    1180
GTGCTGGTGTCTGAGAAATGTCGGGCCCCAACCACTACATCACCTTGGGGGCAACCTCAGCGACCAATGTTCTCAGCCTAGCTGAAATGCCACAGGTACCTGGTAAG
lyAlaGlyAlaValArgMetSerGlyProAsnAsnTyrIleThrLeuGlyAlaThrSerAlaThrAsnValLeuSerLeuAlaGluMetProGlnValProGlyLys
BanI      KpnI

```

FIG. 9. Nucleotide sequence of the antigenic determinant region of *spaA* in pYA770. The nucleotide sequence of the anticoding strand from the *PvuII* site (proximal to the *SstI* site) until 7 nucleotides past the second *KpnI* site is presented. This 1,187-nucleotide region permits the synthesis of a polypeptide of 395 amino acids.

determinant is unclear, since our results indicate that the immunodominant determinant(s) of the SpaA protein is discontinuous. However, being highly charged and located at a β -turn, the heptapeptide is likely to form a distinctive feature on the surface of the polypeptide, constituting, perhaps, a component of the immunodominant determinant(s).

Molecular masses determined from mobilities in SDS-polyacrylamide gels for SpaA polypeptides encoded by pYA170, pYA176, pYA177, pYA178, pYA184, and pYA190 significantly exceeded the values predicted from the DNA sequences of their respective *spaA* regions. Thus, whereas the predicted sizes for the SpaA polypeptides synthesized by pYA176, pYA190, pYA184, pYA170, pYA177, and pYA178 were 17.4, 34.8, 23.7, 44.2, 61.6, and 79.0 kDa, respectively, the measured values were instead 46.8, 54.2, 37, 64, 84, and 113 kDa as determined from either pulse-radiolabeled polypeptides (Fig. 8) or bands with the slowest mobility on Western immunoblots (Fig. 7). Conditions that aggressively promote protein denaturation (such as adding urea and increasing the SDS concentration in the gel, or incorporating formamide into the electrophoresis buffer) did not significantly affect the abnormal migration of the polypeptides (Goldschmidt, unpublished results). Because the vector contains repeated termination codons in all three reading frames near the insert-vector junction (1, 11), translational readthrough cannot explain the anomalous molecular mass.

Analogous situations have been described (12, 33, 37) and have been interpreted as a result of the presence of either a high negative charge (12, 37) or a proline-rich region (33). Neither explanation is applicable for the *Ptrc*-expressed SpaA polypeptide. We speculate that the accumulation within short polypeptide segments either of strings of adjacent charged residues or of multiple β -turns imposes configurational constraints that slow down its migration on SDS-polyacrylamide gels.

SpaA polypeptides appear to be subject to extensive *in vivo* degradation. This was apparent both from Western blot determinations and from radioactive labeling of polypeptides synthesized in minicells and can be a problem for molecular mass measurements. However, it is also possible that some of these polypeptides may reflect aberrant transcription or translation events affecting *S. sobrinus* genes in *E. coli*.

A consistent discrepancy was observed between the results obtained by Western immunoblot and those obtained by immunoprecipitation. Thus, polypeptides expressed from the *spaA* promoter whose molecular mass was smaller than 110 kDa (i.e., affected by a mutation that terminated protein synthesis promoter proximal to the first *KpnI* site) were not immunoprecipitated. Similarly, almost all polypeptide degradation products failed to immunoprecipitate. This may well reflect that the perturbations of polypeptide chain folding caused by the different treatments involved in the

two assays resulted in differential accessibility of epitopes to the antibodies.

DNA sequencing of the region of *spaA* encoding the immunodominant determinant(s) of antigen I indicated that codon occurrence frequencies were characteristic, differing from those determined both for *E. coli* genes (30, 41) and for a compilation of genes from *Staphylococcus aureus* and *Streptococcus* sp. (33). Also, certain amino acids occurred at much higher (such as proline) or much lower (such as methionine) frequencies in the encoded polypeptide than expected from a simple random distribution. This difference was clearly apparent in radioautographs of SDS-polyacrylamide gel electrophoresis analyses of proteins from extracts of cells that had been labeled in vivo with either ^{14}C -proline or ^{35}S -methionine: the amount of SpaA relative to other cell proteins in the former was almost 10-fold higher than in the latter.

The function of the SpaA protein is still unknown, though genetic evidence suggests that it may be a virulence determinant, possibly involved in the attachment of *S. sobrinus* to the tooth surface (17, 23). Indeed, for *S. mutans* serotype c, antiserum against purified SpaA partially inhibits the attachment of the cells to saliva-coated hydroxylapatite (23)—a tooth adherence model for *S. mutans*. However, the significance of this result for *S. sobrinus* is unclear, since *S. mutans* and *S. sobrinus* may use different mechanisms to adhere to saliva-coated hydroxylapatite (28). A possibly different role for SpaA was suggested by the recent cloning into *E. coli* of a *Streptococcus sanguis* gene encoding a cell surface receptor for a salivary agglutinin (22). The recombinant polypeptide produced by *E. coli* harboring the cloned gene strongly cross-reacted with antiserum prepared against recombinant SpaA from *S. sobrinus* (22). However, some salivary agglutinins also seem to be able to adsorb to the tooth surface and, as part of the tooth pellicle, promote the adherence of *S. mutans* (3, 10). Thus, the SpaA protein may be functioning both as a cell receptor for a salivary agglutinin and as an adhesin for a receptor on the saliva-coated tooth surface. Clearly, ultimate elucidation of SpaA function will require examination of agglutinating and tooth-colonizing abilities of mutants of *S. sobrinus* containing in vitro-generated deletions of well-defined ends, resulting from replacing the resident gene with altered recombinant copies.

ACKNOWLEDGMENTS

We are grateful to Ron Esser for helpful discussions and to Paul Gulig, Patricia Haber, Michael Hudson, and Koji Nakayama for their useful comments on the manuscript.

This research was supported by Public Health Service grant DE06673 from the National Institutes of Health.

LITERATURE CITED

- Amann, E., and J. Brosius. 1985. 'ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene* 40:183-190.
- Ayakawa, G. Y., L. W. Boushell, P. J. Crowley, G. W. Erdos, W. P. McArthur, and A. S. Bleiweis. 1987. Isolation and characterization of monoclonal antibodies specific for antigen P1, a major surface protein of mutans streptococci. *Infect. Immun.* 55:2759-2767.
- Babu, J. P., E. H. Beachey, D. L. Hasty, and W. A. Simpson. 1986. Isolation and characterization of a 60-kilodalton salivary glycoprotein with agglutinating activity against strains of *Streptococcus mutans*. *Infect. Immun.* 51:405-413.
- Beighton, D., H. Hayday, R. R. B. Russell, and R. A. Whaley. 1984. *Streptococcus macacae* sp. nov. from the dental plaque of monkeys (*Macaca fascicularis*). *Int. J. Syst. Bacteriol.* 34:322-335.
- Benjamin, D. C., J. A. Berzofsky, I. J. East, F. R. N. Gurd, C. Hannum, S. J. Leach, E. Margoliash, J. G. Michael, A. Miller, E. M. Prager, M. Reichlin, E. E. Sercarz, S. J. Smith-Gill, P. E. Todd, and A. C. Wilson. 1984. The antigenic structure of proteins: a reappraisal. *Annu. Rev. Immunol.* 2:67-101.
- Bergmeier, L. A., and T. Lehner. 1988. Separation and characterization of a 14,000-dalton cyanogen bromide-generated peptide from a 185,000-dalton streptococcal antigen. *Infect. Immun.* 56:2424-2429.
- Birnboim, H. C. 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.* 100:243-255.
- Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline-phosphatase conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175-179.
- Boyer, H., and D. Rouland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* 41:459-472.
- Brack, C. M., and E. C. Reynolds. 1987. Characterization of a rat salivary sialoglycoprotein complex which agglutinates *Streptococcus mutans*. *Infect. Immun.* 55:1264-1273.
- Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.* 148:107-127.
- Burton, Z., R. P. Burgess, J. Lin, D. Moore, S. Holder, and C. A. Gross. 1981. The nucleotide sequence of the cloned *rpoD* gene for the RNA polymerase sigma subunit from *E. coli* K12. *Nucleic Acids Res.* 9:2889-2903.
- Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* 134:1141-1156.
- Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. *Biochemistry* 13:222-245.
- Chou, P. Y., and G. D. Fasman. 1978. Empirical predictions of protein conformation. *Annu. Rev. Biochem.* 47:251-276.
- Clark-Curtiss, J. E., W. R. Jacobs, M. A. Docherty, L. R. Ritchie, and R. Curtiss III. 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. *J. Bacteriol.* 161:1093-1102.
- Curtiss, R., III. 1986. Genetic analysis of *Streptococcus mutans* virulence and prospects for an anticaries vaccine. *J. Dent. Res.* 65:1034-1045.
- Curtiss, R., III, R. Goldschmidt, J. Barrett, M. Thoren-Gordon, D. J. Salzberg, H. M. Murchison, and S. Michalek. 1987. Genetic analysis of surface proteins essential for virulence of *Streptococcus sobrinus*, p. 212-216. In J. J. Ferretti and R. Curtiss III (ed.), *Streptococcal genetics*. American Society for Microbiology, Washington, D.C.
- Curtiss, R., III, R. M. Goldschmidt, N. B. Fletchall, and S. M. Kelly. 1988. Avirulent *Salmonella typhimurium* Δ cyt Δ crp oral vaccine strains expressing a streptococcal colonization and virulence antigen. *Vaccine* 6:155-160.
- Czerkinsky, C., M. W. Russell, N. Lycke, M. Lindblad, and J. Holmgren. 1989. Oral administration of a streptococcal antigen coupled to cholera toxin B subunit evokes strong antibody responses in salivary glands and extramucosal tissues. *Infect. Immun.* 57:1072-1077.
- Dagert, M., and S. D. Ehrlich. 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* 6:23-28.
- Demuth, D. R., C. A. Davis, A. M. Corner, R. J. Lamont, P. S. Leboy, and D. Malamud. 1988. Cloning and expression of a *Streptococcus sanguis* surface antigen that interacts with a human salivary agglutinin. *Infect. Immun.* 56:2484-2490.
- Douglas, C. W. I., and R. R. B. Russell. 1984. Effect of specific antisera upon *Streptococcus mutans* adherence to saliva-coated hydroxylapatite. *FEMS Microbiol. Lett.* 25:211-214.
- Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon. 1981. A reliable method for the recovery of DNA fragments

- from agarose and acrylamide gels. *Anal. Biochem.* **112**:295-298.
25. Forester, H., N. Hunter, and K. W. Knox. 1983. Characteristics of a high molecular weight extracellular protein of *Streptococcus mutans*. *J. Gen. Microbiol.* **129**:2779-2788.
 26. Frazer, A. C., and R. Curtiss, III. 1975. Production, properties and utility of bacterial minicells. *Curr. Top. Microbiol. Immunol.* **69**:1-84.
 27. Glasuddin, A. S., T. Lehner, and R. W. Evans. 1983. Identification, purification and characterization of a streptococcal protein antigen with a molecular weight of 3800. *Immunology* **50**: 651-658.
 28. Gibbons, R. J., L. Cohen, and D. I. Hay. 1986. Strains of *Streptococcus mutans* and *Streptococcus sobrinus* attach to different pellicle receptors. *Infect. Immun.* **52**:555-561.
 29. Goldschmidt, R. M., and R. Curtiss III. 1990. Cross-reactivity between the immunodominant determinant of the antigen I component of *Streptococcus sobrinus* SpaA protein and surface antigens from other members of the *Streptococcus mutans* group. *Infect. Immun.* **58**:2276-2282.
 30. Grantham, R., C. Gautier, M. Gouy, M. Jacobzone, and R. Mercier. 1981. Codon catalog usage is a genome strategy modulated for gene expressivity. *Nucleic Acids Res.* **9**:r43-r74.
 31. Hamada, S., and H. D. Slade. 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol. Rev.* **44**: 331-384.
 32. Helfman, D. M., J. R. Feramisco, J. C. Fiddes, G. P. Thomas, and S. H. Hughes. 1983. Identification of clones that encode chicken tropomyosin by direct immunological screening of a cDNA expression library. *Proc. Natl. Acad. Sci. USA* **80**: 31-35.
 33. Hollingshead, S. K., V. A. Fischetti, and J. R. Scott. 1986. Complete nucleotide sequence of type 6 M protein of the group A *Streptococcus*. *J. Biol. Chem.* **261**:1677-1686.
 34. Holt, R. G., Y. Abiko, S. Saito, M. Smorawinska, J. B. Hansen, and R. Curtiss III. 1982. *Streptococcus mutans* genes that code for extracellular proteins in *Escherichia coli* K-12. *Infect. Immun.* **38**:147-156.
 35. Holt, R. G., and J. O. Ogundipe. 1987. Molecular cloning in *Escherichia coli* of the gene for a *Streptococcus sobrinus* surface protein containing two antigenic determinants, p. 217-219. In J. J. Ferretti and R. Curtiss III (ed.), *Streptococcal genetics*. American Society for Microbiology, Washington, D.C.
 36. Hopp, T. P., and K. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* **78**:3824-3828.
 37. Ishii, S., M. Ihara, T. Maekawa, Y. Nakamura, H. Uchida, and F. Imamoto. 1984. The nucleotide sequence of the cloned *nusA* gene and its flanking region of *Escherichia coli*. *Nucleic Acids Res.* **12**:3333-3342.
 38. Jacobs, W. R., J. F. Barrett, J. E. Clark-Curtiss, and R. Curtiss III. 1986. In vivo repackaging of recombinant cosmid molecules for analyses of *Salmonella typhimurium*, *Streptococcus mutans*, and mycobacterial genomic libraries. *Infect. Immun.* **52**: 101-109.
 39. Jen, G., and R. Thach. 1982. Inhibition of host translation in encephalomyocarditis virus-infected L cells: a novel mechanism. *J. Virol.* **43**:250-261.
 40. Knecht, D., and R. L. Dimond. 1984. Visualization of antigenic proteins on Western blots. *Anal. Biochem.* **136**:180-184.
 41. Konigsberg, W., and G. N. Godson. 1983. Evidence for use of rare codons in the *dnaG* gene and other regulatory genes of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **80**:687-691.
 42. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**:105-132.
 43. Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* **80**:575-599.
 44. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ³H and ¹⁴C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**:335-341.
 45. Lee, S. F., A. Progulsk-Fox, and A. S. Bleiweis. 1988. Molecular cloning and expression of a *Streptococcus mutans* major surface protein antigen, P1 (I/II), in *Escherichia coli*. *Infect. Immun.* **56**:2114-2119.
 46. Lehner, T., M. W. Russell, J. Caldwell, and R. Smith. 1981. Immunization with purified protein antigens from *Streptococcus mutans* against dental caries in rhesus monkeys. *Infect. Immun.* **34**:407-415.
 47. Lennox, E. S. 1955. Transduction of linked genetic characters of the host *Escherichia coli* by bacteriophage P1. *Virology* **1**: 190-206.
 48. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*, p. 97-148. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 49. Manoil, C., and J. Beckwith. 1985. *tnpHoA*: a transposon probe for protein export signals. *Proc. Natl. Acad. Sci. USA* **82**: 8129-8133.
 50. Modrow, S., and H. Wolf. 1986. Characterization of two related Epstein-Barr virus-encoded membrane proteins that are differentially expressed in Burkitt lymphoma and *in vitro*-transformed cell lines. *Proc. Natl. Acad. Sci. USA* **83**:5703-5707.
 51. Moro, I., and M. W. Russell. 1983. Ultrastructural localization of protein antigens I/II and III in *Streptococcus mutans*. *Infect. Immun.* **41**:410-413.
 52. Neidhardt, F. C., P. L. Bloch, and D. F. Smith. 1974. Culture medium for enterobacteria. *J. Bacteriol.* **119**:736-747.
 53. Ogundipe, J. O., and R. G. Holt. 1989. Molecular and immunochemical characterization of recombinant *Escherichia coli* containing the *spaA* gene region of *Streptococcus sobrinus*. *Infect. Immun.* **57**:1906-1915.
 54. Oliver, D. B., and J. Beckwith. 1981. *E. coli* mutant pleiotropically defective in the export of secreted proteins. *Cell* **25**: 765-772.
 55. Rusche, J. P., and P. Howard-Flanders. 1985. Hexamine cobalt chloride promotes intermolecular ligation of blunt end DNA fragments by T4 DNA ligase. *Nucleic Acids Res.* **13**:1997-2008.
 56. Russell, M. W. 1986. Protein antigens of *Streptococcus mutans*, p. 51-59. In S. Hamada, S. Michalek, H. Kiyono, L. Menaker, and J. R. McGhee (ed.), *Molecular microbiology and immunobiology of Streptococcus mutans*. Elsevier Biomedical Press, Amsterdam.
 57. Russell, M. W., L. A. Bergmeier, E. D. Zanders, and T. Lehner. 1980. Protein antigens of *Streptococcus mutans*: purification and properties of a double antigen and its protease-resistant component. *Infect. Immun.* **28**:486-493.
 58. Russell, M. W., E. D. Zanders, L. A. Bergmeier, and T. Lehner. 1980. Affinity, purification, and characterization of protease-susceptible antigen I of *Streptococcus mutans*. *Infect. Immun.* **29**:999-1006.
 59. Russell, R. R. B. 1979. Wall associated protein antigens of *Streptococcus mutans*. *J. Gen. Microbiol.* **114**:109-115.
 60. Russell, R. R. B. 1980. Distribution of cross-reactive antigens A and B in *Streptococcus mutans* and other oral streptococci. *J. Gen. Microbiol.* **118**:383-388.
 61. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
 62. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. *Experiments with gene fusions*, p. 147-148. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 63. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76-85.
 64. Sommer, P., T. Bruyère, J. A. Ogier, J.-M. Garnier, J.-M. Jeltsch, and J.-P. Klein. 1987. Cloning of the saliva-interacting protein gene from *Streptococcus mutans*. *J. Bacteriol.* **169**: 5167-5173.
 65. Takahashi, I., N. Okahashi, C. Sasakawa, M. Yoshikawa, S. Hamada, and T. Koga. 1989. Homology between surface protein antigen genes of *Streptococcus sobrinus* and *Streptococcus mutans*. *FEBS Lett.* **249**:383-388.

66. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
67. Whiley, R. A., R. R. B. Russell, J. M. Hardie, and D. Beighton. 1988. *Streptococcus downei* sp. nov. for strains previously described as *Streptococcus mutans* serotype *h*. *Int. J. Syst. Bacteriol.* **38**:25-29.
68. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103-119.