

Streptococcus sanguis adhesins mediating attachment
to saliva-coated hydroxyapatite beads

by

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ABSTRACT

Streptococcus sanguis 12 adhesins mediating attachment to saliva-coated hydroxyapatite beads (S-HA) were isolated and characterized. Cell surface fibrils were released from this organism by a method of freeze-thawing followed by brief homogenization. Fibrils in the homogenate were precipitated by ultracentrifugation or ammonium sulphate precipitation. This precipitate was shown to contain fibrils by electron microscopy. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of fibrils showed a single band which stained with Coomassie blue and periodate-Schiff. The molecule had a M_r in excess of 300,000. This protein has been given the name long-fibril protein (LFP). Antibody raised against the LFP reacted with long fibrils of *S. sanguis* 12. LFP was degraded by subtilisin, pronase, papain, and trypsin, but not by chymotrypsin and muramidases. Fibrils were hydrolyzed by subtilisin into discrete lower M_r protein bands which reacted with both anti-fibril and anti-LFP serum. $F(ab')_2$ prepared from anti-fibril IgG inhibited adhesion of *S. sanguis* 12 to pH modified S-HA, indicating that fibrils were acting as an adhesin mediating attachment via the neuraminidase-sensitive receptor on S-HA.

Five recombinant clones expressing surface antigens of *S. sanguis* 12 were isolated by ligating a partial digest of *S. sanguis* 12 chromosomal DNA with the plasmid vector pUC 18, and transforming into *Escherichia coli* JM83. Recombinant clones were screened by a colony immunoassay with antisera raised against either *S. sanguis* 12 whole cells or with anti-fibril serum. Positive clones were then analyzed by SDS-PAGE, Western blotting and restriction endonuclease digestion of recombinant plasmids. One recombinant plasmid, pSA2 expressed two proteins of M_r s of 20,000 and 36,000. The 36,000- M_r protein has been designated as SsaB (*S. sanguis* adhesin B). Both proteins were purified to homogeneity by gel filtration and ion exchange chromatography. Anti-SsaB serum was used in an immunogold bead labelling experiment to demonstrate that this protein was present on the surfaces of *S. sanguis* 12 and in the non-saliva-aggregating variant 12na, but not on the non-adhering non-aggregating hydrophilic variant 12L. Western blot analysis with anti-SsaB and anti-20 kd sera showed that both SsaB and the 20 kd proteins were present in cell extracts of *S. sanguis* 12 and its variants. SsaB inhibited adhesion of *S. sanguis* 12na to S-HA, indicating that it was the adhesin which mediates the binding to the pH-sensitive

receptor. SsaB was found to be present on all *S. sanguis* strains tested, but not on other oral streptococci. Chemical cross-linking studies of SsaB on *S. sanguis* 12 cell surface suggested that this protein may be present in a higher M_r complex.

This study provides direct evidence that binding of *S. sanguis* 12 to S-HA involves at least two adhesin-receptor interactions. The adhesin mediating binding to the neuraminidase-sensitive receptor on S-HA involves the long fibrils and the adhesin binding to the acid labile receptor is a 36,000 M_r protein.

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ABBREVIATIONS, NOMENCLATURE AND SYMBOLS

Anti-Fibril	Antiserum raised against the native fibrils
Anti-LFP	Antiserum raised against the denatured long fibril protein
ATCC	American Type Culture Collection
Ap	Ampicillin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulphonic acid
kb	1000 base pairs
kd	1000 daltons
LFP	Long fibril protein
LTA	Lipoteichoic acid
M _r	Relative molecular mass
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline/Tween 20
r	Resistant
SDS	Sodium dodecyl sulphate
S-HA	Saliva-coated hydroxyapatite beads
SsaA	<i>Streptococcus sanguis</i> adhesinA
SsaB	<i>Streptococcus sanguis</i> adhesinB
TAE	Tris/Acetate/EDTA buffer
TE	Tris/EDTA buffer
TES	Tris/EDTA/NaCl buffer
TSB	Trypticase soy broth

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Introduction

In this section of the thesis there will be a general discussion on bacterial adherence followed by a detailed description of adherence to human host surfaces. The latter will include methods of study, mechanisms of adherence, and bacterial structures involved in adherence. This will be followed by a detailed description of adherence in the oral cavity with a particular emphasis on *Streptococcus sanguis* and its interactions with the saliva derived tooth pellicle.

Bacterial adherence

Bacterial adherence can be described as a state of irreversible attachment of bacteria to a surface. Marshall *et al.*, (1971), postulated that when a bacterium approaches a receptor it binds via a reversible interaction before a permanent bond is established. Doyle *et al.*, (1982), further hypothesized that subsequent to the initial adhesion, other interactions may stabilize or contribute towards the process of irreversible attachment. This interaction is mediated by macromolecules on the bacterial surface that combine with complementary structures present on the surface to which it binds. Bacterial surface molecules which mediate attachment are defined as adhesins and molecules to which they bind are referred to as receptors (Beachey, 1981).

Bacterial colonization is a complex process which depends initially on adherence, and subsequently on the ability to withstand competition and accommodate to the environment within a particular ecological niche. A pathogenic flora can evolve when the niche is populated by microorganisms with virulence properties. When a microorganism is able to form a harmonious relationship with the host and other resident microflora, it becomes part of an indigenous flora.

The diversity of the microbial flora is due to in part to the specificity of adherence reactions. For example, bacterial colonization of the mouth and the intraoral surface distribution of bacteria reflects the remarkable diversity and specificity of adhesins which

recognize the multiplicity of receptors available on host tissues and other bacteria. A number of *in vivo* and *in vitro* studies (Gibbons *et al.*, 1980, and 1984; Ellen, 1982, and 1985; Mergenhagen *et al.*, 1987), have shown that the specificity in attachment to various oral surfaces corresponds to the natural localization of the organism.

Attachment of an organism modifies the environment and in effect creates a new ecosystem which may have either a positive or a negative effect on the host. A positive example is provided by the indigenous human flora which serves to protect the host from invading pathogenic organisms (Gibbons *et al.* 1975a,1975b). The negative effects of adherence are evident in studies of a number of pathogenic organisms which selectively adhere to host tissues (Jones *et al.*, 1977). For example, enteropathogenic and uropathogenic *Escherichia coli* possess multiple adhesins mediating attachment to a number of epithelial surfaces (Levine, 1987).

Adhesion prevents the colonizing bacteria from being swept away by mechanical forces and cleansing mechanisms such as sneezing, coughing, peristalsis, and fluid flow. In order to colonize the mucosal surfaces, the organisms must not only adhere but also multiply at a sufficiently rapid rate to replenish newly exposed epithelial cell surfaces as old epithelial cells, along with their adherent bacteria, are exfoliated and swept away. In addition, the oral cavity contains nonshedding tooth surfaces which circumvent problems associated with exfoliation of epithelial cells allowing a stable non-renewing flora to establish. Nevertheless, attachment to this nonshedding surface must be sufficiently strong to resist mechanical and cleansing mechanisms such as mastication, swallowing and salivary flow.

Methods of study

A number of different methods have been employed in an attempt to gain a better understanding of how bacteria bind to a surface. The technique chosen has depended on whether the objective is to, (a) characterize the adhesive interactions, (b) identify, isolate and characterize the adhesin or receptor, (c) study the regulation and expression of the genes involved, or (d) study the *in vivo* activity of the adhesin.

A classical approach to studying adherence interactions has involved chemical or enzymatic modification of specific cell surface components. For example, Weerkamp *et al.*, (1980a) implicated *Streptococcus salivarius* cell surface protein in the coaggregation of *Fusobacterium nucleatum* using protease treated streptococci. McBride *et al.*, (1977) and subsequently Levine *et al.*, (1978) demonstrated that *S. sanguis* reacted, via terminal sialic acid residues with salivary glycoproteins which could be removed by neuraminidase.

Another widely used traditional approach has been the inhibition of adherence interactions with receptor analogues. One of the best studied examples is the mannose-sensitive haemagglutination adhesin of enterobacteriaceae (Duguid *et al.*, 1980). Another example is the lactose-sensitive coaggregation among oral plaque bacteria (McIntire *et al.*, 1985).

The isolation and characterization of many adhesins has proven to be a difficult task. One approach is to isolate structures involved in adhesion from the whole cells by physical procedures and visualize them microscopically (Duguid *et al.* 1955, 1966, and 1980; Gaastra *et al.*, 1982; Cisar *et al.*, 1978; Fives-Taylor *et al.*, 1982, and 1985). Another approach has been to isolate adhesion negative mutants by selective enrichment and/or by chemical mutagenesis and comparing the mutant and parent cell walls (Weerkamp *et al.*, 1980b; Fives-Taylor *et al.*, 1982, and 1985; Cisar *et al.*, 1983; Kolenbrander *et al.*, 1985; Morris *et al.*, 1985). Monospecific polyclonal anti-adhesin serum has been obtained by absorbing anti-wild type serum with the mutant. Such antiserum can be used to specifically inhibit adhesive interactions (Cisar *et al.*, 1984; Clark *et al.*, 1984; Fachon-Kalweit *et al.*, 1985).

Recombinant DNA technology has afforded the possibility of systematically isolating the genes involved in adhesion. These methods have simplified our access to genes that encode for structures that mediate adhesion. Once the genes have been identified, a number of questions can be asked, these include: (1) how many genes are associated with the expression of the adhesin on the bacterial surface; (2) what are the structural and regulatory nucleotide sequences; (3) can overproduction of a protein in a heterologous host facilitate purification; (4) can site specific mutagenesis localize amino acid sequences

involved in adhesin-receptor interactions; (5) can the genes of interest be reintroduced to obtain isogenic mutants to evaluate the role of a particular adhesin in colonization and/or pathogenesis; and finally (6) can the cloned genes be used as probes to study the dissemination, gene origin, and gene copy number?

Studies directed to determine the mechanisms of adherence have involved quantitating the number of bacteria adhering to a surface and subsequently analyzing the data kinetically (Gibbons *et al.*, 1976; Clark *et al.*, 1978). By using either competitors that inhibit or alter the adherence (Nesbitt *et al.*, 1982b; Gibbons *et al.*, 1983a), or by using bacteria with altered cell surface properties (Gibbons *et al.*, 1982; Morris *et al.*, 1984), the role specific components involved in adherence can be deduced.

Mechanisms of adherence

Based on studies described above, adherence has been postulated to involve ionic (Rolla, 1976; Heckels *et al.*, 1976), lectin-like (Gibbons *et al.*, 1982; Morris *et al.*, 1984) or hydrophobic interactions (Beachey, 1981; Doyle *et al.*, 1982; Svanberg *et al.*, 1984). The ionic and hydrophobic bonds are thought to be relatively nonspecific whereas the lectin-like interactions are highly specific. However, ionic or hydrophobic regions can be organized in such a way that they could interact in a specific manner with appropriate receptor molecules.

Cell surface of bacteria

Bacterial cytoplasm is surrounded by a rigid, highly structured layer called the cell wall. The mechanical strength and shape of the cell wall is derived from the unique highly cross-linked aminosugar polymer, called peptidoglycan. Peptidoglycan consists of glycan strands composed of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid joined by a 1,4- β glycosidic linkage. A chain of four amino acids joined by peptide bonds is attached to the carboxyl group of each muramic acid residue. The tetrapeptide chains on adjacent glycan strands are linked by various means to give a cross-linked polymer. Cross-linking

of the tetrapeptide strand may be through a direct peptide linkage or by another peptide chain. The degree and diversity of cross-linking, and the amino acids involved in cross-linking varies depending on the organism.

In Gram-positive bacteria, peptidoglycan may account for as much as 50% of the weight of the cell wall. The remaining 50% is made up of accessory polymers such as teichoic acids, teichuronic acids, proteins, carbohydrates, lipoteichoic acids (LTA) and in encapsulated bacteria, an external layer of polysaccharide.

Teichoic acids are linear polymers of glycerol or ribitol phosphate joined by phosphodiester bonds linked covalently to the peptidoglycan. The teichoic acids are substituted at their free hydroxyl ends by D-alanine and other sugar substituents which are attached by an α or β glycosidic linkage. The high phosphate content makes this polymer acidic and confers a negative charge to the bacterial cell surface.

Teichuronic acids are similar to the teichoic acids except that they are linear polymers containing uronic acids covalently linked to the peptidoglycan by phosphodiester bonds. The negative charge is due to the carboxyl groups of the uronic acids.

Neutral polysaccharides which are covalently linked to the peptidoglycan are found in the streptococcal cell wall. Group A streptococci contain a neutral polysaccharide containing L-rhamnose and N-acetylglucosamine (Krasse *et al.*, 1961). In streptococci, the composition of neutral polysaccharides confers antigenicity, which allows the organisms to be grouped serologically into Lancefield Groups A to O (Lancefield, 1933 and 1968).

Other components are present in the cell wall which are not covalently linked to the peptidoglycan. One of these is lipoteichoic acid. It is a polyglycerol phosphate covalently linked to a glycolipid molecule (Wicken *et al.*, 1980). The latter is presumed to anchor the molecule to the outer surface of the cytoplasmic membrane. This polymer protrudes through the matrix of the cell wall so that it can be detected on the outer surface of the cells. The polyglycerol phosphate backbone is substituted with ester-linked alanine and

contains a glycerophosphoryl diglucosyl diglyceride moiety at one end.

The capsule is an accessory wall component. It is usually a large polysaccharide molecule which envelops the cell wall. It may be a hetero- or homopolysaccharide. Glucan is an example of a glucose homopolymer produced by *Streptococcus mutans*. In this case glucan may exist in a linear soluble form or in a branched insoluble form which is tightly associated with the cell (Hamada *et al.*, 1980).

A variety of proteins have been recognized as cell wall components. A family of molecules known as the M proteins are found in the cell wall of Group A streptococci. They have been shown to be involved in adherence and to have anti-phagocytic properties (Ellen *et al.*, 1972). In another section of this thesis the molecular structure of M protein and its involvement in adhesion will be discussed in detail.

Gram-negative bacterial cell walls are more complex than those of Gram-positive bacteria. The cell wall peptidoglycan appears to be thinner and not as closely associated with the cytoplasmic membrane. A second lipid bilayer membrane known as the outer membrane is located exterior to the peptidoglycan. The outer membrane contains lipopolysaccharide (LPS), carbohydrates and proteins. LPS consists of a membrane anchor called lipid A, core (R) and O polysaccharide side chain. The O side chain which is found outside the cell surface exhibits gross differences in composition and structure between and within species. It is not surprising that it is a major antigenic determinant conferring an antigenic uniqueness which has been exploited in microbial taxonomy.

The region between the outer membrane and peptidoglycan is known as the periplasmic space. This area contains proteins involved in catalytic function, nutrient binding, detoxification and transport of specific molecules to and from the cytoplasmic membrane.

Radiating from the cell surface are a variety of structures which can be seen only by electron microscopy. These include flagella, fimbriae, and fibrils. The flagella, are filamentous structures averaging 20 nm in width and 10-20 μ m in length, and they are responsible for cell motility. Flagella are composed of protein subunits known as flagellin

(Silverman *et al.*, 1977). Fimbriae are thin (6-7 nm), hair-like structures which are frequently involved in attachment of cells to surfaces. They are made up of fimbrillin subunits which are linked to form arrays varying in length from 0.2 to 20 μm . The terms fimbriae and pili are used interchangeably although it has been suggested that pili should be reserved for those structures which are involved in conjugation (Ottow, 1975). Fibrils or fibrillae are similar to fimbriae, but they differ in that they have variable width and may be wiry and flexible. Like fimbriae they have been implicated in adherence.

Bacterial surface components involved in adherence

As indicated in the preceding brief review, bacterial cell surfaces are complex entities composed of a variety of different constituents. Any of these components may be capable of acting as an adhesin.

Fimbriae

Fimbria (latin for thread, fiber and fringe) was the term used to describe proteinacious, non-flagellar appendages that radiate outwards in a fairly rigid and filamentous fashion (Houwink *et al.*, 1950; Duguid *et al.*, 1955). In Gram-negative bacteria, fimbriae were classified by variation in morphology and haemagglutinating properties. For example, type 1 fimbriae present on enterobacteriaceae are about 7 nm wide and 2 μm long. They hemagglutinate red blood cells in a reaction which can be inhibited by D-mannose, hence these adhesins are termed mannose-sensitive (MS). In addition, many enterotoxigenic strains of *E. coli* possess morphologically similar fimbriae, but which mediate mannose-resistant (MR) haemagglutination (Ottow, 1975).

Functions attributed to fimbriae are adherence (Swanson *et al.*, 1975; Gaastra *et al.*, 1982; Cisar *et al.*, 1984; Fachon-Kalweit *et al.*, 1985), twitching mobility (Henriksen *et al.*, 1975), and pellicle formation (Old *et al.*, 1968). Haemagglutination caused by Gram-negative organisms is an example of a well studied adherence phenomenon which can be frequently attributed to fimbriae. Haemagglutination reactions mediated by fimbriae can be

differentiated on the basis of inhibition by simple sugars (Duguid *et al.*, 1966), size of fimbriae (Ottow, 1975), species of bacteria (Gaastra *et al.*, 1982), phage specificity and the type of red blood cells to which they will bind (Pearce *et al.*, 1980).

Fimbriae isolated from Gram-negative organisms have been shown to be made up of protein subunits having M_r s in the range of 15-25 kd (Pearce *et al.*, 1980; Jones *et al.*, 1983). The monomers contain a high percentage of nonpolar amino acids, suggesting that they are hydrophobic or possess major regions of hydrophobicity. The N-terminal amino acid sequence is highly conserved among a number of fimbriae from different species (Jones *et al.*, 1983).

If fimbriae are involved in adhesion, a question arises as to whether these structures can be categorized as virulence determinants. In order to determine this, studies were done to compare infectivity of fimbriate and afimbriate strains of non-invasive enterotoxigenic *E. coli* (Gaastra *et al.*, 1982). This organism is known to selectively attach to small intestinal epithelium, where it causes diarrheal diseases in humans and domestic animals. Fimbriate strains bound and caused disease, but afimbriate strains did not bind and were not pathogenic. Anti-fimbrial serum prevented colonization. These experiments demonstrate that fimbriae are true virulence determinants.

One must realize that all fimbriae cannot be termed as virulence determinants, because enterobacteria have the capacity to produce a number of morphologically and antigenically diverse fimbriae which exhibit different receptor specificity. This indicates that individual bacteria may express distinct adhesins under different environmental conditions and only some of these may promote colonization.

Fimbrial adhesins present on enteropathogenic strains of *E. coli* which cause diarrhea in humans are called human colonization factors (CF) (Levine, 1987). Four human colonization factors have been identified. They are CFA/1, CFA/11 (Evans *et al.*, 1975, 1978), E8775 (Thomas *et al.*, 1982), and PCF 0159 (Tacket *et al.*, 1987). CFA/11 consists of three antigenically distinct fimbriae designated CS1, CS2, and CS3 (Cravioto

et al., 1982; Smyth *et al.*, 1982). Expression of these antigens varies depending on the strains. Some strains express CS1 and CS3, and others express CS2 and CS3. Still others express only CS3. Subunit sizes of these antigens are 16.3 (CS1), 15.3 (CS2) and 14.7 (CS3) kd respectively. They are encoded by a single plasmid (Smith *et al.*, 1983). Morphologically CS1 and CS2 are 6-7 nm diameter rigid fimbriae, CS3 is a 2-3 nm flexible fibrillar structure (Mullany *et al.*, 1983; Smyth *et al.*, 1984; Levine *et al.*, 1984; Knutton *et al.*, 1984). From these studies a number of conclusions were made: (1) bacteria are able to produce multiple fimbrial adhesins with different antigenicity; (2) expression of these fimbrial adhesins varies between strains; and (3) the antigen common to all strains appears to be a fibril (CS3) rather than a fimbriae. The CFA results resembled observations made with K88 (K88ab, K88ac and K88ad) antigens of enteropathogenic *E. coli* of porcine strains. (Gaastra *et al.*, 1982). Colonization factor E8775 exhibited characteristics (McConnell *et al.*, 1985) similar to CFA/11.

It has become apparent with genetic analysis of fimbriation that the receptor binding component can be different from the fimbrial structural subunit. For example, it has been shown that, (Maurer *et al.*, 1985; Minion *et al.*, 1986) a distinction exists between the adhesin molecule and the type I fimbriae of *E. coli*. Clegg *et al.* (1987) have demonstrated that mutants can be constructed from *Klebsiella pneumoniae* which possess morphologically intact fimbriae which lack the ability to agglutinate guinea pig erythrocytes. This implies that adhesin responsible for recognition of receptors is a moiety separate from that of the fimbrial structural element. This could lead to the production of fimbriae which differed in the structural subunit but which had a common adhesive subunit (Purcell *et al.*, 1987).

Among Gram-positive bacteria *Corynebacterium renale* has been shown to possess fimbriae (Kumazawa *et al.*, 1972). They are produced in bundles (0.4 x 10 μ m), made up of a number of individual fimbriae each 3 nm in diameter. Others have identified structures which appear to be fimbriae from *Actinomyces viscosus*, *Actinomyces naeslundii*, *S. salivarius*, and *S. sanguis* (Cisar *et al.*, 1984; Handley *et al.*, 1984 and 1985; Fachon-Kalweit *et al.*, 1985) .

Fimbrial subunits from most Gram-positive organisms are not well characterized, because

they do not dissociate into monomers (Wheeler *et al.*, 1980; Weerkamp *et al.*, 1981), which can be analysed by standard biochemical techniques. An approach to overcome this problem has been to clone the structural genes. This technique has been used to show that the subunits structure of type 1 and type 2 *A. viscosus* fimbriae have M_r s of 65,000 (Yeung *et al.*, 1987) and 59,000 (Donkersloot *et al.*, 1985) respectively.

Type 1 fimbriae

Type 1 fimbriae of *E. coli* mediate the mannose-sensitive (MS) adherence to eucaryotic cells and the agglutination of guinea pig erythrocytes. The fimbriae are 7 nm in diameter, approximately 2 μ m long in length, and are composed of protein subunits having M_r of 17,000 (McMichael *et al.*, 1979).

Genes responsible for type 1 fimbriation have been cloned from uropathogenic *E. coli* J96 (Orndorff *et al.*, 1984a, 1984b, 1985a, and 1985b, Maurer *et al.*, 1985, and 1987) and an *E. coli* K-12 derivative PC31 (Klemm *et al.*, 1984, 1985, and 1987). The genes responsible are arranged in similar gene clusters as operons. The genes are designated as *pilA-E* in *E. coli* J96 and *fimA-H* in *E. coli* PC31. *pilA* and *fimA* genes encode for an identical protein subunit with M_r of 15,706.

The order of organization of the genes is similar. In the *pil* operon the genes are arranged as *pil* 'E-F-D-C-B-A-hyp'. The *pilB* and *pilC* genes code for 30,000 and 86,000 M_r proteins that are involved in fimbrial assembly and anchorage. The 86,000 M_r protein is thought to be the membrane anchor. The *hyp* region codes for a 23 kd protein which has been shown to be involved in the negative regulation of transcription of the *pilA* gene. Hyp⁻ mutants exhibited a forty fold increase in the number of pili per cell. Maurer and Orndorff *et al.*, (1987) using isogenic mutants generated by transposon mutagenesis showed that mutations in *pilE* (which codes for a 31,000 M_r protein) resulted in a nonadhesive pilated phenotype, whereas *pilF* mutations (*pilF* codes for a 18,200 M_r protein) showed a striking increase in pilus length. The authors suggested that the *pilF* gene product acts as a competitive inhibitor of pilus polymerization.

In the *fim* operon the genes are arranged as *fim* 'B-E-A-C-D-F-G-H' . *FimB* and *fimE*, code for 25,000 and 23,000 M_r proteins which have been found to control the phase dependent expression of fimbriae, by directing the periodic inversion of a 300 bp DNA segment that contains the promoter of the *fimA* gene. The *fimD* genes code for a 89,000 M_r protein believed to anchor the fimbriae to the cell membrane.

Klemm *et al.*, (1987) used deletion and complementation analysis to show that the *fimF*, *fimG*, and *fimH* genes that code for 17,500, 16,000 and 32,000 M_r proteins played a role in the fimbrial morphology as longitudinal modulators. They suggested that these genes appeared to control the length and the number of the fimbriae. The DNA sequence of *fimF*, *fimG*, and *fimH* exhibited homologies with the fimbrial subunit gene *fimA*. Subsequently Abraham *et al.*, (1987) using antibodies raised against synthetic oligopeptides of the NH₂ terminal regions of *fimG* and *fimH* gene products demonstrated that antibodies to *fimH* proteins bound to fimbrial tips and at long intervals along the fimbrial filament. The antibody inhibited the adherence of type 1 fimbriated *E. coli* to epithelial cells.

It has become clear from studies on type 1 fimbriation that, (a) the receptor binding component is different from that of the fimbrial subunit, (b) the control of expression is regulated metastably at the transcriptional level (Eisenstein, 1981) due to periodic inversion of a 300 bp DNA segment , and (c) fimbrial length and fimbrial number are negatively regulated by associated genes.

Pap pili

A chromosomal DNA fragment which encodes for proteins involved in MR haemagglutination and uroepithelial attachment was isolated from the uropathogenic clinical *E. coli* strain J96 (Normak *et al.*, 1983; Norgren *et al.*, 1984; Uhlin *et al.*, 1985; Lindberg *et al.*, 1986). The *pap* genes were arranged in similar fashion to the type 1 fimbriae described earlier.

The role of type 1 fimbriae and *pap* pili were investigated in urinary tract infections using

mutants lacking either or both fimbriae. The type of pili expressed by the organism affected its ability to colonize mice (Hagberg *et al.*, 1983). Organisms with type 1 fimbriae were recovered in large numbers from the bladder. The bacteria colonizing the kidneys showed a loss of type 1 fimbriae while retaining the pap pili. Bacteria having only the pap pili were less effective colonizers. This is because type 1 fimbrial receptors are present in bladder epithelium and pap pili receptors in kidney tissues. When the bacteria reaches the kidney tissues it loses type 1 fimbriae which are no longer necessary. In addition phagocytes in the kidney tissues can remove type 1 fimbriated bacteria because they have receptors on the surface which can bind to the type 1 fimbriae. This clearly demonstrates that a single strain of bacteria has evolved mechanisms to evade host defenses by possessing two fimbrial adhesins with different adherence specificities.

Most pyelonephritis-associated *E. coli* strains have other types of fimbriae in addition to pap pili and type 1 fimbriae. These include S fimbriae (Korhonen *et al.*, 1984), type 1C fimbriae (Klemm *et al.*, 1982), M fimbriae (Jokinen *et al.*, 85), O75X fimbriae (Vaisanen-Rhen, 1984a) and a variety of X fimbriae (Vaisanen *et al.*, 1981; Vaisanen-Rhen *et al.*, 1984b) which can act as adhesins. The O75X fimbrial adhesin is specific for Bowman's capsule and interstitial areas of the kidney tissues (Nowicki *et al.*, 1987). The S fimbriae are specific for sialic acid residues of human erythrocytes. DNA hybridization studies with S fimbriae genes have shown that there is a high degree of homology between S fimbriae, and type 1C fimbriae and to a lesser extent with pap fimbriae, and the type 1 fimbriae (Ott *et al.*, 1987).

Fibrils/fibrillae

Among streptococci, the term "fuzzy coat" was created to describe extracellular surface appendages in *S. sanguis* (Mouton *et al.*, 1980), *S. mutans* (Nalbandian *et al.*, 1974), *Streptococcus mitis* (Liljemark *et al.*, 1972) and *Streptococcus pyogenes* (Ellen *et al.*, 1972). More detailed analysis of these structures in *S. sanguis* and *S. salivarius* resulted in the following observations (Handley *et al.* 1984, and 1985). The surface of these organisms possessed structures which were long wiry, flexible, and did not have a

constant width. They were categorized as fibrils, and not fimbriae because they did not have a defined width. The fibrils were peritrichous, polar or on the sides (tufted) of the bacteria. They were divided into long fibrils (150-210 nm) and short fibrils (70-110 nm). The location and the presence of fibrils were used to subdivide various strains of *S. salivarius* and *S. sanguis*. The authors associated the lack of different fibrils with specific adhesive properties in *S. sanguis*. Fibrils present on *S. salivarius* have now been divided into four distinct groups depending on their adhesive properties (Weerkamp *et al.*, 1986). In addition to the fibrils, some of the strains possessed fimbriae similar in structure to those found in Gram-negative organisms.

As mentioned earlier, adhesins which have a fibrillar structures have been identified in enterotoxigenic *E. coli* (Jacobs *et al.*, 1987) and *Yersinia* (Kopperud *et al.*, 1987).

Carbohydrates

Adherence can be mediated by carbohydrates which associate with the cell wall. This material has been called capsule and more recently glycocalyx (Costerton *et al.*, 1978). Extracellular glucan synthesized by *S. mutans* is involved in mediating the attachment of this organism to teeth and to other *S. mutans* (Hamada *et al.*, 1980). *S. mutans* possesses a glucan binding protein as well as a variety of extracellular enzymes which synthesize water soluble and water insoluble glucans.

S. sanguis 34 has carbohydrate receptors which mediate coaggregation with *A. viscosus* and *A. naeslundii*. The receptor has been isolated and found to consist of repeating subunits of hexasaccharides containing *N*-acetylglucosamine, galactose, glucose, and rhamnose in a ratio of 2:2:1:1 joined together by phosphodiester bonds (McIntire *et al.*, 1985).

Teichoic acids mediate the binding of *S. aureus* to the amino and carboxy terminal regions of the fibronectin on squamous epithelium. (Kuusela *et al.*, 1984; Aly *et al.*, 1987). This finding was based on the observation that teichoic acid coated fibronectin failed to inhibit attachment while teichoic acid alone inhibited attachment of this organism to nasal

epithelium. It was also noted that neonatal nasal epithelium lost its binding ability with age because the tissues became devoid of fibronectin as they matured into adult nasal epithelium.

Lipoteichoic acids

The cell wall amphiphiles, LTA and LPS which are found in Gram-positive and Gram-negative bacteria respectively have been shown to function as adhesins in some species of bacteria. LTA has been suggested to function as an adhesin in *S. pyogenes* and Group B streptococci. LPS is recognized as an adhesin mediating binding of *Agrobacterium tumefaciens* (Lippincott *et al.*, 1980) and *Rhizobium trifoli* (Dazzo *et al.*, 1975, and 1983) to plant cell walls and clover root hair.

Nealon *et al.*, (1984) have shown that LTA mediates the attachment of Group B streptococci to neonatal epithelial cells. Studies using deacylated and acylated LTA, they have shown that the polyglycerol backbone of the LTA binds to the fibronectin receptor (Butler *et al.*, 1987). Group B streptococci adhere preferentially to neonatal rather than adult epithelial cells. Organisms freshly isolated from patients possess higher level of LTA than laboratory strains. Neonatal cells have receptors for the LTA adhesin which are subsequently lost as the infant ages (Broughton *et al.*, 1983; Teti *et al.*, 1987). This is thought to be one reason for the high susceptibility of infants to Group B streptococcal infections.

A number of workers (Beachey *et al.*, 1975, 1976, and 1987; Ofek *et al.* 1982, and 1983; Simpson *et al.*, 1987) have concluded that the binding of *S. pyogenes* to epithelial cells is mediated by LTA. This conclusion was based on the following observations: (1) Purified LTA or anti-LTA serum inhibited attachment to buccal epithelial cells; (2) Proteolytic treatment removed LTA from the bacterial surface and reduced binding to buccal epithelial cells; (3) Removal of the lipid portion of LTA renders it incapable of binding to the cell and destroys its ability to inhibit attachment of *S. pyogenes*. (4) Restoration of the lipid moiety restores the binding and inhibitory functions. (5) Bacteria

grown on sublethal concentration of antibiotics lost both LTA and the ability to bind. (6) LTA was associated with M protein fibrils on the surface of *S. pyogenes*; (7) LTA forms complexes with purified M protein *in vitro* even after the removal of the lipid moiety; and (8) The cell surface of *S. pyogenes* is hydrophobic (Moirner *et al.*, 1983). Beachey *et al.*, (1987), have suggested that the polyglycerol phosphate backbone component of the LTA intercalates with M protein leaving the lipid moiety free to interact with the buccal epithelium.

The hypothesis put forward by Beachey *et al.*, (1987) has been the subject of debate. It is generally believed that the lipid moiety of LTA is inserted into the streptococcal cytoplasmic membrane and thus is not available to interact with epithelial cell receptors as postulated. The studies mentioned in the preceding paragraph were done with buccal epithelial cells, whereas pharyngeal epithelium is the natural habitat of the organism. Early studies by Ellen *et al.*, (1972) had shown that the loss of M protein reduced binding of the organism to pharyngeal epithelium. Recently Tylewska *et al.*, (1988) have shown that binding of *S. pyogenes* to pharyngeal epithelial cells is mediated by M protein and not by LTA. The evidence against LTA being the adhesin is as follows: (1) M⁺ *S. pyogenes* cells bound in higher numbers to pharyngeal epithelium than M⁻ phenotypes; (2) Adhesion to pharyngeal epithelium involved a lectin-like interaction because binding was inhibited by fucose and galactose. (in contrast, these sugars have no effect on the binding of LTA to epithelial cells); (3) Lectins specific for fucose and galactose bound in higher numbers to human pharyngeal epithelium than to buccal epithelium; and (4) The binding of LTA does not reflect the specificity seen in the binding of whole cells.

M protein of *S. pyogenes*

Attempts to isolate M protein by conventional methods have proven difficult. Antigenic variation among the M proteins has given rise to more than 70 serotypes (Lancefield, 1968). There is considerable confusion about the size of the molecule. Type 12 serotype M protein isolated from *S. pyogenes* missing its cell wall had a M_r of 58 kd (Van de Rijn *et al.*, 1981). But when this protein was secreted into the medium, it was found to have been

cleaved into a soluble 32 kd molecule. Others have characterized proteolytic digests of M protein (Beachey *et al.*, 1974). which gave rise to various molecular weights depending on the method, enzyme and the serotype strain used. Analysis by recombinant DNA techniques has proven to be the most useful procedure for gaining insight into the molecular nature of the protein. The type 12 M protein gene has been shown to encode for a 73 kd protein, a molecule considerably larger than that indicated by other techniques. (Scott *et al.*, 1983).

The gene coding for type 6 M protein was cloned into *E. coli* and the DNA sequence has been determined (Hollingshead *et al.*, 1986). The protein has a M_r of 53,466. The carboxy terminal end contains a membrane anchor region homologous to other Gram-positive surface proteins. Adjacent to the anchor region there is a proline rich region which is believed to be important in traversing through the thick cell wall of the Gram-positive bacteria. Between the proline rich region and the N-terminal region there are two extended tandem repeat regions, A and B, followed by a non-tandem repeat region C. Region A has five repeats of 42 nucleotides and region B has two repeats of 75 nucleotides. The non-tandem repeat C has two regions of 81 nucleotides. Duplication and/or deletion of these regions could give rise to diversity in size, sequence and antigenicity of the M protein in a manner similar to that seen in *N. gonorrhoeae* pilin genes (Hagbolm *et al.*, 1985)

Recently, the partial sequence including the regulatory regions of the type 12 M protein has been determined (Robbins *et al.*, 1987). There were similarities between type 12 and type 6 M proteins in the sequenced regions. The signal peptides were nearly identical (41 amino acids), and the carboxy terminal end showed 98 % homology. There were similar membrane anchor and proline rich regions. The repeat A and B regions varied in homology and in the number of tandem repeats. There was 99% homology in the C repeat regions.

The type 5 M protein gene has been cloned and found to produce a number of proteins ranging in M_r from 64-50 kd. All these proteins reacted with type 5 M antiserum (Kehoe *et al.*, 1985). Type 5 M protein DNA probes hybridized with a number of other regions in the chromosome giving rise to the possibility that there are multiple copies of the genes.

Transcripts of the 3' third of the gene of type 6 M protein hybridized with 10 different serotypes suggesting conservation in the region which functions essentially to anchor the protein to the cell wall (Hollingshead *et al.*, 1987).

Deletions of sequences upstream from the type 12 M protein genes resulted in the loss of expression of M protein. Such spontaneously occurring variants were isolated at high frequencies (Simpson *et al.*, 1987). Some M⁻ phenotypes exhibited no deletions but the phenotypes were unstable and reversible. Two regulatory regions were identified in type 12 M protein DNA sequences. The authors suggested these sequences may be involved in regulating the M⁻ phenotypes.

Genes encoding for similar M proteins have been cloned from a clinical isolate of Group G streptococci (Bisno *et al.*, 1987). The proteins cross-reacted with some of the monoclonal antibodies raised against Group A streptococcal M protein. DNA hybridization studies between cloned M protein genes of both groups exhibited homologies suggesting a common evolutionary linkage (Jones *et al.*, 1987).

Non-fimbrial proteinaceous adhesins

A number of non-fimbrial proteins have been identified and characterized in microorganisms that mediate attachment to human tissue. Cytoadherence of *Mycoplasma pneumoniae* is mediated by a 165 kd protein, P1 (Baseman *et al.*, 1982). This adhesin binds to respiratory epithelial cell receptors via terminal sialic acid residues. Monoclonal antibodies, which block this adhesion, have been shown to cross-react with a 140 kd protein from *Mycoplasma genitalium* isolated from urethral specimens (Plummer *et al.*, 1987; Hu *et al.*, 1987). An 18 kd cytoadherence protein has been cloned from *Chlamydia trachomatis* (Kaul *et al.*, 1987). Opacity-associated protein II from *N. gonorrhoeae* has been shown to be involved in binding to cultured human cell lines (Bessen *et al.*, 1987). A 190 kd protein from *S. mutans* has been shown to mediate binding to salivary coated tooth pellicle *in vitro* (Douglas *et al.*, 1985). Two different non-fimbrial adhesins from *E. coli* were found to bind specifically to human kidney epithelial cells

(Goldher *et al.*, 1987). The adhesins designated NFA-1 and NFA-2 were made up from 21 and 19 kd subunits.

Phase variation

The composition and organization of the cell surface varies markedly from species to species and indeed from strain to strain making it important to clearly define the strain being studied. In fact, some of the confusion surrounding studies of adherence originate because the experiments are done with strains having different surface properties (Gibbons *et al.*, 1982). Sometimes phase and antigenic variation associated with adhesins has led to confusion about the occurrence of these structures, and hence their specific roles in adherence.

Phase variation of surface components in some bacteria is regulated by a process that involves rearrangement of DNA. Phase variation of *Salmonella* flagella is due to reversible "flipping" of a specific DNA sequence located close to the flagellar structural genes (Simon *et al.*, 1980). Phase variation of *E. coli* type 1 fimbriae is regulated transcriptionally and is due to reversible inversion of a novel sequence associated with the fimbrial structural gene (Eisenstein, 1981). The extent of fimbriation is also affected by a *trans*-acting 23 kd protein as described above (Orndorff *et al.*, 1984b). Phase and antigenic variation of gonococcal pili are not due to an "on-off" switch in orientation of a "flipper" sequence, but rather to a complex interaction between the silent and expressed *pil* sites (Mayer *et al.*, 1982 and 1984)

One of the characteristics which contributes towards changes in surface components has the growth environment of bacteria (Knox *et al.*, 1985). Rosan *et al.* (1982b) have demonstrated that growth rate, growth pH, and carbohydrate source will influence the ability of *S. sanguis* to bind to saliva derived tooth pellicle. Fimbrial structures of enterotoxigenic *E. coli* ((Gaastra *et al.*, 1982) and fibrillae of *Yersinia* species are not expressed at 18°C, but expressed at 37°C *in vitro* (Kapperud *et al.*, 1985). Changes in ultrastructural and surface protein profiles were observed in a periodontal pathogen, *Actinobacillus actinomycetemcomitans*, when it was cultured, with and without oxygen

(Scannapieco *et al.*, 1987)

Receptors

The identification and characterization of receptors were initially based on inhibition of haemagglutination by specific sugars and lectins. α -D-mannose (Duguid *et al.* 1980), D-galactose (McIntire *et al.*, 1978), sialic acids (Baseman *et al.*, 1982) and L-fucose (Jones *et al.*, 1977) are among the sugars that have been identified as receptors. These studies gave valuable, but limited information about the molecules which play a role in adhesion. Although a number of adhesins bind to the same terminal sugar moiety (eg. mannose), adjacent carbohydrates, and the configurations of these carbohydrates provide specificity and receptor diversity.

A. naeslundii haemagglutination is inhibited by the galactose containing disaccharide, lactose, but not by the monosaccharide α -D-galactose (McIntire *et al.*, 1985). Globotetraosylceramide, glycolipids with the trisaccharide galactose-N-acetyl-galactosamine-galactose, act as receptors for P fimbriae of uropathogenic *E. coli* (Swanborg-Eden *et al.*, 1985). Either modification or replacement of any of the monosaccharides affected the ability of the glycolipid efficiently to bind the adhesin.

Receptors from human cells have been characterized by enzymatic, chemical, and physical means. Type 2 fimbriae of *A. naeslundii* WVU45 attached to epithelial cells glycosphingolipids separated on thin layer silica gel plates (Brennan *et al.*, 1987). By treating the chromatogram with appropriate enzymes, the Actinomyces lectin was shown to recognize the Gal β GalNAc termini of gangliosides and the GalNAc β 3gal terminus of globosides present on mammalian cells. Radiolabelled bacteria have been used as probes to identify receptors from cell extracts that have been blotted onto nitrocellulose paper. Using this technique, Korhonen *et al.* (1984) identified a fimbrial binding sialic acid receptor from pathogenic *E. coli*. Removing the terminal sialic acid with neuraminidase, and then replacing it at different positions with various sialyl transferases showed that the receptor was a sialoglycophorin with a terminal O-linked NeuAc α 2-3Gal β 1-3GalNAc sugar moiety (Parkkinen *et al.*, 1986).

S. sanguis

A detailed discussion of *S. sanguis* will be presented in the following section. *Streptococcus sanguis* is the name given to the species of alpha hemolytic streptococci originally isolated from the blood of patients with subacute bacterial endocarditis (White *et al.*, 1946). Before the advent of appropriate taxonomic criteria the organisms were grouped together with other oral streptococci as *Streptococcus viridans* (Carlsson, 1968). The primary habitat of *S. sanguis* is the tooth surface where it colonizes in large numbers and forms an important part of the indigenous oral flora (Gibbons *et al.* 1975a, and 1975b). It was demonstrated by Carlsson *et al.* (1970) that *S. sanguis* appear, in the mouth only after the eruption of teeth and begins colonization of teeth before other streptococci appear on this surface.

S. sanguis hydrolyzes arginine and esculin and produces glucan from sucrose (Facklam, 1977). These metabolic characteristics differentiate *S. sanguis* from other oral, alpha hemolytic streptococci. The organism can also be differentiated from other oral streptococci by colonial morphology on Mitis Salivarius agar. Typical *S. sanguis* colonies are small, hard firmly attached to the agar and surrounded by a small moat (Hamada *et al.*, 1980).

The classification of strains of *S. sanguis* has been confused by conflicting serological and biochemical data. Even today some authors classify certain strains of *S. sanguis* as *Streptococcus mitior* (Rosan, 1978). The confusion surrounding the nomenclature of *S. mitior* derives from the fact that the organism has been classified by negative criteria. i.e.: because an isolate does not possess a certain characteristic it is presumed to be *S. mitior*.

The characterization of the different *S. sanguis* serotypes is summarized in Table I. Five serotyping antigens have been identified (Rosan, 1973). Serotype I lacks b antigen and the

Table I. Classification of *S. sanguis*.

Characteristics	Serotyping			References
	I	II	heterogeneous	
<u>Antigens</u>				
constant	a	a,b	-	Rosan, 1973
variable	c,d,e	c,d,e	b,c,d	Rosan, 1976a Rosan <i>et al.</i> , 1976b
<u>Cell wall</u>				
rhamnose	high	high	low	Slade <i>et al.</i> , 1962
phosphorus	low	low	high	Cole <i>et al.</i> , 1976
ribitol	low	low	high	Coleman <i>et al.</i> , 1965
glucose	+	+	-	Facklam, 1977
galactose	+	-	+	Hardie <i>et al.</i> , 1976
LTA	+	+	-	Rosan <i>et al.</i> , 1982c
<u>Acid from</u>				
inulin	+	+	-	Facklam, 1977
arginine	+	+	-	Hardie <i>et al.</i> , 1976
esculin	+	+	-	Coleman <i>et al.</i> , 1965
sucrose	+	+	+ / -	
%GC ¹	41-43	44-46	43-44	Coykendall <i>et al.</i> , 1975
Biotype	I	I	II	Farmer, 1954
Reaction with group H antisera ²	+	+ / -	-	Porterfield, 1950

¹, 41-43, 43-44, and 44-46 are referred to as group 1, 11, and 111 respectively.

², +, -, and +/- are referred to as type 1, 11, and 1/11 respectively.

heterogeneous group which is considered by some to be *S. mitior* lacks antigens a and e. Serotype antigen a was identified as LTA (Rosan *et al.*, 1982c). Antigen b is a polysaccharide which was originally thought to contain glucose, rhamnose and phosphorus (Appelbaum *et al.*, 1978) but was subsequently shown to contain glucose:rhamnose:*N*-acetylglucosamine (1.4:2.5:1.0) (Okahashi *et al.*, 1983).

Serotype I and II contain relatively large amounts of rhamnose, no ribitol and low levels of phosphorus in their cell wall (Cole *et al.*, 1976; Hardie *et al.*, 1976). This group is also designated as biotype I (Farmer, 1954). Galactose is found in serotype I but not in serotype II strains (Cole *et al.*, 1976; Hardie *et al.*, 1976). The heterogeneous group had low levels of rhamnose and larger amounts of ribitol and phosphorus (biotype II) in their cell wall.

Nutritionally serotypes I and II were similar to but distinct from the heterogeneous group. The %GC content provided convincing evidence that the heterogeneous group was distinct from serotype I and II (Coykendall *et al.*, 1975). Also serotype I and II have peptidoglycan linked by di- or tri- alanyl cross bridges while the heterogeneous group contained alanyl-lysyl cross bridges (Rosan, 1976a). The various strain designations that have been reported in the literature are summarized at the bottom of Table I.

Viridans streptococci are the most prevalent bacteria causing subacute bacterial endocarditis (SABE). *S. sanguis* has been isolated from 30-40% of diagnosed cases (Bayliss *et al.*, 1983; Roberts *et al.*, 1979). The mechanisms involved which enable usually non-invasive inhabitants of the oral cavity to cause SABE is unknown. Damaged heart valves are presumed to be a predisposing factor. Such damaged tissue would expose components which would induce platelet aggregation and thrombus formation. Herzberg *et al.*, (1985) has proposed that the SABE thrombi serve as a substrate for *S. sanguis* platelet aggregation and new thrombi formation. They initially isolated two *S. sanguis* antigens which mediated adherence to platelets via a class 1 antigen and induced platelet aggregation through a class 11 antigen. Both antigens were proteins. The class 1 antigen was isolated from a three minute tryptic digest of whole cells. The class 11 antigen was

(M_r 65 kd) the only antigen present after a 30 min tryptic digestion. By screening dental plaque isolates, they were able to isolate *S. sanguis* L74 which adhered to platelets, but was unable to aggregate platelets. This confirmed the previous observation that the two components were distinct antigens. Subsequently they isolated another component which is involved in platelet aggregation. This was designated as class 111 antigen and was identified as a *S. sanguis* cell surface ecto-ATPase.

In the following sections of this thesis, the general characteristics of the oral cavity as they affect microbial adherence will be discussed. This will be followed by a detailed discussion of the adherence properties of *S. sanguis*.

Microbial adherence in the oral cavity

Three types of surfaces exist in the oral cavity. They are shedding surfaces of keratinized or non-keratinized epithelium and nonshedding mineralized tissue (teeth). The chemical composition of the epithelial surfaces will be modified by keratinization and probably differs from one ecosystem to another thus providing a multitude of potential receptors.

Oral surfaces are subjected to a number of modifying influences including the effect of saliva, crevicular fluid and ingested food. Saliva secreted by major and minor salivary glands continuously rinses oral surfaces to remove non-adhering organisms. Crevicular fluid plays the same role in the gingival crevice. Both fluids supply nutrients to the indigenous microflora and remove waste products.

The food we ingest can alter the pH and depending on its composition, it favors the colonization of certain organisms (Gustafsson *et al.*, 1954). Fermentation of carbohydrates leads to the production of acids and the selection of an acidogenic flora. This can result in the acid decalcification of teeth. The most profound effect is caused by sucrose which in addition to being fermented, is converted to high M_r glucans which bind *S. mutans* to the teeth (Clark *et al.*, 1977).

The indigenous flora of the oral cavity is a complex mixture of bacteria. Socransky *et al.* (1982) has estimated that at least 300 species will be eventually identified. Moore *et al.* (1982) have identified over 264 species in the gingival crevice alone. These organisms are arranged into unique ecosystems. They modify their environment by consuming oxygen, altering pH, removing nutrients, releasing metabolic end products, and elaborating enzymes which may change the nature of the bacterial receptors on immobilized surfaces.

The most studied ecosystems of the oral cavity include the tongue, buccal surfaces, tooth and gingival crevice. The tongue is a relatively aerobic environment which is thought to be the area of the most rapid cell proliferation (Gibbons *et al.*, 1972; Weerkamp *et al.*, 1980a). *S. salivarius* is found here and not on the tooth probably because of its ability to attach to tongue epithelium. Buccal surfaces are a sparsely populated aerobic ecosystem colonized by a restricted number of organisms (Gibbons *et al.*, 1972).

The area between the tooth and the gum, known as the gingival crevice supports the growth of a strict anaerobic population. The gingival crevice is relatively isolated from other oral ecosystems (Gibbons *et al.*, 1964, 1975a, and 1975b). Organisms have the possibility of binding to non-keratinized epithelium or to the subgingival regions of the tooth. Food is supplied by the crevicular fluid, a nutrient rich serum transudate which seeps into the crevice from the gingiva. The particular bacterial species that are present in the crevice reflects the state of health of the gingival tissues. In the healthy state the predominant organisms are Gram-positive facultative rods and cocci. In the diseased state there is a change to Gram-negative anaerobic rods (Socransky, 1970).

Another unique site in the oral cavity is the tooth. The exposed surface of the tooth is enamel which is composed primarily of hydroxyapatite (Rolla, 1977). Hydroxyapatite is amphoteric due to both phosphate groups and calcium ions being exposed on its surface and therefore both acidic and basic components bind to this material. Since many more phosphate groups than calcium ions are exposed, hydroxyapatite crystals have a net negative charge.

The dental enamel is covered by a thin organic layer termed the acquired pellicle (Leach,

1967; Meckel, 1968). The pellicle is generally less than a micron thick and is thought to be formed by the selective adsorption of salivary constituents (Hay *et al.*, 1967).

Dental plaque

Bacterial accumulations on the tooth surfaces are commonly referred to as dental plaque. Plaque consists of dense masses of bacteria embedded in an amorphous matrix which is thought to contribute to its structural integrity (Gibbons *et al.*, 1975a, 1975b, and 1984). The matrix consists of bacterially synthesized polymers and components derived from saliva, crevicular fluid and the diet. Plaque tends to form most rapidly on protected areas of the teeth but in time will cover all smooth surfaces as well.

The bacterial composition of the dental plaque differs with plaque age and with various stages of the dental diseases associated with the tooth. When dental plaque is associated with teeth above the gingival tissue it is designated as supragingival plaque, and when it is below the gingiva it is called subgingival plaque. Mature dental plaque consists of Gram-positive filaments, *Fusobacterium*, *Neisseria*, *S. sanguis*, *S. mitis*, and small percentages of *Lactobacillus*, *Veillonella* and *Bacteroides*. *S. mutans* may comprise 0-50% of the bacteria cultivable from dental plaque (Gibbons *et al.*, 1975a, 1975b, and 1984; Hamada *et al.*, 1980).

Cell proliferation and surface interactions are overtly involved in the development of dental plaque. Three types of cell to surface interactions are possible. Cells can absorb selectively to the acquired pellicle on the tooth surface. Subsequently, by means of homotypic cell-cell interactions, cell of one species may accumulate and maintain their position in the growing plaque. Heterotypic cell-cell interactions leading to coaggregation between species are also important. With regard to the latter it has been shown in *in vitro* studies that there are many combinations of oral organisms which can coaggregate when mixed together (Gibbons *et al.*, 1970, 1975a, 1975b; Hay *et al.*, 1971; Kolenbrander *et al.*, 1985). McBride *et al.*, (1981) have demonstrated that coaggregation supports colonization of *Veillonella* in gnotobiotic rats infected with *Streptococcus*.

The ability of salivary constituents to form aggregates with a variety of oral streptococci is well documented and has been extensively reviewed by Bowden *et al.*, (1979) and Gibbons *et al.*, (1970, 1975a, 1975b, and 1984). Such interactions may promote homotypic bacterial aggregation in the plaque matrix as well as promote the removal of unattached bacteria by masking bacterial adhesins and blocking attachment to immobilized receptors. It is also thought that unattached aggregates of bacteria are more readily removed by salivary flow from the oral cavity. Salivary constituents bound to bacterial cell surfaces may also promote heterotypic bacterial aggregation (coaggregation) thus contributing to the species diversity seen in plaque (Ellen, 1985; Hamada *et al.*, 1980).

Aggregation

A number of salivary constituents have been implicated in the self-aggregation of *S. sanguis*. These include lysozyme (Laible *et al.*, 1982), immunoglobulin A (IgA) (Liljemark *et al.*, 1979) and mucinous glycoproteins (Levine *et al.*, 1978; Hogg *et al.*, 1979; Prakobphol *et al.*, 1982). Salivary mucins involved in aggregation carry blood group reactivity, although the bacterial receptor sites are apparently distinct from the oligosaccharides responsible for the A and B antigenic determinants (Gibbons *et al.*, 1978; Hogg *et al.*, 1982). Others have shown that aggregation of some oral streptococci can be mediated by non-mucinous glycoproteins (Eggert, 1979). IgA and other salivary constituents have been found to form complexes with mucins as well with high molecular weight non-mucinous glycoproteins (Clamp, 1977).

Unlike other oral streptococci, *S. sanguis* aggregation in saliva is sensitive to treatment with neuraminidase (McBride *et al.*, 1977; Levine *et al.*, 1978). Aggregation appears to be caused by one or more sialic-acid containing high molecular weight mucinous glycoproteins (Hay *et al.*, 1971; Hogg *et al.*, 1979) as well as by a sialic-acid containing monomeric mucin with a M_r of 200-250,000 (Prakobhol *et al.*, 1982). Murray *et al.*, (1982) have isolated a sialic acid binding lectin from *S. sanguis*, which is involved in hemagglutination. The role of the lectin in binding to saliva-coated hydroxyapatite (S-HA)

has not been studied. *S. sanguis* 12, the organism studied in our laboratory does not hemagglutinate red blood cells, thus the lectin identified by Murray *et al.*, (1982) is unlikely to be present in strain 12.

Different *S. sanguis* strains were tested for their ability to aggregate in saliva and bind to S-HA (Rosan *et al.*, 1982d). Salivary aggregation was quantitated by adding radiolabelled cells to saliva, and removing the aggregates by low speed centrifugation. The number of cells aggregated varied from 3% to 67%. There was no correlation between salivary aggregation and serotype of the organism. The question as to whether aggregation and binding to S-HA involved different salivary components was resolved by showing that when the aggregating activity of saliva was depleted by repeated absorption with an aggregating bacteria, the resulting saliva retained factors that were able to mediate adherence to S-HA.

It has been shown that non-immune serum and crevicular fluid can cause neuraminidase sensitive aggregation of certain strains of *S. sanguis*. Some strains aggregated by a neuraminidase sensitive reaction in both saliva and serum whereas others reacted only with saliva (Morris *et al.*, 1983). This raises the possibility that some strains may possess more than one sialic-acid recognizing adhesin. Crevicular fluid by itself was shown to inhibit attachment of *S. sanguis* 12 to S-HA (Cimasoni *et al.*, 1987).

Coaggregation

A number of investigators have shown that *S. sanguis* can coaggregate with various species of Gram-positive and Gram-negative oral bacteria in a reaction mediated by cell surface lectins. McIntire *et al.*, (1978) discovered lactose-sensitive coaggregation between *A. viscosus* T14V and *S. sanguis* 34. It was subsequently found that this coaggregation was mediated by *A. viscosus* T14V type 2 fimbriae binding to repeating units of hexasaccharides on *S. sanguis* 34 (McIntire *et al.*, 1985). Monoclonal antibodies raised against *A. viscosus* T14V type 2 fimbriae cross reacted with type 2 fimbriae on *A. naeslundii*. *A. naeslundii* exhibited similar lactose-sensitive coaggregation with *S. sanguis* 34 (Cisar *et al.*, 1982). Other studies have demonstrated that the phenomenon of

lactose-sensitive coaggregation is widespread among oral bacteria. More than 90% of fresh isolates of *A. viscosus*, *A. naeslundii*, and *S. sanguis* were found to coaggregate in such a manner (Kolenbrander *et al.*, 1982, and 1983). *S. sanguis* has been divided into six coaggregation groups based on the effect of lactose and heat on the coaggregation with *A. viscosus* and *A. naeslundii*. Other bacteria that have been shown to coaggregate with *S. sanguis* are *Bacteroides loeschii* (Weiss *et al.*, 1987b), *Haemophilus parainfluenzae* (Liljemark *et al.*, 1985), *S. mutans* (Schachtele *et al.*, 1976), *Propionibacterium acnes* (Ciardi *et al.*, 1987), *F. nucleatum* (Lancy *et al.*, 1983), *Capnocytophaga ochracea* (Weiss *et al.*, 1987a) and *Bacterionema matruchotii* (Mouton *et al.*, 1977; DiRienzo *et al.*, 1985).

Bacteria that are unable to bind to teeth by themselves can bind via coaggregation with other bacteria already on the tooth surface. Studies with *S. sanguis* DLI and *P. acnes* PK93 demonstrated that, the latter organism binds poorly to artificial tooth pellicle (Ciardi *et al.*, 1987). When the assay is done with *S. sanguis* DL1 bound to the artificial tooth pellicle adherence of *P. acnes* PK93 increased. The increased adherence was inhibited by lactose and *N*-acetylglucosamine indicating that the increased binding involved a lectin-like coaggregation. Weiss *et al.*, (1987b) have isolated a fimbrial adhesin on *B. loescheii* PK1295 which mediates lactose-sensitive coaggregation with *S. sanguis* 34.

Dental plaque has structures known as cornucobs (Jones, 1972). These structures develop due to streptococci forming an ordered array along a filamentous microorganism. *S. sanguis* is able to form cornucobs *in vitro* with *B. matruchotii* and *F. nucleatum* (Lancy *et al.*, 1983; DiRienzo *et al.*, 1985).

The ability of *S. sanguis* to form cornucobs with *F. nucleatum* and *B. matruchotii* has been investigated by DiRienzo *et al.*, (1985). *S. sanguis* CC5A isolated from a cornucob found *in vivo* (Mouton *et al.*, 1977) possessed three adhesins which bound specifically to *F. nucleatum* 364, *F. nucleatum* ATCC 10953 and *B. matruchotii* ATCC 14266. The *S. sanguis* CC5A - *F. nucleatum* 364 coaggregation was inhibited by *S. sanguis* LTA but not by deacylated LTA. Coaggregation with *F. nucleatum* ATCC 10953 was not affected by

LTA. The *S. sanguis* - *B. matruchotii* coaggregation was inhibited by both LTA and deacylated LTA suggesting that the polyglycerol phosphate portion of the molecule was the binding site.

In summary it can be seen that oral microorganisms have the possibility of binding to host tissue or to other bacteria. The interaction between the microorganism and the surface to which it is attached can be attributed to host synthesized polymers (e.g. saliva) or to bacterially derived polymers (e.g. LTA). Regardless of the mechanism the phenomenon of adherence is characterized by a high degree of specificity.

Adherence to saliva-coated hydroxyapatite

S. sanguis binds to tooth surface in the oral cavity, forming an important component of human dental plaque (Van Houte *et al.*, 1970; Gibbons *et al.*, 1975a). Adherence of *S. sanguis* to the tooth surface is believed to be mediated by salivary glycoproteins, which form a pellicle coating the tooth enamel (Clark *et al.*, 1978). For each salivary receptor, there must be a complementary bacterial adhesin, although little is known about the nature or variety of such components.

Adhesion to the tooth surface is studied in an *in vitro* adherence model which consists of adding increasing numbers of radiolabelled bacteria to a fixed number of hydroxyapatite beads (HA), or hydroxyapatite beads that are first treated with human saliva (S-HA) to form the *in vitro* equivalent of the acquired pellicle. These experimental pellicles have similar properties to a naturally acquired pellicle (Gibbons *et al.*, 1976; Clark *et al.*, 1978). Buffer treated hydroxyapatite beads are used as a control in these experiments.

This model system has been used to study the kinetics of binding. The binding isotherm is obtained by plotting the number of unbound cells (U) at equilibrium against the bound cells (B). In the absence of cell-cell binding, the binding isotherm (B vs U) will show saturation kinetics. Such adherence data can be fitted to the Langmuir adsorption isotherm (Langmuir, 1918), $U/B = K/N + (1/N) U$, where U is the number of unbound cells at equilibrium; B is

the number of bound cells; K is the dissociation constant and N is the maximum number of binding sites on the S-HA beads. A plot of U/B vs U allows an estimation of the dissociation constant K and its reciprocal, the affinity constant $1/K$ from the x intercept. The theoretical number of binding sites (N) on the S-HA beads can be estimated from the slope.

A mathematical representation of the Langmuir adsorption isotherm is the Scatchard equation: $[B/U = K(N-B)]$. The shape of the Scatchard plot will provide information relating to the nature of the interaction between bacteria and the receptor, and will indicate whether there is negative or positive cooperativity.

Binding and Langmuir isotherms were used to compare the adherence of different species of oral bacteria to S-HA and HA (Clark *et al.*, 1978). Certain species of bacteria (e.g. *S. sanguis*, *S. mitis*, *A. viscosus*) adhered better to S-HA than others (e.g. *S. mutans*, *S. salivarius*). These adherence patterns reflected the percentages of these bacteria isolated from human dental plaque.

Significantly more *S. sanguis* adhered to S-HA than HA (Clark *et al.*, 1978). Analysis of the Langmuir adsorption isotherm revealed a 36 fold increase in the number of binding sites when compared to the buffer treated control. This observation confirmed that saliva was acting as a modulator of the ecology of the teeth. Binding to HA was found to be relatively non-specific whereas binding to S-HA was a specific reaction and therefore selective. There was no competition for binding sites on S-HA between species of oral streptococci (Liljemark *et al.*, 1977). This data provided evidence that bacterial adhesins were recognizing specific receptors on the salivary constituents adsorbed to HA and demonstrated that different bacterial species do have unique adhesins recognizing specific S-HA receptors.

Molecular analysis has revealed that the binding of *S. sanguis* to S-HA involves a number of adhesins (Doyle *et al.*, 1982; Gibbons *et al.*, 1982, 1983a, and 1983c; Nesbitt *et al.*, 1982a, and 1982b; Morris *et al.*, 1984). Enzymatic modification of receptors on S-HA has shown that different strains of *S. sanguis* bind to different receptors. Treatment of S-HA with neuraminidase reduced its ability to bind *S. sanguis* C5, did not affect binding of

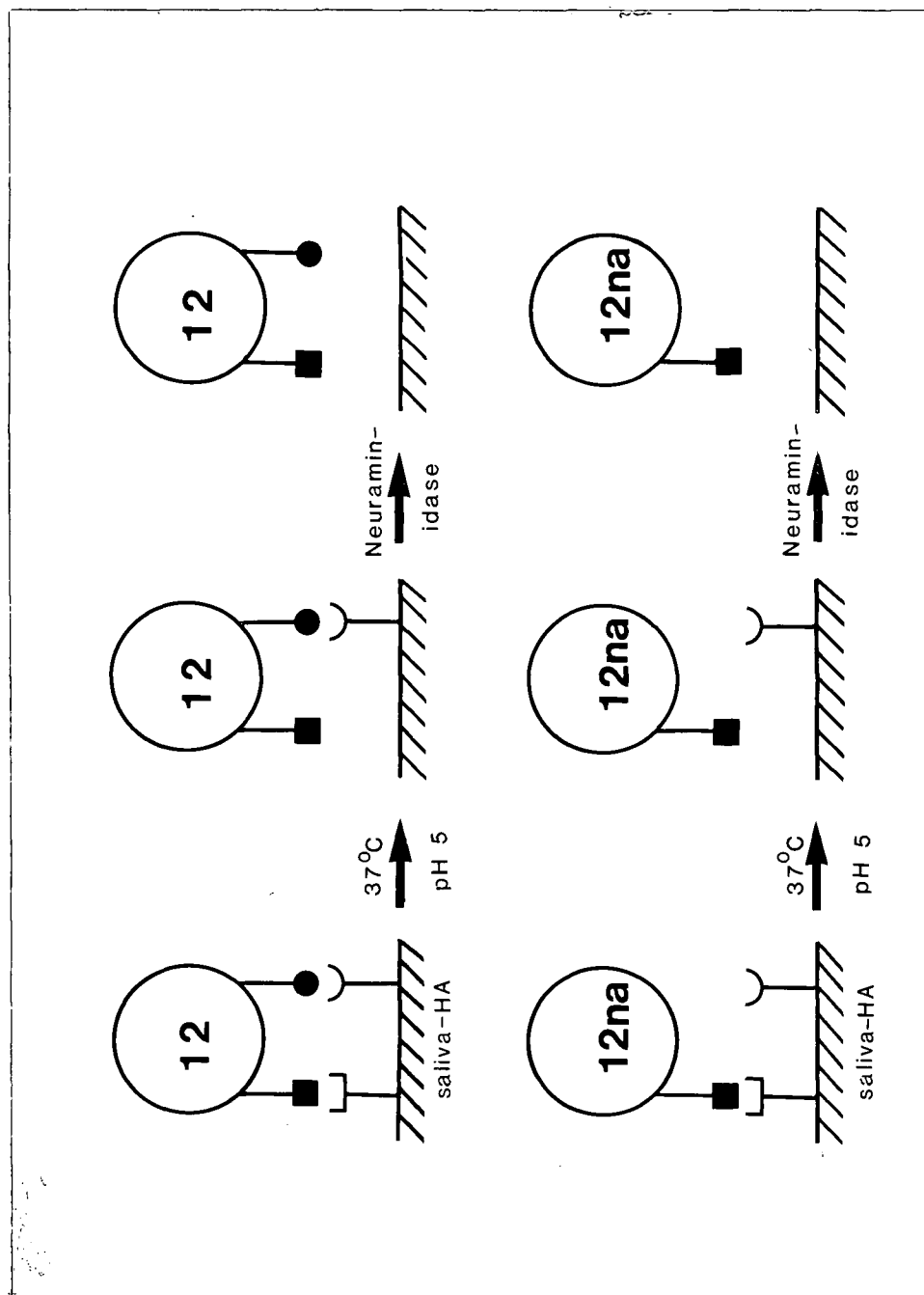
strain FC-1 (Gibbons *et al.*, 1982), and completely eliminated binding of strain 12 (Morris *et al.*, 1984). Kinetic analysis of the binding of *S. sanguis* C5 has shown that there are a small number of high affinity neuraminidase-sensitive salivary receptors and a larger number of lower affinity neuraminidase-insensitive receptors (Gibbons *et al.*, 1983a). Binding at both sites is sensitive to reagents which interfere with the formation of hydrophobic bonds (Gibbons *et al.*, 1983d). High affinity neuraminidase-sensitive sites were also described by Cowan *et al.* (1987) with *S. sanguis* ATCC 10556. These authors suggested these sites were responsible for the formation of a stable cell-substratum complex which was difficult to desorb. Doyle *et al.*, (1982) postulated that binding is dependent on the formation of lectin like or ionic bonds which are stabilized by hydrophobic interactions. Scatchard analysis of adsorption isotherms suggested that binding of *S. sanguis* to S-HA exhibited characteristics of positive cooperativity (Nesbitt *et al.*, 1982a; Morris *et al.*, 1984).

Morris and McBride (1984) compared the adherence properties of *S. sanguis* 12 and *S. sanguis* 12na (a variant deficient in salivary aggregation) to S-HA. Their results showed that strain 12 bound to S-HA via two types of salivary receptors. One receptor was sensitive to neuraminidase and the other receptor was sensitive to preincubation at pH 5.0 (37°C). Strain 12na has lost the adhesin which binds to the neuraminidase sensitive receptor, but retains the adhesin binding to the pH sensitive receptor. They proposed a two site binding model (Fig. 1).

These studies leave little doubt that adherence is a complex process involving a minimum of two types of cell-to surface interactions. The complexity is compounded by different strains having different adhesins is exemplified by a comparison of the effect of neuraminidase on the binding of *S. sanguis* 12, and *S. sanguis* FC-1 and *S. sanguis* C-5.

The nomenclature of the fibril-like surface structures present on *S. sanguis* were discussed by Handley *et al.*, (1985). They screened 36 strains of *S. sanguis* biotype 1 and 8 strains of *S. sanguis* biotype 11 for the presence fibrils and their ability to

Fig. 1. Model for adherence of *S. sanguis* 12 and 12na to S-HA. Symbols: ■, adhesin which binds to the pH-sensitive S-HA receptor; ●, adhesin which binds to the neuraminidase-sensitive S-HA receptor.



coaggregate *A. viscosus* , *A. naeslundii* and *F. nucleatum*. Generally biotype 1 strains carried long fibrils (159 nm) and shorter fibrils (72 nm) while biotype 11 strains had shorter fibrils (52 nm). While another group had tufts of fibrils at their lateral or polar ends. Out of 44 strains only one had fimbriae which had a width of 5 nm and were 0.7 μ m long. Coaggregation with *Actinomyces* was not observed with tufted strains but was observed with fibrillar strains.

The role of fibrils in the adherence of *S. sanguis* has remained ambiguous until recently. Henriksen *et al.*, (1975) demonstrated a correlation between polar fibrils and twitching motility (a type of surface translocation) in *S. sanguis*. Fives-Taylor (1982), postulated a role for peritrichous fibrils present on *S. sanguis* FW213 in mediating adherence to S-HA. Recently these workers have (Fachon-Kalweit *et al.*, 1985; Fives-Taylor *et al.*, 1987) confirmed their hypothesis with the following observations: (1) non-fibrillar mutants do not adhere, (2) anti-fibril antibody inhibits adherence, and (3) a cloned fibrillar peptide of M_r 30,000 which reacts with anti-fibril antibody. Hogg *et al.*, (1981) reported a role for *S. sanguis* peritrichous surface fibrillar components in adherence to erythrocytes and blood group-reactive glycoproteins isolated from human saliva.

Fives-Taylor *et al.*, (1985) isolated 17 mutants of *S. sanguis* FW213 which were unable to bind to S-HA. The non-adherent mutants were categorized into 6 groups based on the following phenotypic characteristics: (1) ability to aggregate in saliva; (2) ability to coaggregate with *Actinomyces* species; (3) surface hydrophobicity; (4) the presence of fibrils; and (5) twitching motility. They found a strong correlation between reduced ability to adhere, loss of fibrils, and decreased surface hydrophobicity. However they were able to isolate mutants which had fibrils but adhered poorly to S-HA. These mutants aggregated in saliva, and coaggregated with *Actinomyces* . This indicated that there was a number of fibrils each with different adherence properties.

Proteins in the cell walls of the streptococci play an important role in mediating attachment to epithelial surfaces (Ellen *et al.*, 1972; Weerkamp *et al.*, 1980a), to salivary pellicle (Liljemark *et al.*, 1981; McBride *et al.*, 1984), and to other bacteria (Weerkamp *et al.*,

1980a, 1980b, and 1981; Cisar, 1982). Proteins with lectin-like activity have been isolated from *S. sanguis* (Murray *et al.*, 1982; Nagata *et al.*, 1983). Nagata *et al.*, (1983) isolated a galactose binding lectin of M_r 20,000 from *S. sanguis* ATCC 10557. Liljemark and Bloomquist (1981) isolated a cell surface protein fraction from sonicated cell walls and whole cells of *S. sanguis* S7 that blocked adherence to S-HA. Treating *S. sanguis* G9B with trypsin results in the loss of salivary adherence properties, coincident with the loss of proteins of M_r s 160,000, 92, 000 and 86,000 (Rosan, 1982a).

Adherence negative mutants of *S. salivarius* lacked certain high M_r proteins (Weerkamp *et al.*, 1980a, 1980b, 1981, and 1982). Cell walls of the parent strain contain three proteins with M_r s greater than 200,000. The proteins were solubilized by digesting the walls with either lysozyme or mutanolysin. *S. salivarius* V5, a mutant unable to coaggregate with *V. alcalescens*, was missing a 320,000 molecular weight protein. The protein binds selectively to *V. alcalescens* and induces aggregation. *S. salivarius* HB-7, a mutant unable to aggregate in saliva or bind to S-HA, was found to be missing two other high molecular weight proteins. Evidence provided immunoelectron microscopy has shown that these high molecular weight components are part of distinct fibrillar structures.

The role of cell surface hydrophobicity in the attachment of oral streptococci has been studied. The most common technique for measuring bacterial hydrophobicity involves the determining the percentage of cells able to bind to a hydrocarbon (e.g. hexadecane) (Rosenberg *et al.*, 1980). Other methods involve either measuring the aggregation in increasing concentrations of ammonium sulphate (Lindhal *et al.*, 1981) or measuring binding to phenyl- and octyl-Sepharose (Olsson *et al.*, 1982). Streptococci which were less hydrophobic by these methods were found to bind poorly to epithelial cells and to salivary pellicles (Westergren *et al.*, 1982; Wadstrom *et al.*, 1984). Fresh isolates of oral bacteria isolated from dental plaque were more hydrophobic than laboratory strains (Weiss *et al.*, 1982; Rosenberg *et al.*, 1983; Svanberg *et al.*, 1984).

Gibbons *et al.*, (1983b) isolated a hydrophilic variant of *S. sanguis* FC-1 which showed reduced ability to bind to S-HA, and to aggregate in saliva. The variant had lost its polar

fibrils. Using similar selective enrichment methods, Morris *et al.*, (1985) isolated variants of *S. sanguis* 12 and its salivary non-aggregating variant 12na which had a reduced ability to bind to hexadecane . These hydrophilic variants adhered poorly to S-HA, and did not aggregate in saliva. Electron microscopic observation and SDS-PAGE cell wall analysis, revealed that these hydrophilic variants had lost a number of components from their cell walls.

McBride *et al.*, (1984) found the proteins missing from the cell walls of a hydrophilic variant of *S. mutans* LK-2 appeared in the culture supernatant. This was not the case for hydrophilic variants of *S. sanguis* 12 and 12na.

Comparison between whole cell extracts of strain 12 and 12na showed a strong correlation between the possession of a M_r 160,000 protein and the ability to aggregate in saliva (Morris *et al.*, 1985). This protein was found to be missing from the whole cells of strain 12 which had been treated with trypsin. Trypsin treatment of whole cells of strain 12 has been shown to destroy salivary aggregating activity and salivary adherence activity in *S. sanguis* G9B (Rosan, 1982a). Trypsin treatment of whole cells also reduced the ability to bind to hexadecane suggesting cell surface proteins were contributing to hydrophobic nature of *S. sanguis* 12 (Morris *et al.*, 85) and *S. sanguis* ATCC 10556 (Oakley *et al.*, 1985). The protease treated *S. sanguis* ATCC 10556 did not exhibit the characteristic positive slopes in Scatchard analysis suggesting that these hydrophobic proteins were playing a role in stabilizing the adherence reaction.

The studies to be described in this thesis were undertaken with the objective of identifying, isolating, and characterizing the S-HA binding adhesins on the cell surface of *S. sanguis* 12.

Materials and Methods

Bacteria

S. sanguis 12 (Biotype 1), its non-salivary aggregating variant 12na (McBride *et al.*, 1977) and its hydrophilic variant 12L (Morris *et al.*, 1985) have been described previously. Strain 12 was isolated from human dental plaque, 12na is a spontaneously occurring non-aggregating variant of 12. Adherence, aggregation, and hydrophobic characteristics of strains 12, 12na, and 12L are shown in Table II.

S. sanguis strains ATCC 10556, 10557, and 10558 were obtained from the American Type Culture Collection (Rockville, Md.), and the strain NY101 was obtained from J. S. van der Hoeven, University of Nijmegen, The Netherlands. Other *S. sanguis* strains were isolated in our laboratory from human dental plaque. *S. salivarius* HB and HBV52 were described by Weerkamp and McBride (1980). *S. mitis* was a human oral isolate. *S. mutans* LK-2 was obtained from B. Krasse, University of Goteborg, Sweden.

E. coli JM83 [*ara* Δ (*lac-proAB*) *rspL* (=strA) ϕ 80 *lacZ* Δ M15] and JM101 [*supE thi* Δ (*lac-proAB*) {F' *traD36 proAB lacI*^q *lacZ* Δ M15}] were used as recipients (Veiera *et al.*, 1982) for the plasmid vector pUC18 [*Ap*^r] (Yanisch-Perron *et al.*, 1985) and the recombinant derivatives.

Bacterial cultures were stored frozen at -70°C in 10% glycerol. Rather than continuously subculturing from agar to broth, inoculum was obtained from the frozen stock culture. To ensure that the *S. sanguis* variants retained their phenotypic characteristics, cultures were routinely analyzed for their adherence and aggregation properties.

Media

S. sanguis strains were grown at 37°C in trypticase soy broth (TSB) (BBL Microbiology

Table II. Adherence characteristics of *S. sanguis* strains.

Strain	adherence to hexadecane (%)	adherence to S-HA	salivary aggregation
12	91	+	+
12na	88	+	-
12L	22	-	-

systems, Cockeysville, MD.) supplemented with yeast extract (3 g/liter).

Cells for adherence assays were radiolabelled by the addition of 1 or 2 μCi of [*Methyl*-1',2',- ^3H]thymidine (The Radiochemical Centre, Amersham, England) per ml of culture medium.

The bacterial cells were harvested by centrifugation, and washed four times in 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer (pH 7.2). The cells were sonicated for 60 s at an output of 5 (Sonifier, Cell disruptor 350, Branson Sonic Power Co., Conn.) to break the chains. Bacteria were examined microscopically to ensure that only singles or pairs were present. The bacteria were then washed twice more in HEPES buffer and suspended as required in HEPES for adherence assays. Cell numbers were determined microscopically with a Petroff-Hauser counting chamber.

E. coli JM83 and JM101 were grown in LB (10 g tryptone, 5 g NaCl, 5 g yeast extract per liter, pH 7.4) medium at 37°C. When solid medium was required, agar (1.5%, wt/vol) was added to LB broth. Ampicillin (50 μg per ml) (Sigma) was added when required. Soft agar contained 10 g tryptone, 5 g NaCl, 7 g agar per liter, pH 7.4.

Saliva

Paraffin stimulated saliva, pooled from a number of donors, was collected at 4°C and clarified by centrifugation at 17,000 x g for 10 min. The clarified saliva was heated at 60°C for 30 min to destroy endogenous enzymes, centrifuged to remove particulate matter, and stored in aliquots at -20°C. When needed, saliva was thawed and then centrifuged to remove sediment.

Adherence assay

Fines were removed from spheroidal hydroxyapatite beads (HA) (BDH Chemical Ltd., Poole,

Eng.) by repeated washing in 500 ml distilled water. After mixing with water the suspension was allowed to settle for 2 min and the supernatant was discarded. This procedure was repeated five times or until all the fines were removed. The beads were dried in an incubator and the dried beads (40 mg) were placed in glass vials. Clarified saliva (0.5 ml) was added and the mixture was shaken on a horizontal shaker (45 cycles/min) (Eberbach Corporation, Michigan) at room temperature for 2 h and then placed at 4°C overnight. After incubation overnight at 4°C, the unbound saliva was removed by aspiration and the saliva-coated hydroxyapatite beads (S-HA) were washed twice with a total of 20 ml of distilled water and a third time with 10 ml of HEPES buffer. Excess buffer was removed by aspiration.

For examination of the effects of neuraminidase on adherence, the pH of the bead-saliva mixture was reduced to 5 by the addition of 0.25 M HCl. Neuraminidase (Sigma; type VI from *Clostridium perfringens*) was dissolved in 0.05 M acetate buffer (pH 5) at a concentration of 50 µg per ml. 10 µl of the enzyme was added to the vials. 10 µl of 0.5% sodium azide was included to prevent microbial growth. The vials containing the mixture were incubated overnight at 37°C. Controls were treated similarly, except that neuraminidase was not included. Beads were washed as described above.

One ml of bacterial suspension (3×10^9 cells per ml) was added to each vial containing S-HA and the vials were shaken on a horizontal shaker at 45 cycles/min for 2 h. For adherence inhibition assays with antibodies, bacteria were preincubated with F(ab')₂ fragments (final concentration, 1 mg per ml) for 1h at room temperature prior to their addition to S-HA. Unbound F(ab')₂ was removed by centrifugation. A buffer control was included. Unattached bacteria were removed by aspiration and the bacteria-S-HA complex was washed three times with a total of 30 ml of HEPES buffer. The washed beads were then transferred to new plastic scintillation vials and 0.5 ml of NCS Tissue Solubilizer (The Radiochemical Centre, Amersham, England) was added and the mixture incubated at 55°C overnight. Scintillation fluid (10 ml) (toluene:methanol:Liquifluor: 6.0:4.0:0.42) was added to each vial and the radioactivity was monitored in a Tracor analytic scintillation counter. Experiments were done in triplicate.

For some adherence inhibition assays, the method was scaled down with the following minor modifications. 5 mg of HA beads were dispensed into a 96 well V-bottom microtitre plate (Flow Laboratories, Inc., McLean, Va.). 62.5 µl of clarified saliva was added to each well and mixed gently for 2 h at room temperature. The mixture was kept at 4°C overnight. When required, the pH of the saliva-HA mixture was adjusted to 5. The beads were washed twice with 0.2 ml distilled water and twice with HEPES buffer. The S-HA was mixed with 0.05 ml of [³H]thymidine labelled cells (7.6×10^9 cells per ml) plus 0.05 ml HEPES buffer for 30 min and then washed five times with 0.2 ml HEPES buffer before transferring to a scintillation vial containing 5 ml of buffer. Excess buffer was aspirated, 0.5 ml NCS tissue solubilizer was added and the vials were incubated at 55°C overnight. Scintillation fluid (10 ml) was added and the radioactivity monitored to determine the numbers of attached bacteria. In some assays, the S-HA was preincubated with 0.05 ml of inhibitor in HEPES buffer for 1 h before the addition of bacteria.

Salivary aggregation

Saliva (0.1 ml) was serially diluted in HEPES buffer in 12 x 75 mm agglutination tubes. An equal volume of bacteria ($A_{660}=3.0$) in HEPES buffer was added and the mixture was shaken on a horizontal shaker (90 cycles per min) at room temperature for 5 min. The aggregation titer was expressed as the highest dilution of saliva which gave microscopically visible aggregation. A control without saliva was included in the assay.

Bacterial hydrophobicity

Bacterial hydrophobicity was measured by determining the number of bacteria adhering to hexadecane as described by Rosenberg *et al.*, (1980) with minor modifications.

Bacteria grown overnight in TSB medium were washed in HEPES buffer to give an absorbance at A_{436} of 0.5. Hexadecane (0.1 ml) was added to a 18 x 150 mm tube

containing 3 ml of the bacterial suspension. The suspension was mixed vigorously on a vortex mixer for 60 s and then was allowed to stand for 20 min. The aqueous phase of the suspension was carefully removed with a pasteur pipette and its absorbance at 436 nm was measured. A control without hexadecane was always included. Experiments were done in triplicate.

Isolation of fibrils

Cells were grown in TSB supplemented with yeast extract in a 100 liter fermentor (LH fermentation 5000 series, L. H. Engineering Co. Ltd., Bells Hill, Stokepoges, Bucks, U. K.). Medium constituents were mixed and sterilized *in situ*. Following sterilization, the medium was rapidly cooled to 37°C and inoculated with 2.0 liters of an overnight culture of *S. sanguis* 12. The culture was incubated at 37°C without stirring until the cells had reached the stationary growth phase. Cells were harvested in a continuous-flow centrifuge (Sharples, Pennawalt). Cell yields were approximately 2.0 g (wet weight) per liter. In some cases cells were grown in the same medium in 20-liter glass bottles. All strains showed similar growth rates under these culture conditions.

Harvested cells were washed once in Tris- MgCl₂ buffer (20 mM Tris, 1 mM MgCl₂, 0.02% NaN₃, pH 6.8), and frozen as a pellet at -20°C. The pellet was thawed, washed once in Tris- MgCl₂ buffer, and then resuspended at a concentration of 40 g (wet weight) of cells in 200 ml of the same buffer. The cell suspension was homogenized in a Waring blender twice for 1 min each, after which the cells were removed by centrifugation and the supernatant containing the fibrils was retained. In initial experiments the fibrils were recovered by centrifugation. The crude extract from homogenization was lyophilized and redissolved in 20 ml of Tris- MgCl₂ buffer and then centrifuged at 80,000 x g for 2 h. The precipitate was resuspended in 20 ml of Tris- MgCl₂ buffer and centrifuged at 12,000 x g for 30 min to remove any cells or cell fragments. The supernatant was then centrifuged at 80,000 x g for 2 h. The fibrillar preparation was obtained as a yellowish, gel-like, translucent pellet which was resuspended in 2.5 ml of Tris- MgCl₂ buffer. In later experiments fibrils were isolated from the crude homogenate by ammonium sulphate precipitation. Ammonium sulphate was added to an initial concentration of 15% and

incubated at 4°C overnight. Precipitated material was removed by centrifugation at 15,000 x g for 40 min. The ammonium sulphate concentration was then raised to 30% and the mixture was incubated at 4°C overnight. At this concentration the fibrils were precipitated. The precipitate was recovered by centrifugation, dialyzed, and resuspended in Tris- MgCl₂ buffer as before.

Enzyme digestion of fibrils

Fibrillar preparations were digested at 37°C for 1 h or overnight in Tris- MgCl₂ buffer with trypsin, chymotrypsin, papain, subtilisin, pronase, or lysozyme at a concentration of 1 mg/ml or with mutanolysin at a concentration of 175 U/ml.

Dissociation of fibrils

Attempts were made to dissociate the fibrils into smaller subunits by treatment with a variety of chemical agents. Fibrillar preparations were dialyzed, freeze-dried, and then treated with 8 M urea, pH 7, 8.6 M guanidine hydrochloride (pH 7), 54 mM HCl (pH 1.8), or 1 M sodium thiocyanate (pH 7); 1 mM EDTA was included with each treatment done at neutral pH to prevent possible reaggregation of fibrillar subunits (Karch *et al.*, 1985). All treatments were carried out at 37°C for 18 h. Treatment with 54 mM HCl was also done at 100°C for 15 min. Reagents were removed by dialysis against distilled water containing 1 mM EDTA before analysis by SDS-PAGE.

ELISA

Fibrillar preparations in carbonate coating buffer (0.05 M, pH 9.6) were dried onto microtiter plates (Immulon 2; Dynatech Laboratories Inc., Alexandria, Va.) by overnight incubation at 37°C.

The enzyme-linked immunosorbent assay (ELISA) was performed as described previously (Engvall *et al.*, 1976; Voller *et al.*, 1976). Coated plates were washed with phosphate-buffered saline containing 0.01% Tween 20 (PBST). Free sites on the plates were blocked by reaction with 5% BSA for 30 min, followed by a further wash with PBST. Antibody at an appropriate dilution in PBS with 1% BSA was added to the wells (0.1 ml per well) and incubated for 2 h at room temperature. This was followed by washing with PBST and then incubation for 1 h with alkaline phosphatase- labeled goat anti-rabbit immunoglobulin G (IgG) (Helix Biotech Ltd.) at a dilution of 1:6000 in PBS-1%BSA. A final wash with PBST was followed by the addition of 0.1 ml of *p*-nitrophenyl phosphate substrate (Sigma) to each well. Color development was monitored after 1 h of incubation at 37°C by the measurement of the absorbance at 405 nm on a Titertek Multiscan (Flow laboratories).

Electrophoretic techniques

Polyacrylamide gels (PAGE) and SDS-PAGE were run with 7, 10 and 12% gels as stated in the text (Laemmli, 1970). Samples for SDS-PAGE were boiled in 2% SDS with or without 5 % β -mercaptoethanol for 10 min unless stated otherwise. Molecular weight standards were: myosin (200,000), β -galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000) trypsinogen (24,000), β -lactoglobulin (18,400), and lysozyme (14,300). Gels were stained with Coomassie brilliant blue or silver nitrate (Oakley *et al.*, 1980). Alternatively, proteins were transferred electrophoretically (Burnette, 1981) to nitrocellulose paper (Bio-Rad Laboratories, Calif.) for Western blotting in 25 mM Tris-192 mM glycine- 20% methanol buffer (pH 8.3). A voltage of 25V was applied for 18 h in a Bio-Rad Trans-Blot cell. The voltage was then increased to 60V for 2 h. Proteins which reacted with specific antiserum were visualized on the nitrocellulose by the procedure described in the Bio-Rad Immuno-Blot (GAR-HRP) assay kit, except that bovine serum albumin was substituted for gelatin. Prestained markers (Bethesda Research Laboratories, Gaithersburg, Md.) were used to calibrate molecular weights in Western blots of recombinant proteins.

Preparation of *S. sanguis* 12 chromosomal DNA

Log phase cells grown in 250 ml of TSB broth were washed twice in TES buffer (20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, pH 7) and resuspended in 3 ml of TES buffer. One ml of mutanolysin (525 U per ml) and 0.25 ml of RNase (125 Kunitz units per ml) were added and the mixture incubated at 37°C. After one hour, 0.25 ml of pronase (700 proteolytic units per ml) and 0.5 ml of 0.5% N-lauryl sarcosine was added and incubation was continued for a further 30 min at 37°C. The lysate was extracted three times with equal volumes of phenol and once with chloroform. DNA was precipitated with ethanol, and purified further by CsCl density gradient centrifugation.

Recombinant DNA methods

Partial digestion for cloning was done as described by Darzins *et al.*, (1984) with minor modifications. To determine the optimal concentration for the partial digestion of *S. sanguis* chromosomal DNA, 50 µg of DNA in a final volume of 50 µl was incubated with 5 units of PstI restriction enzyme at 37°C. Aliquots of digested DNA were removed at various times. Reactions were stopped with 0.5 M EDTA and the entire volume was loaded onto 0.7% agarose gels. Once the appropriate digestion time had been determined, aliquots of partially digested chromosomal DNA were combined with an alkaline phosphatase treated pUC18 plasmid vector which had been linearized with PstI enzyme (vector DNA 0.1 mg per ml: chromosomal DNA 0.5 mg per ml). T4 DNA ligase was added with ATP and dithiothreitol to a final concentration of 0.1 and 1 mM respectively. The ligation reaction was performed at 12°C in a 20 µl reaction volume. The ligated DNA was used to transform *E. coli* JM83 which had been made competent by CaCl₂ treatment (Maniatis *et al.*, 1982). After incubation at 37°C for 60 min in 1 ml of LB broth, the transformed cells were plated on LB agar with ampicillin and 2% Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 2% IPTG (isopropyl-β-D-thiogalactopyranoside).

Plasmids were isolated from recombinant clones by SDS-lysis or alkaline lysis methods as

described by Maniatis *et al.*, (1982) and further purified by CsCl density gradient centrifugation. Plasmids were analysed by the alkaline lysis miniprep procedure after subcloning (Maniatis *et al.* 1982). DNA restriction fragments were resolved by 0.7 % agarose gel electrophoresis in 50 mM Tris, 50 mM borate, 0.002 M Na₂EDTA buffer containing 0.5 µg per ml of ethidium bromide.

Subcloning was done with DNA fragments eluted from low melting point agarose (Bio-Rad) gels run with 0.04 M Tris-acetate, 0.001 M Na₂EDTA buffer after appropriate restriction enzyme digestion of pSA2. The DNA was recovered by heating the gel slices at 65°C for 5 min in five times the volume of the gel slice and extracting the agarose solution with phenol and phenol- chloroform- isoamy alcohol (25:24:1, v/v/v). The DNA remaining in the aqueous phase was precipitated with ethanol and resuspended in TE (20 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer. The extracted DNA was either self ligated or ligated to pUC18 linearized with the appropriate restriction enzyme and transformed into *E. coli* JM83. Expression of the desired recombinant proteins was checked by lysing the cells with SDS-PAGE solubilization mixture and analyzing the lysate by SDS-PAGE followed by Western blotting. The orientation of the DNA insert in the deletion derivatives was determined by restriction enzyme digest analysis.

The 1.9 kb BamH1 fragment of pSA2 was used as the probe in Southern hybridization studies. The 1.9 Kb fragment was isolated after digesting pSA2 with BamH1 enzyme and separating the fragments on an agarose gel. The fragment was visualized with UV and a trough was cut in front of the fragment. A DEAE strip was placed inside the cut gel and the gel electrophoresed until the fragment was completely bound to the strip. Bound DNA was eluted from the DEAE strip as recommended by the manufacturer (Schleicher & Schuell, Inc., Keene, N.H.) The probe was labelled with [γ -³²P]ATP by polynucleotide kinase as described by Maniatis *et al.*, (1982). DNA fragments to be probed were separated in agarose gels and transferred to nitrocellulose by the method of Southern, (1975) with 20x SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 7) as the transfer buffer. Hybridization was done overnight at 42°C in hybridization buffer (50% (v/v) deionized formamide, 5x SSC- 50 mM sodium phosphate (pH 6.5), heat denatured salmon sperm DNA (250 µg per ml), and 5x Denhart's solution). The blot was washed three times in 2x SSC containing

0.1% SDS at room temperature and twice at 50°C in 0.1x SSC, 0.1% SDS, and then dried and autoradiographed.

Colony immunoassay

White colonies carrying recombinant plasmids were replica plated on LB agar containing ampicillin and XGal and incubated overnight at 37°C. The cells were lysed *in situ* by overlaying with soft agar containing lysozyme (0.5 mg per ml) and SDS (0.25 mg per ml) and then incubating for 1 h at 37°C (Whittle *et al.*, 1982). Plates were inverted over chloroform vapor for 15 min to complete lysis. Nitrocellulose filters (BA85; Schleicher & Schuell, Inc., Keene, N.H.) were blotted onto the agar plate for 1 h and air dried. 5 µl of an overnight grown culture of *S. sanguis* 12 cells were spotted onto the filters as positive controls. The filters were placed in a blocking solution of 3% bovine serum albumin in TBS (20 mM Tris, 0.5 M NaCl, pH 7.5). Blots were then treated as described for Western blotting (see electrophoretic techniques). Duplicate blots were treated with antisera raised against either *S. sanguis* 12 (anti-12) or a fibrillar preparation of strain 12 (anti-fibril) at a dilution of 1:300. (Cross-reactive antibodies were removed by adsorption with *E. coli* JM83 carrying the pUC18 plasmid. 10 ml of antisera were adsorbed two times with 13 g wet weight of cells at 4°C.) Blots were then incubated with alkaline phosphatase conjugated anti-rabbit sera (Helix Biotech Ltd., Richmond, B.C.) for 1 h and then stained with fast red (20 mg) in 50 ml of 50 mM Tris (pH 8.0) and 10 mg of naphthol AS-MX phosphate (Sigma). Positive colonies were picked from a master plate for further study.

Antisera

Antisera were raised in New Zealand white rabbits against formalinized *S. sanguis* 12 as described previously (Morris *et al.*, 1985).

(i) Anti-LFP sera. To obtain anti-long-fibril protein (LFP) antibody, rabbits were immunized with purified protein from an SDS-PAGE gel. Gels were stained with Coomassie

blue, and the required band was excised from the gel. This strip was completely destained in 25% isopropanol-10% acetic acid and then equilibrated overnight in PBS. The gel was homogenized in PBS and injected intramuscularly on days 1, 7, and 28. Booster injections were given at approximately monthly intervals thereafter.

(ii) Anti-fibril sera. Rabbits were immunized by intramuscular injection with a fibrillar preparation in complete Freund's adjuvant. This was followed on days 7, and 28 with intramuscular injections of a fibrillar preparation in incomplete Freund's adjuvant. Booster injections were given at approximately monthly intervals thereafter.

(iii) Anti-SsaB and anti-20 sera. Antisera against SsaB and the 20 kd protein were prepared in New Zealand white rabbits. Intramuscular injections were given with complete Freund's adjuvant on day one and with incomplete Freund's adjuvant on days 7 and 28 (0.25 mg protein). Rabbits were bled one week after the last injection. The rabbits were given booster injections at approximately monthly intervals thereafter.

(iv) Anti *E. coli* lysate sera. Antisera to cell lysate containing recombinant proteins were raised in Balb/c mice. Cell lysates were injected intraperitoneally into Balb/c mice on day one with Freund's adjuvant and without adjuvant on days 14 and 28. Mice were bled through the tail.

IgG was obtained by passage of antisera over a column of Protein A-Sepharose CL4B (Sigma) to obtain IgG. The 5 ml sample was applied to a [2 x 6cm] column and exhaustively washed with 0.1 M borate-0.5 M NaCl buffer, pH 8.4. IgG was then eluted with 0.1 M glycine-0.5 M NaCl buffer, pH 2.5, and then dialyzed against an appropriate buffer for subsequent use.

Purification of SsaB and the 20 kd proteins

Overnight grown cultures of *E. coli* SA2 were harvested and washed twice with phosphate-buffered saline (PBS). The cells were resuspended in PBS to give 0.2 g wet

weight per ml. DNase and RNase were added at 0.02 mg per ml final concentration. The cells were passed through a French Press four times at 20,000 psi and subsequently sonicated for 1 min. The lysate was separated from unbroken cells by differential centrifugation at 20,000 x g for 20 min. The pellet was washed twice, the supernatants pooled along with the lysate, and dialyzed against Tris buffer (50 mM Tris, 0.5 M NaCl, 0.02% NaN₃, pH 8.0). The dialyzed lysate was applied to a Sephadex G-75 column [2.5 x 85cm] at 4°C and eluted with the same buffer. All the fractions were analysed for the presence of SsaB and the 20 kd protein by SDS-PAGE.

The fractions containing the 20 kd protein were purified further through a Mono Q column (Pharmacia FPLC system, Pharmacia, Uppsala, Sweden) with 0.15-1.0 M NaCl gradient. Fractions containing SsaB were dialyzed against distilled water and subsequently against 10 mM Tris, pH 8.0 containing 0.02 % NaN₃ and applied to a DE-52 [Whatman, 1.5 x 30cm] column. The column was eluted by a step gradient of 0.1 M NaCl, 0.15 M NaCl and 1.0 M NaCl in 10 mM Tris, 0.02% NaN₃, pH 8.0 buffer. Fractions were analyzed by SDS-PAGE for the presence and purity of the SsaB and the 20 kd proteins. The fractions containing purified 20 kd protein and SsaB were dialyzed against distilled water and lyophilized.

Immunological techniques

S. sanguis cells were washed twice in PBS and resuspended in PBS to give an absorbance of 1 at 660nm. 0.2 ml of cells were incubated with an equal volume of the appropriate IgG (1 mg per ml for 1 h). Unbound IgG was removed by two washings with PBS. Anti-LFP and anti-Mab 32.33 antibody conjugated to gold beads were a gift from Dr. E. J. Morris.

For immunogold labelling, cells were then resuspended in 0.2 ml PBS and mixed with 25 µl of gold-bead (5nm) conjugated to goat anti-rabbit serum (EM GAR G5; Janssen Life Sciences Products, Beerse, Belgium.), and incubated at 4°C overnight. Unbound secondary antiserum was removed by washing twice with PBS. Cells were resuspended in PBS and negatively stained with 5% uranyl acetate in 70% ethanol. Observations were made with a Philips EM 300 electron microscope.

For immunofluorescence, cells were incubated with FITC conjugated secondary antiserum for 1 h. Unbound antibody was removed by washing in PBS and visualized by epifluorescence microscopy.

For thin-section immunoelectron microscopy, *S. sanguis* cells ($OD_{660}=1$) were resuspended in 1 ml of 2.5% glutaraldehyde in PBS and allowed to shake for 1 h at 4°C. The cell suspension was centrifuged and the supernatant removed by decanting. The pellet was washed in PBS for 5 min, resuspended in PBS and kept at 4°C. Cells were fixed in 1% OsO_4 for 30 min at 4°C, washed two times for 5 min in distilled water and then dehydrated with ethanol and embedded in epon-adaldite resin for 2-3 days at 57°C before cross-sectioning. Cross-sectioned preparations were picked up on copper grids. The grids were immersed in a solution of 3% hydrogen peroxide for 10 min and gently washed in filtered Tris hydrochloride buffer (0.02 M, pH 7.2). Grids were then placed in anti-SsaB IgG for 2 h, and washed in Tris hydrochloride buffer and immersed for 1 h in a solution containing 50 μ l colloidal gold (conjugated to anti-IgG) in 1 ml of Tris hydrochloride buffer. Grids were then washed in buffer and subsequently with distilled water and stained with 1% uranyl acetate in 70% ethanol. Observations were made with a Philips EM 300 electron microscope. (Cole *et al.*, 1987)

Cross-linking of whole cells of *S. sanguis*

Cells for cross-linking experiments were grown in TSB overnight, harvested, and washed in PBS and resuspended in the appropriate buffer. For cross-linking experiments with DSP (dithio-bis-succinimidyl propionate), the cells were resuspended in 0.2 M triethanolamine buffer, pH 8.5 following the specifications of Reithmeier *et al.* (1977) and Angus *et al.*, (1983). DSP was dissolved in dimethylsulphoxide and added to the samples to give the optimum final concentration of DSP as indicated for each experiment. After two minute reaction time, excess 1 M Tris-HCl pH 8.5 was added to stop the cross-linking reaction. The cells were centrifuged and washed in Tris-MgCl₂ buffer, resuspended in the same buffer and digested with mutanolysin as indicated below.

For cross-linking experiments with DTBP (dimethyl-dithiobis-propioimide) cells were resuspended in 50 mM triethanolamine, 1 mM MgCl_2 , pH 8.0 buffer (Wang *et al.*, 1974) DTBP was added to give the optimal concentration as indicated for each experiment. After one hour of incubation at room temperature the reaction was stopped by adding 50 μl per ml of 1 M ammonium acetate, incubating for 10 min at room temperature and then adding 50 μl per ml of 2 mM N-ethyl maleimide. Cells were then centrifuged and washed in Tris- MgCl_2 buffer and resuspended in the same buffer and digested with mutanolysin.

The cross-linked preparations were diluted 1:1 into sample buffer containing 2% (w/v) SDS, 0.5 M Tris-HCl pH 6.8, and 20% (v/v) glycerol without reducing agent and the sample was incubated for 30 min at 37°C and electrophoresed. Strips containing the electrophoresed samples were cut from the first dimension gel, and incubated with 10% β -mercaptoethanol (v/v) for 15 min at room temperature. These strips were then sealed on top of a second dimension SDS-polyacrylamide gel (7%) with 1.2% (w/v) agarose and electrophoresed. The gels were silver stained. Alternatively, the gels were Western blotted and probed with anti-SsaB serum as described previously.

Mutanolysin digests of *S. sanguis*

Mutanolysin (Sigma) digests of *S. sanguis* strains were done as recommended by the manufacturer. The digestion was carried out in Tris- MgCl_2 buffer, pH 6.8 for 4 h or overnight at 37°C.

Analytical procedures

Protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad Laboratories) with BSA or bovine gamma globulin as the standard. Total carbohydrate was measured by the phenol-sulphuric acid method (Dubois *et al.*, 1956) with glucose as the standard. Amino acid analyses of fibrillar preparation was performed by high-pressure liquid chromatography with a Waters sulphonated polystyrene cation exchange resin by Dr.

D. Mackenzie of Agriculture Canada, Vancouver, B.C. Samples were hydrolyzed with 6 N HCl in vacuo for 24 h at 110°C. Eluted amino acids were detected by post-column reaction with α -phthalaldehyde and monitored by fluorescence. Amino acids containing secondary amines were detected by hypochlorite oxidation prior to α -phthalaldehyde derivatization.

The NH₂-terminal amino acid sequence was determined by automated Edman degradation using an Applied Biosystems model 470A gas sequenator utilizing the resident sequencing program. The amino acid residues were analyzed by reverse phase HPLC chromatography. These analyses were provided by the University of Victoria protein sequence facility. The amino acid composition of intact recombinant proteins (20 kd and SsaB) were determined at the protein sequencing facilities at the University of Victoria and the University of Calgary.

Cell fractionation

The cellular location of 20 kd and SsaB in *E. coli* SA2 was determined by the chloroform shock method described by Ames *et al.*, (1984). 30 ml of culture were used in this experiment. Overnight grown cells were centrifuged at 1,100 x g for 10 min. Cells were resuspended in 10 μ l of CHCl₃ per ml of culture by vortexing and left at room temperature for 15 min, and then 0.1 ml of 0.01 M Tris hydrochloride (pH 8.0) per ml culture was added, and cells were separated by centrifugation at 6,000 x g for 20 min. The supernatant fraction containing periplasmic proteins was removed and the cells were sonicated twice for 1 min at 4°C to obtain the cytoplasmic fraction. Each fraction was assayed for β -lactamase (O'Callaghan *et al.* 1972) and glucose-6-phosphate dehydrogenase to assess the extent of contamination with cytoplasmic or periplasmic constituents.

F(ab')₂ preparation

Protein A-Sepharose CL4B-purified IgG was digested with pepsin at a concentration equal to 2% of total IgG by weight. The digestion was carried out in 0.1 M sodium acetate buffer (pH 4.5) at 37°C for 18 h and stopped by adjusting the pH to 8.0 with 1 M Tris. The digest

was assayed for F(ab')₂ fragments by SDS-PAGE.

Enzymes and reagents

All restriction enzymes, polynucleotide kinase, T₄ DNA ligase and lambda DNA were from Pharmacia Fine Chemicals, Uppsala, Sweden. Calf intestinal phosphatase was from Boehringer-Mannheim. IPTG, XGal, fast red, naphthol AS-MX phosphate, mutanolysin, lysozyme, salmon sperm DNA, DNase, trypsin, papain, subtilisin, and RNase were from Sigma Chemical Co., St. Louis, Mo. Pronase was obtained from Calbiochem-Behring, La Jolla, Calif. and chymotrypsin was obtained from Worthington Diagnostics, Freehold, N.J.

RESULTS

Section A: Fibrils

Fimbriae or fimbriae like structures (fibrils) have been implicated in the adhesion of Gram-positive and Gram-negative organisms to human tissue. Studies with other *S. sanguis* strains have implicated similar structures in adhesion to S-HA (Gibbons *et al.*, 1983; Fives-Taylor *et al.*, 1985), in coaggregation (Handley *et al.*, 1985) and in salivary aggregation (Hogg *et al.*, 1981).

Isolation of fibrils

Studies were initiated to determine if fibrillar structures were present in *S. sanguis* 12. The electron micrograph in Figure 2 shows structures which can be classified as fibrils as described by Handley *et al.*, (1985). *S. sanguis* 12 has short and long fibrils. The shorter fibrils which often appeared as a distinct dense layer surrounding the cell were approximately 70 nm long. The long fibrils, which were more prevalent at the poles, had a length of at least 200 nm.

A technique of gently shearing the cells in a Waring blender followed by precipitation or ultracentrifugation was found to be the most successful method in isolating the fibrils. The presence of *S. sanguis* 12 fibrils prepared either by ultracentrifugation or by ammonium sulphate precipitation (30%) after freeze-thawing and homogenization was confirmed by electron microscopy of negatively stained samples. Fibrillar preparations appear turbid, and electron microscopy revealed them to be composed of large aggregates with relatively few individual fibrils (Fig. 3). Electron microscopy of preparations obtained by the same treatment of *S. sanguis* 12na, *S. sanguis* 12L and *S. sanguis* N did not show any fibrillar structures. It seems likely that this technique may release only a particular class of fibrils, which are not universally distributed among the strains of *S. sanguis*, since no fibrils were obtained from *S. sanguis* N, an organism that was shown by electron microscopy to possess surface fibrils. It was less surprising that fibrils were not obtained

Fig. 2. Electron micrograph of *S. sanguis* 12 negatively stained with uranyl acetate, showing two lengths of fibrils on the cell surface. Bar, 0.5 μm .

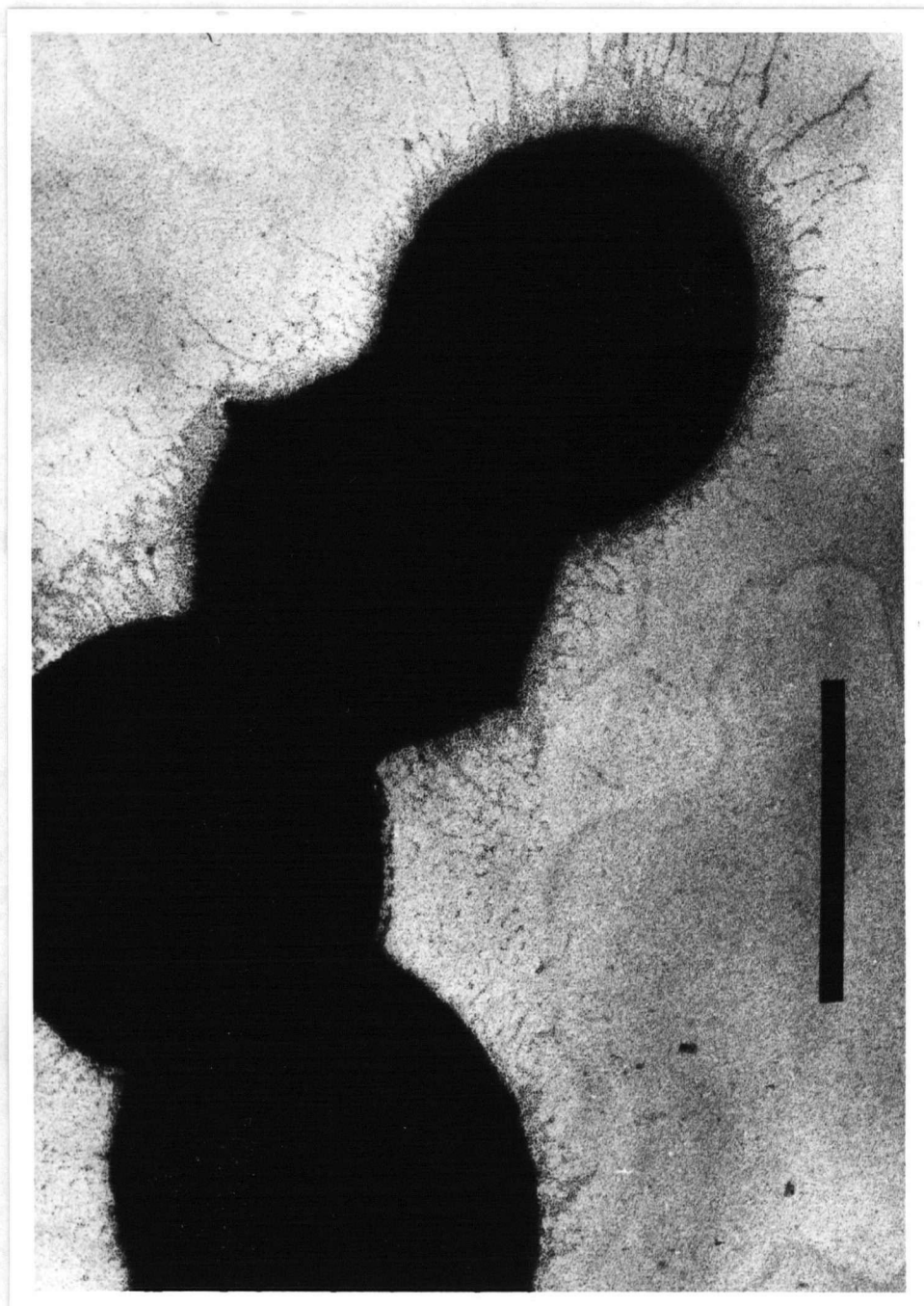
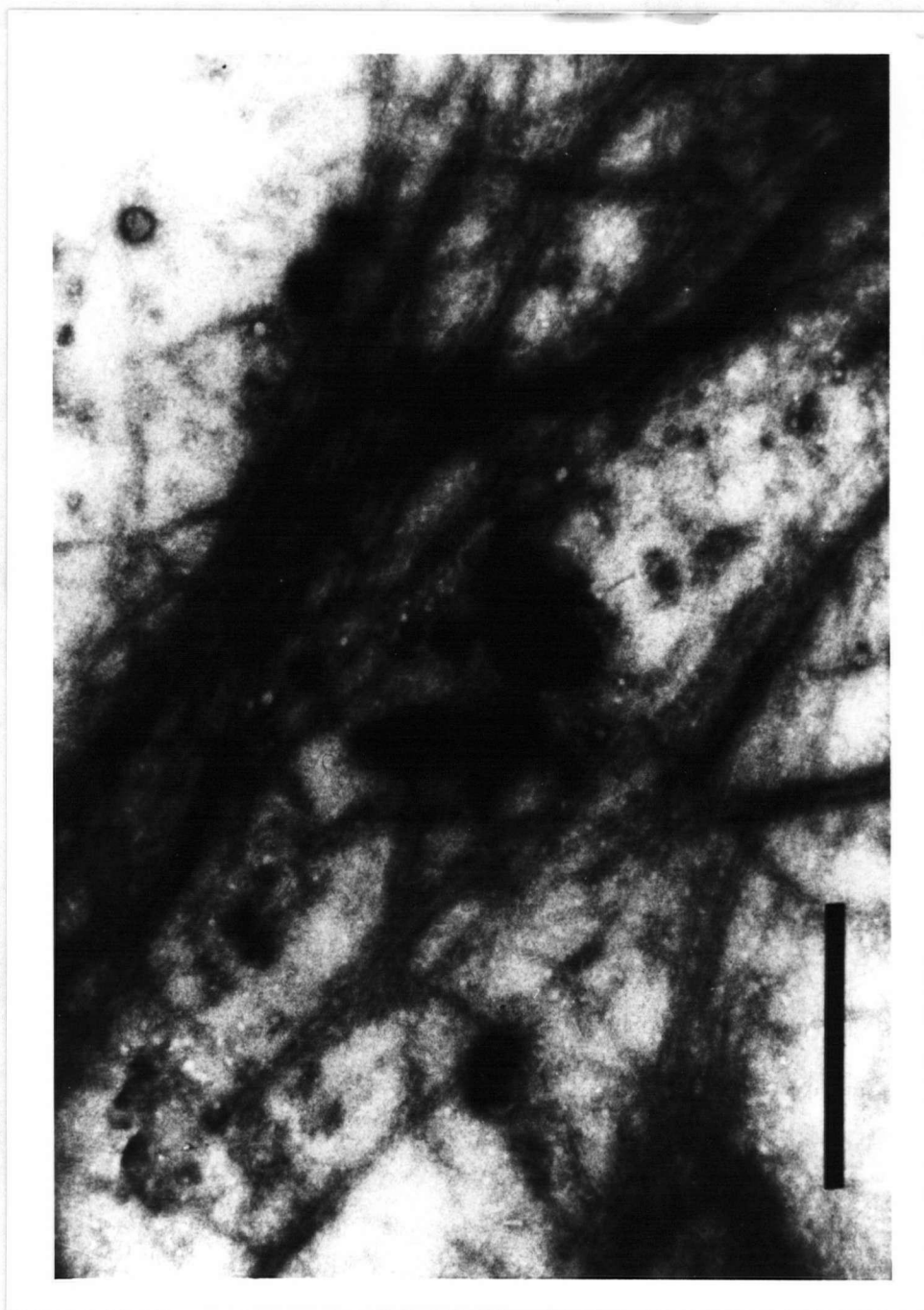


Fig. 3. Electron micrograph of a fibrillar preparation from *S. sanguis* 12 obtained by ultracentrifugation. Negatively stained with phosphotungstic acid. Bar, 0.5 μm .



from strain 12na, since it is known that this strain has lost a number of cell surface molecules (Morris *et al.*, 1985). An alternative explanation for the failure to release fibrils from strain N might be that the fibrils are linked more tightly to the cell wall and cannot be removed as easily from this strain.

The preliminary freezing of cells appeared to be essential for the subsequent release of fibrils, because no fibrils were obtained when this step was omitted. Bacteria examined in the electron microscope after freezing and homogenization still possessed considerable amount of fibrillar material on their cell surface. Passing the cells through a 25 gauge 5/8" needle or sonicating for 10 min or grinding them in a mini-mill for 2 min with glass beads did not result in an increased yield of fibrils.

Cells which had been used for preparing fibrils were tested for their hydrophobicity and salivary aggregation properties. These cells retained their hydrophobicity but showed a somewhat reduced salivary aggregation titer. 91% of untreated cells adhered to hexadecane and exhibited a salivary aggregation titer of 256. 90% of frozen and homogenized cells adhered to hexadecane but the salivary aggregation titer was reduced to 64.

In early studies, glucose was sterilized separately and added to the sterilized growth medium after cooling. Cells harvested from this medium had a reduced ability to bind to S-HA and yielded only a small amount of fibrils. In addition, these fibrillar preparations contained large quantities of contaminating material which was not seen in preparations obtained from cells grown in medium in which glucose was included prior to heat sterilization. Similar results were obtained when an enriched medium was used to grow cells. Although the yield of cells was increased two times, the amount of fibrils that could be isolated was very much reduced.

Composition of fibrillar preparations

Fibrillar preparations from *S. sanguis* 12 were found to contain approximately equal

quantities of protein and carbohydrate. The recovery of both protein and carbohydrate was 3 µg/g (wet weight) of cells. ELISA with antiserum raised against lipoteichoic acid (LTA) from *Lactobacillus casei* (anti-LTA serum was a generous gift from K. Knox, Institute of Dental Research, United Dental Hospital of Sydney, Surrey Hills, New South Wales, Australia 2010) indicated that fibrillar preparations did not contain LTA. Amino acid analysis of the fibrils is shown in Table III.

Electrophoretic Characterization of the fibrils

SDS-PAGE (7%) analysis of fibrillar preparations of *S. sanguis* 12 showed a single strong band with a M_r greater than 300,000, with very little contaminating material (Fig. 4). Similar results were obtained regardless of whether fibrils were prepared by the ultracentrifugation or the ammonium sulphate purification procedures. This high M_r band has been designated the long fibrillar protein (LFP). There were no silver-staining bands when the fibrillar preparation was analyzed by 7% polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions.

Fibrillar preparations were routinely boiled in 2% SDS-5% βME prior to PAGE analysis. Boiling was not required to dissociate the protein, as fibrils mixed with 2% SDS at room temperature were dissociated to form the LFP. When the SDS-treated fibrillar preparation was fractionated on Sephacryl S-400, the LFP was found to elute in the void volume, indicating an M_r in excess of 300,000. The LFP band was not found in preparations obtained from strains 12na, and N (Fig. 4, lanes 2 and 3) nor was it found in preparations of strain 12L (data not shown). A band of M_r 145,000 was seen in the preparations from 12na (Fig. 4, lanes 2) . This band was sometimes seen as a minor contaminant in fibrillar preparations from *S. sanguis* 12 which had been obtained by the ultracentrifugation method. When ammonium sulphate precipitation was used to collect fimbriae, this band was found in the material which precipitated at 15% saturation, whereas the LFP appeared in 30% ammonium sulphate fraction. The LFP band stained positively with both Coomassie blue and periodate-Schiff, indicating that it was a glycoprotein. Both staining activities were lost after incubation of the fibrils with subtilisin (1 mg/ml) for 5 min.

Fig. 4. SDS-PAGE of a fibrillar preparation obtained by ultracentrifugation. Lanes: 1, *S. sanguis* 12; 2, *S. sanguis* 12na; 3, *S. sanguis* N; 4, Molecular weight markers. Molecular weight markers are myosin (200,000), β -galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200) and ovalbumin (45,000). The gel was silver stained.

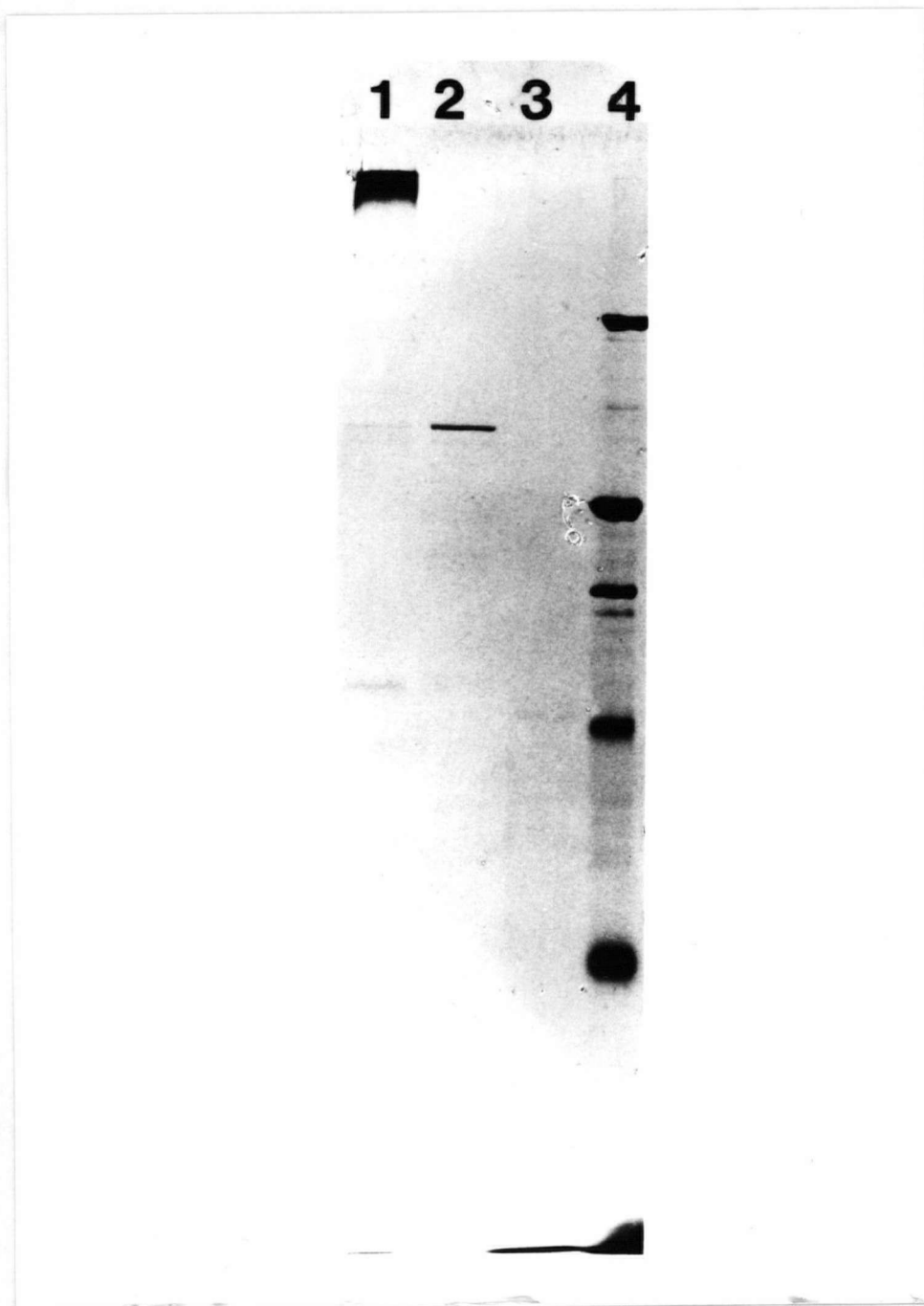


Table III. Amino acid composition of the *S. sanguis* 12 fibrillar preparation.

Amino acid	content in whole fibril (mol%)
Asx	9.3
Thr	6.4
Ser	14.5
Glx	15.7
Pro	3.6
Gly	15.0
Ala	9.9
Cys	0
Val	5.1
Met	0.3
Ile	3.6
Leu	4.8
Tyr	2.2
Phe	2.3
His	1.7
Lys	3.3
Arg	2.0

Enzymatic modification of fibrils

Information on the chemical nature of the fibrils was sought by digesting purified fibrillar preparations with a number of hydrolytic enzymes. The fibrils were incubated with the enzyme, and at appropriate intervals samples were removed from the digestion mixture and mixed with SDS- β ME and placed in a boiling waterbath for 5 min. The SDS-treated samples were then analysed by SDS-PAGE (7%). Subtilisin, pronase and papain completely hydrolyzed the silver nitrate and the periodate-Schiff staining LFP band.

Exposure of the fibrils to 100 μ g of subtilisin for 5 min was sufficient to cause complete loss of the protein (Fig. 5, lane 5). Incubation with smaller amounts of the enzyme reduced the rate at which the LFP disappeared, but at no time were any degradation products visible in silver nitrate-stained gels. The fibrils were moderately sensitive to trypsin (Fig. 5, lane 2); incubation with 1 mg of the enzyme led to a slight reduction in the M_r of LFP. Chymotrypsin did not hydrolyze the LFP. The fibrils were insensitive to mutanolysin (Fig. 5, lane 3) and lysozyme (Fig. 5, lane 4), suggesting that peptidoglycan is not associated with the glycoprotein .

The fibrillar protein was released from whole cells incubated for short periods with mutanolysin. However, after continued incubation, the LFP band disappeared. This suggests that other lytic enzymes, presumably proteases, are also released by mutanolysin and that it is these enzymes that degrade the LFP.

Immunological analysis of the fibrillar preparation

The fibrillar preparation was subjected to SDS-PAGE and then examined for its ability to react with a variety of antisera (Fig. 6). Antisera raised against either formalinized whole cells of *S. sanguis* 12 or the fibrillar preparation reacted with the LFP. The reaction of LFP with anti-12 serum could be eliminated by adsorbing the serum with whole cells of *S. sanguis* 12. This suggests that the LFP is located on the bacterial cell surface. Antisera raised against LFP which had been eluted from a gel also reacted with the glycoprotein

Fig. 5. SDS-PAGE of a fibrillar preparation of *S. sanguis* 12 treated with various enzymes. Lanes: 1, untreated; 2, trypsin; 3, mutanolysin; 4, lysozyme; and 5, subtilisin. Molecular weight markers as in fig. 4. are shown by arrows. The gel was silver stained. The lower molecular weight bands seen in lanes 4 and 5 are lysozyme and subtilisin, respectively. Proteins bands of M_r s of approximately 70,000 and 45,000 in lane 4, were contaminants present in lysozyme preparations.

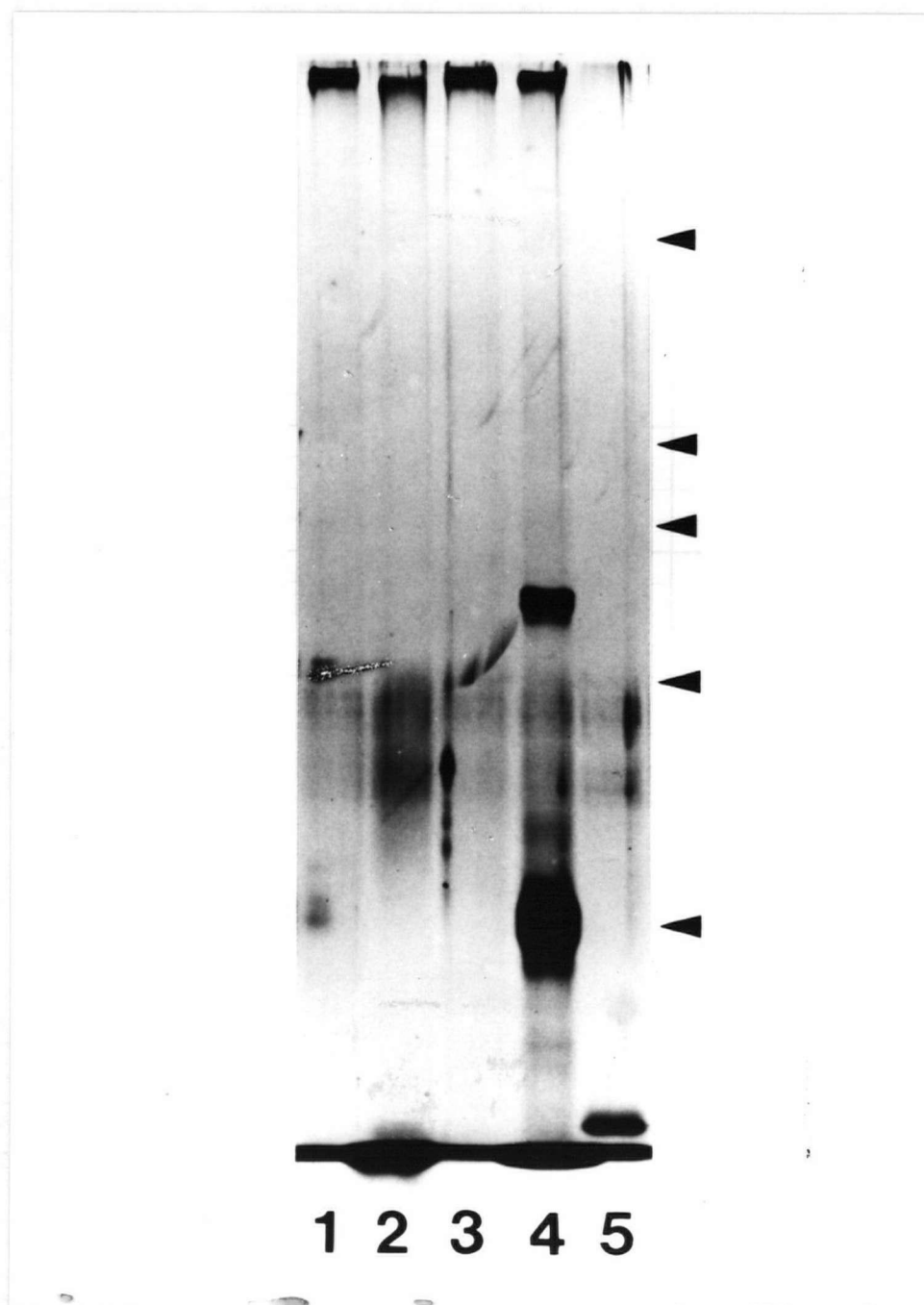
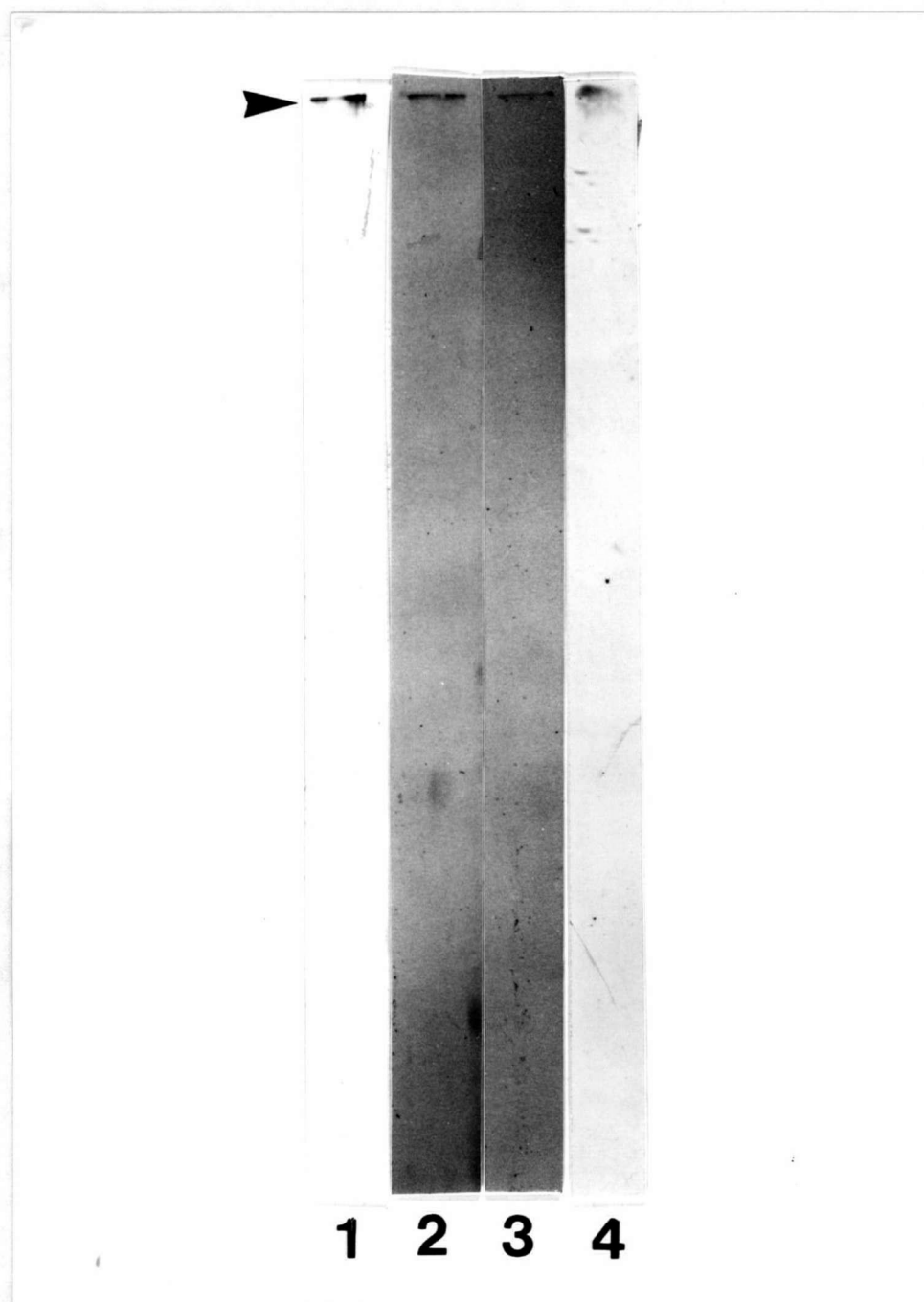


Fig. 6. Western blot of fibrillar preparations of *S. sanguis* 12. Lanes: 1, anti-12 serum; 2, anti-fibril serum; 3, anti-LFP serum; and 4, Mab 32. 33. Arrow indicates reaction with LFP.



(Fig. 6, lane 3). The antiserum did not react with any protein in culture supernatants from the parent strain 12, from the non-aggregating strain 12na or the non-adhering strain 12L. Monoclonal antibody 32.23 derived from mice immunized with whole cells of *S. sanguis* (gift from M. Song), reacted with LFP (Fig 6, lane 4). This Mab also reacted with the fibrillar preparation, as determined by ELISA. The poor reactivity observed with the Western blots is because the high M_r LFP protein is not efficiently transferred to the nitrocellulose paper.

Immunogold electron microscopy

S. sanguis 12 possesses two lengths of surface fibrils, as shown in Fig. 2. The shorter distinct capsular layer approximately 70 nm long, and the long polar fibrils which have a length of 200 nm.

Anti-LFP antibody coated to gold beads reacted strongly with the long polar fibrils. Figure 7 shows the beads clearly aligned along the long fibrils, they apparently did not adhere to or were unable to penetrate the shorter fibrillar layer.

The ability of anti-LFP-coated gold beads to bind to *S. sanguis* 12na was also studied. Although no fibrils could be isolated from this strain, which has lost the ability to aggregate in saliva and has altered adherence properties (Morris *et al.*, 1984). Some gold beads were found attached to a surface layer around 12na cells (Fig. 8). The position of the beads suggested that they are located at the external ends of the short fibrillar layer seen on strain 12. The beads often adhered more densely at the poles of the cell, as seen in Fig. 9; this may represent adherence to some residual or defective form of long fibrils.

Anti-LFP conjugated to gold beads bound to aggregated masses of fibrils indicating that these fibrils were the same as the long fibrils on the surface of strain 12. Gold beads coated with non-immune IgG did not adhere either to whole cells of *S. sanguis* 12, or to the isolated fibrils.

Fig. 7. Electron micrograph of *S. sanguis* 12 labelled with anti-LFP serum. Cells were incubated with gold beads coated with antiserum raised against the LFP band excised from an SDS-PAGE gel. Negatively stained with uranyl acetate. Bar, 0.5 μm .

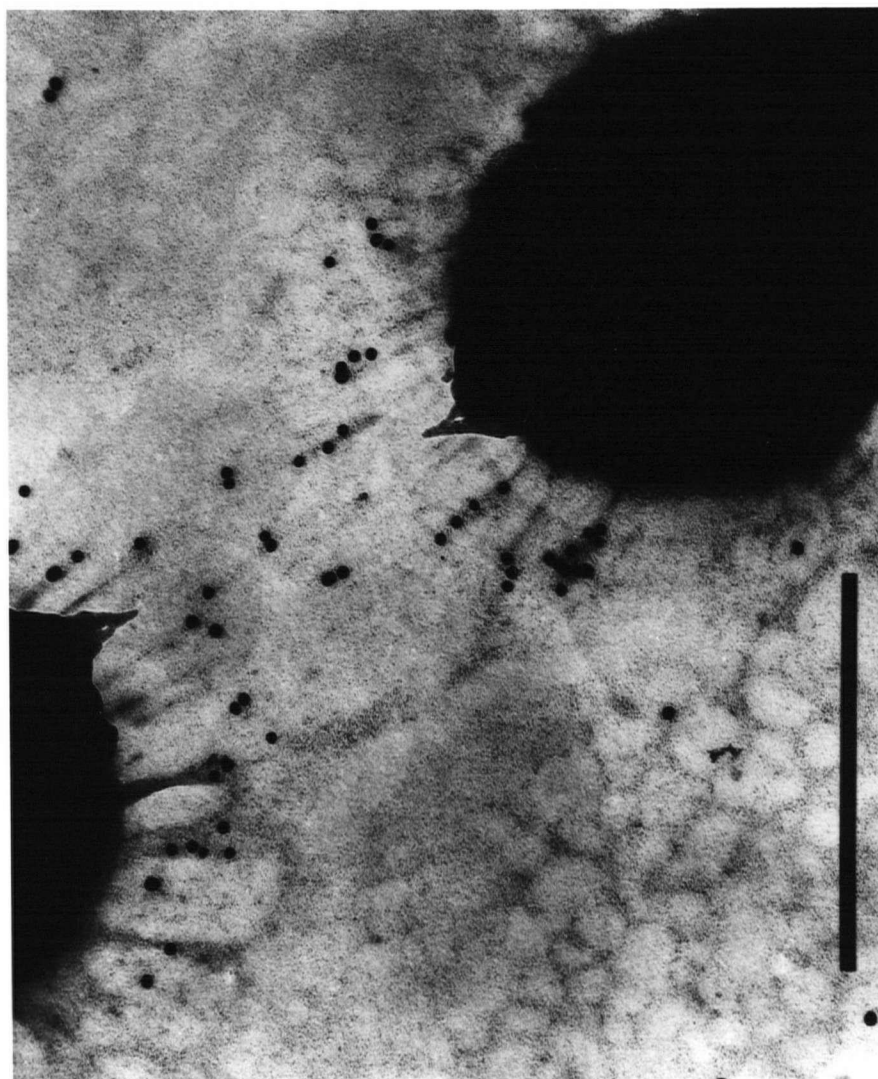
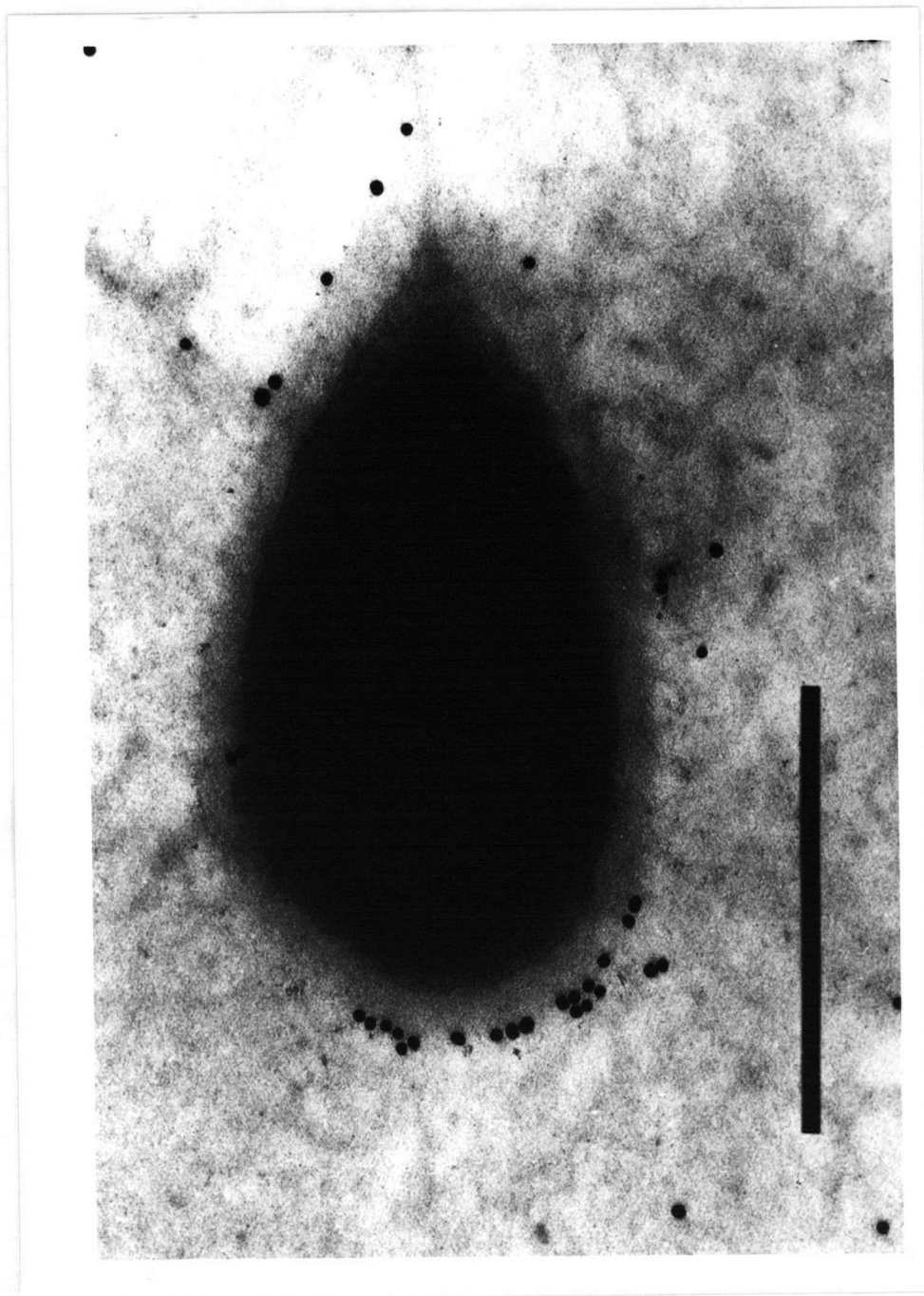


Fig. 8. Electron micrograph of *S. sanguis* 12na labelled with anti-LFP serum. Cells were incubated with gold beads coated with anti-LFP serum. Negatively stained with uranyl acetate. Bar, 0.5 μ m.



Gold beads coated with the LFP-reacting Mab 32.33 formed a halo around the cells (Fig. 9), suggesting that Mab 32.23 recognized an epitope in the terminal subunit of the fibril. The position of the gold bead suggests that it was at a point consistent with the end of the long fibril.

Anti-fibril antibody reacted with whole cells of *S. sanguis* 12 (Fig. 10) in a similar fashion. However, in this experiment, cells were first incubated with the anti-fibril serum and then reacted with gold bead conjugated goat anti-rabbit serum. The size of the gold beads in these studies were 5nm in diameter and the amount of gold bead conjugated serum used was higher than in earlier experiment (Fig. 7-8). In the earlier experiments the gold beads were conjugated directly to the antibody of interest and the size of the gold beads were 20 nm in width. This accounts for the much higher number of gold beads associated with cells in Fig. 10.

Dissociation of fibrils

It was considered possible that the LFP might be made up of subunits, as found for the fimbrial proteins of a number of Gram-negative bacteria (Jones *et al.*, 1983; Karch *et al.*, 1985). Consequently, various dissociating agents were tested for their ability to break down the LFP band into smaller units. Urea (8 M), guanidine hydrochloride (8.6 M), and sodium thiocyanate (1 M) were found to be ineffective. HCl treatment caused partial disappearance of the band when the sample was boiled in HCl at pH 1.8 for 5 to 15 min and total disappearance after overnight incubation at 37°C. However, no bands of lower M_r were formed which were visible in either a 7 or 12% silver-stained SDS-PAGE gel. Boiling the fibrillar preparation in SDS- β ME for 1 h instead of 5 min prior to SDS-PAGE analysis resulted in some degradation of the glycoprotein. A long silver-staining smear was seen on the gel, but discrete subunits could not be detected. This was the case even when samples were applied to 5 to 20% gradient gels. The smear reacted with the anti-LFP serum.

Attempts to hydrolyze fibrils with subtilisin were found to be effective. On prolonged incubation (18 h) with subtilisin at a concentration of 100 μ g/ml, the fibrillar material

Fig. 9. Electron micrograph of *S. sanguis* 12 labelled with Mab 32.33. Cells were incubated with gold beads coated with Mab 32.33. Negatively stained with uranyl acetate. Bar, 0.5 μm .

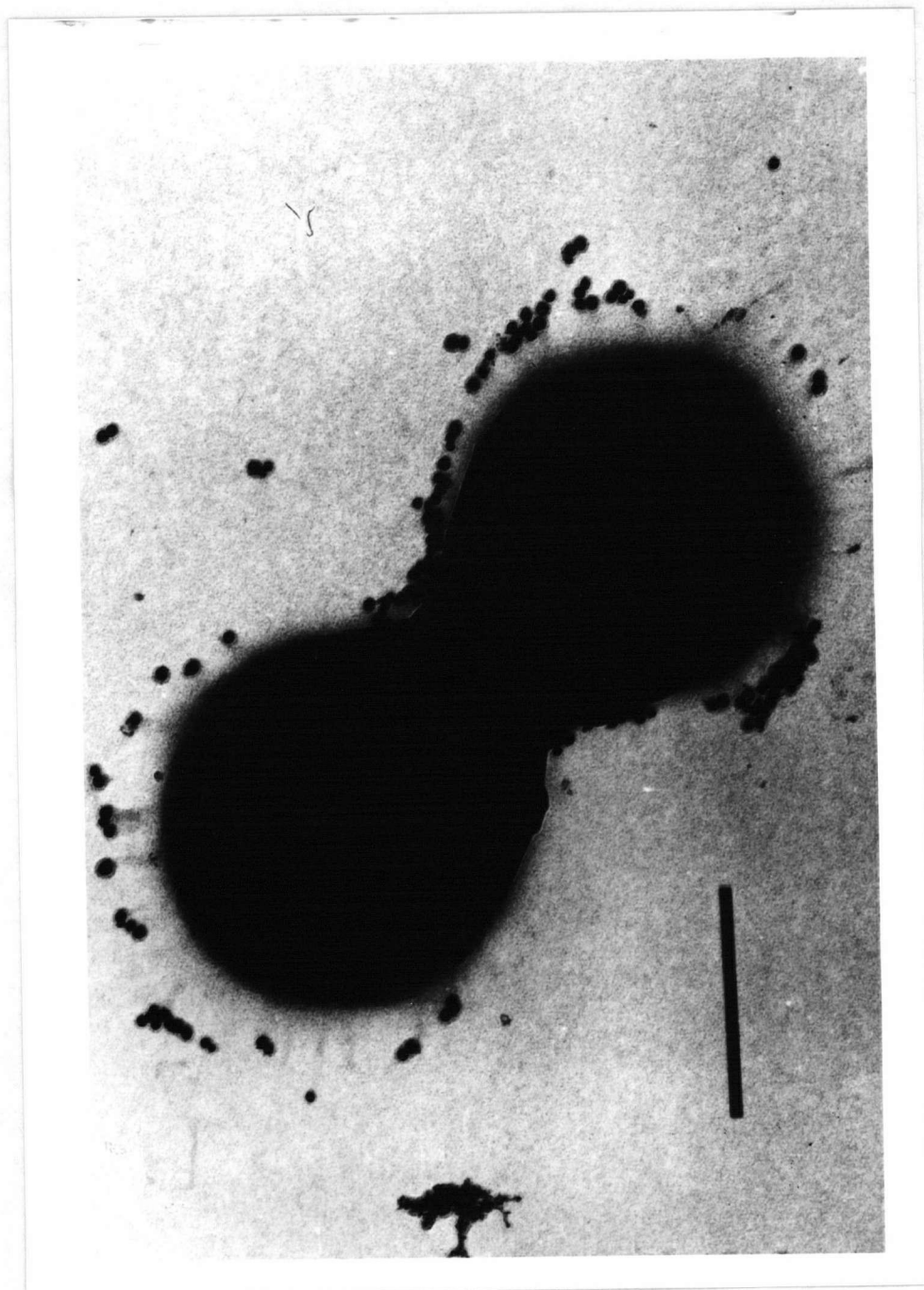
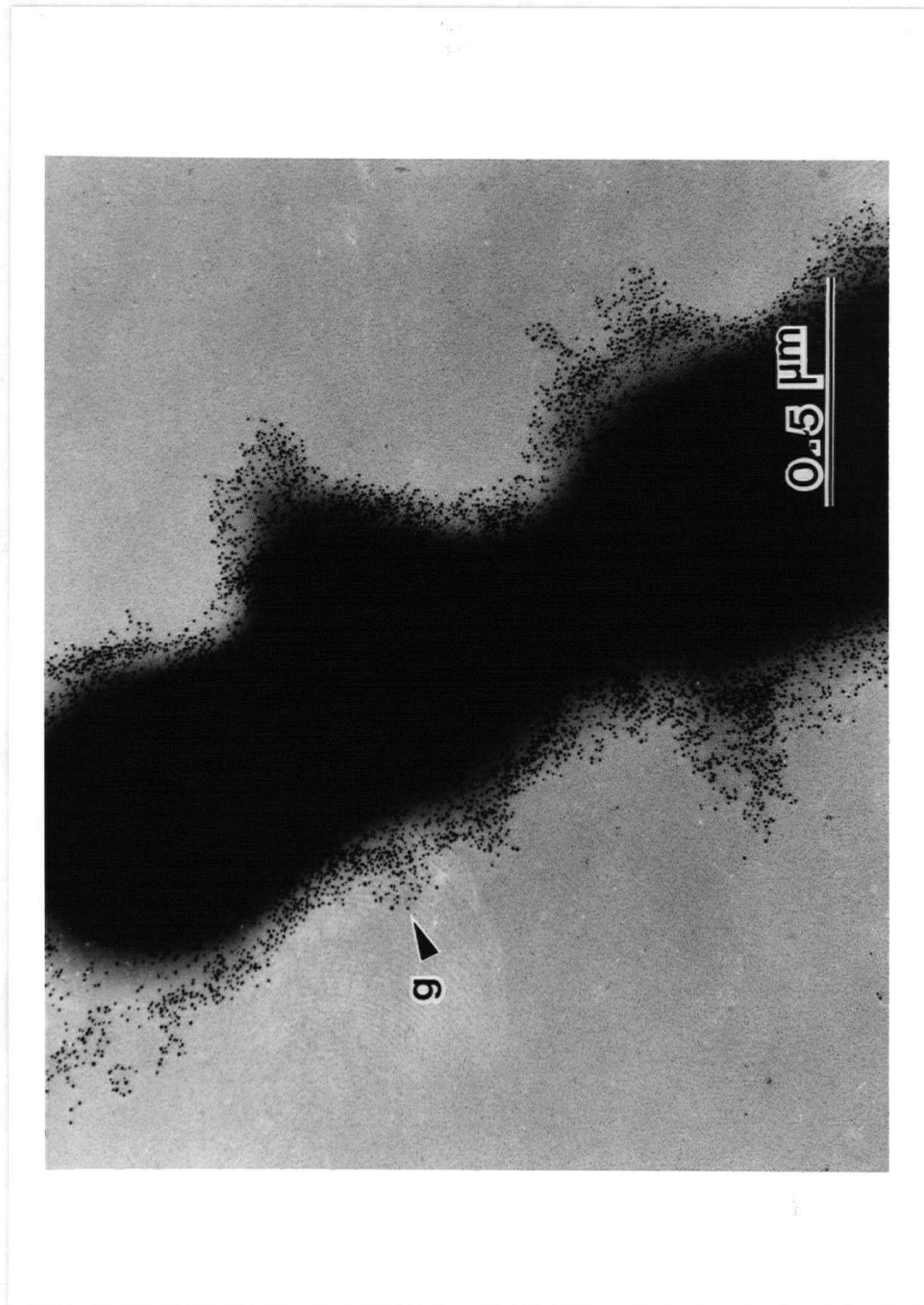


Fig. 10. Electron micrograph of *S. sanguis* 12 labelled with anti-fibril serum. Cells were reacted with anti-fibril serum and then incubated with gold beads conjugated to goat anti-rabbit serum. Negatively stained with uranyl acetate.



broke down into a series of discrete lower M_r bands which could be seen on 5-20% gradient gels (Fig. 11). The LFP band disappeared within 5 min (Fig 11, lane 1) of incubation with subtilisin. No bands could be seen until after 18 h incubation with the enzyme (Fig. 11. lane 6). These new bands were spaced at regular intervals and reacted with both anti-LFP (data not shown) and anti-fibril serum (Fig. 11). At no time did the fibrils break down to form a single monomeric band. It is possible that the monomer is sensitive to protease and, hence not visualized on the gel. The low M_r bands appear at regular intervals suggesting a constant region of the molecule of the fibrils is being cleaved (Fig. 11, lane 6). The other lower M_r bands seen on the gel are residual subtilisin and minor contaminating bands seen with the fibrils. These do not react with anti-fibril serum on Western blot. The lack of reactivity of LFP band to anti-fibril serum is due to the poor transfer of this high M_r protein to nitrocellulose paper as noted earlier.

Inhibition of adherence

The possibility that the fibrils were involved in binding to S-HA was assessed in an indirect antibody blocking assay (Table IV). In this assay, cells were first preincubated with the appropriate $F(ab')_2$ fragments for 1 h prior to mixing with S-HA. Adherence inhibition experiments with strain 12 were done with untreated S-HA beads and S-HA beads treated at pH 5.0 to remove the pH sensitive receptor as described previously (Morris *et al.*, 1984). As seen in Table 4, $F(ab')_2$ fragments prepared from anti-fibrillar antibody inhibited binding by 85%. These results were obtained only when the S-HA beads had been treated at pH 5. This would leave the S-HA beads with a single neuraminidase-sensitive receptor. When both the receptors were present the adherence inhibition could not be detected. This indicated that each adhesin-receptor interaction was functioning as an independent entity. $F(ab')_2$ fragments prepared from antisera raised against the LFP band which had been excised from an SDS-PAGE gel did not inhibit binding. This may be because antibody was raised against a denatured protein which did not express the appropriate epitopes. $F(ab')_2$ fragments prepared from non-immune IgG had no effect.

Neither anti-fibrillar nor anti-LFP $F(ab')_2$ fragments inhibited the binding of strain 12na.

Fig. 11. Gradient SDS-PAGE (5-20%) and Western blot analysis of a fibrillar preparation treated with subtilisin for increasing lengths of time. The left hand panel represents a silver stained gel, and the right hand panel represents the Western blot of the gel reacted with anti-fibril serum. Lanes: 1, enzyme alone; 2, 5 min; 3, 30min; 4, 1 h; 5, 5 h; 6, 18 h; and 7, untreated fibrils. Each lane contained approximately 6 μ g of fibrils.

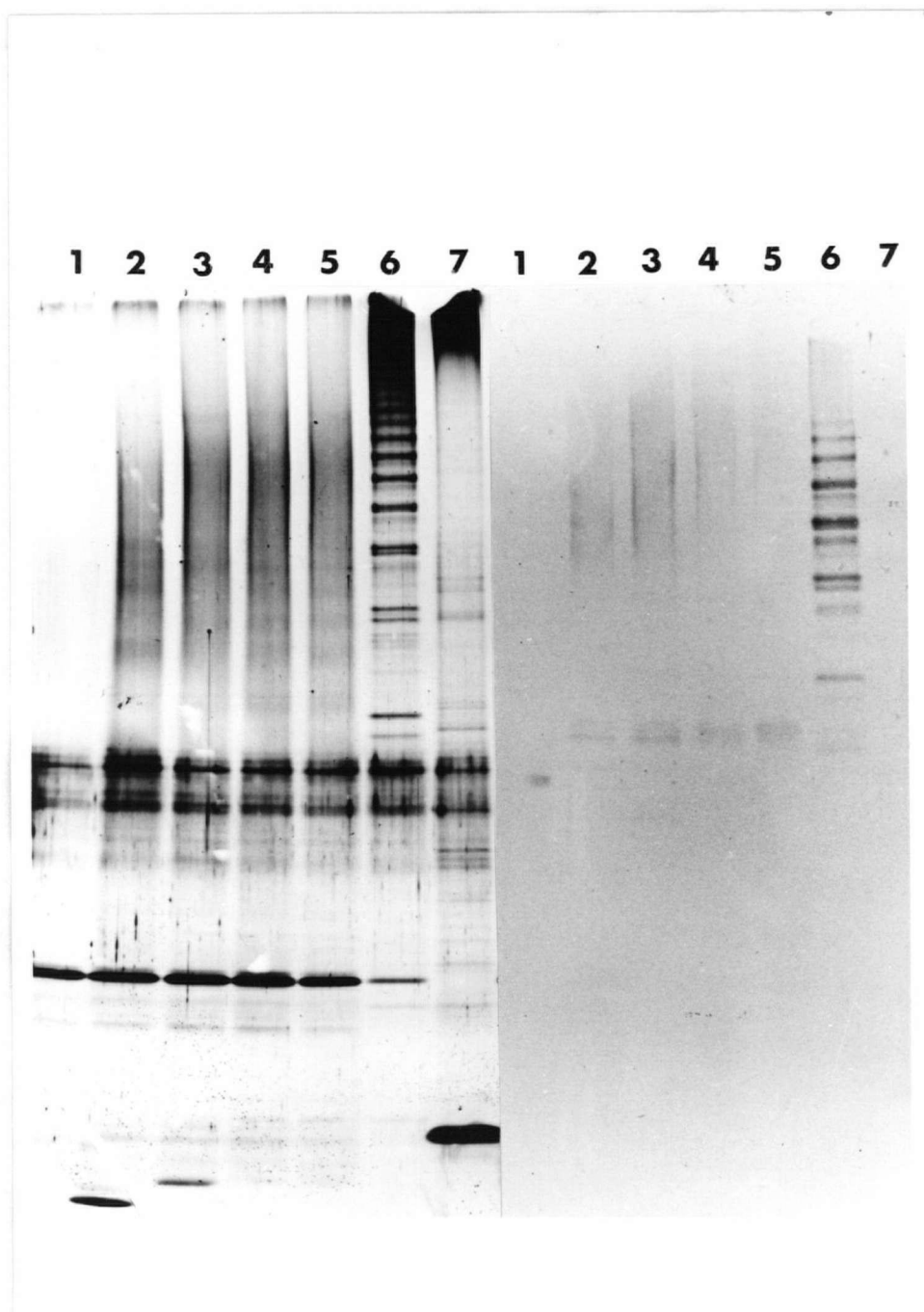


Table IV. Adhesion-blocking activity of F(ab')₂ fragments.

IgG source	Mean adhesion ^a to S-HA (10 ⁸ cells per ml) ± SD	Relative Adhesion (%)
Non-immune	1.32 ± 0.06	100
Anti-fibril	0.20 ± 0.29	15
Anti-LFP	1.20 ± 0.01	91
HEPES buffer	1.49 ± 0.19	113

^a 3 x 10⁹ cells were added to 40 mg of S-HA.

This was not unexpected as this strain lacks the adhesin which binds to the neuraminidase-sensitive receptor. It was not possible to determine if fibrils would inhibit binding because of the extensive aggregation as visualized in phase contrast microscopy.

The studies of *S. sanguis* fibrils demonstrated: (1) that long fibrils are composed of in part a high M_r glycoprotein (LFP); (2) antibody raised against the native fibrils inhibited adherence of the organism to pH modified S-HA through the neuraminidase-sensitive receptor; and (3) subtilisin hydrolyzed fibrils yield a series of lower M_r proteins which were reactive with anti-fibril and anti-LFP antiserum.

Further characterization of LFP was discontinued because of the : (1) difficulty in obtaining adequate yields of LFP; (2) complexity of the fibrillar preparation; and (3) difficulties associated with working with a large complex molecule which could not be dissociated into subunits.

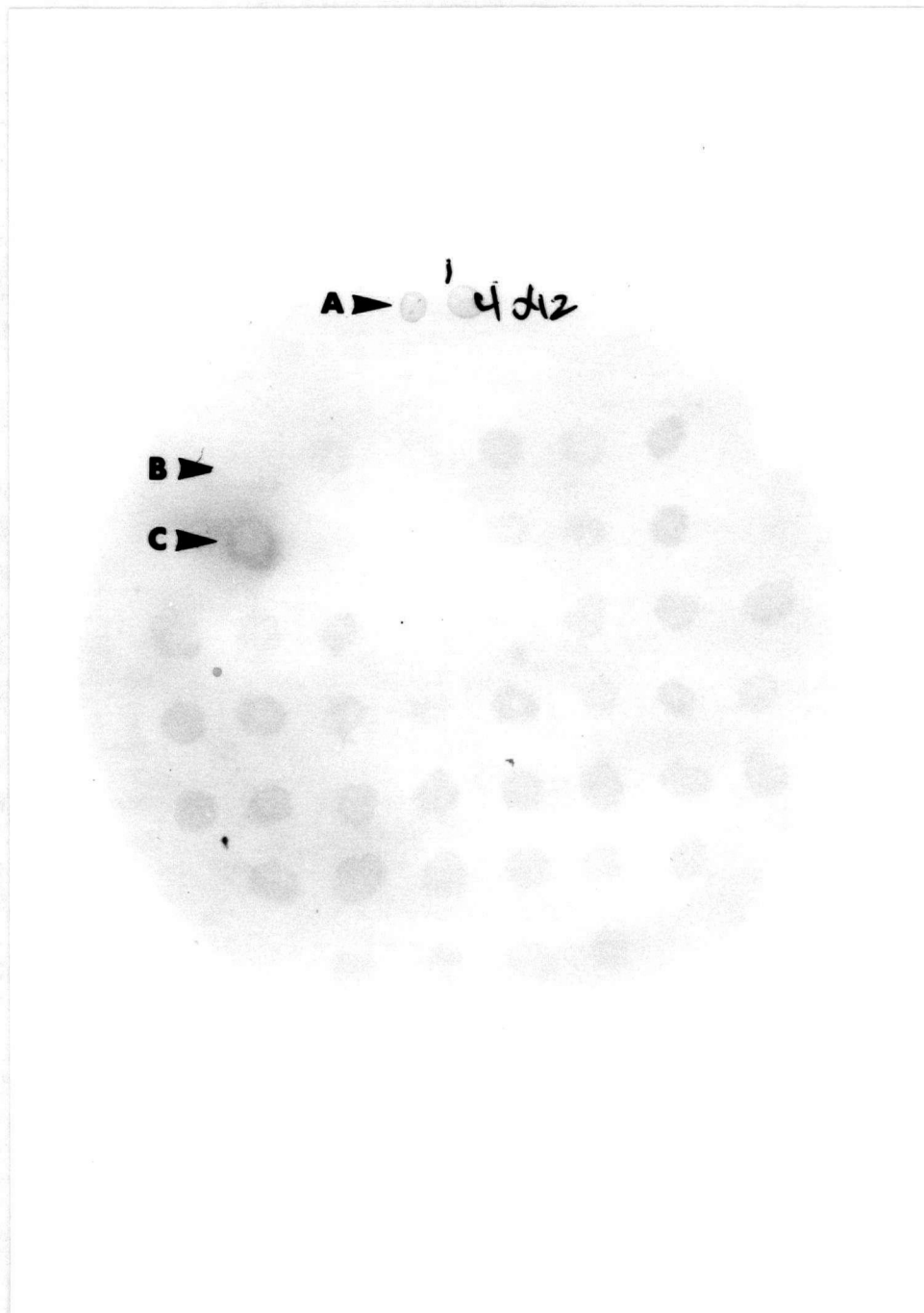
Section B: Cloning

In order to circumvent the problems described in the previous section, it was decided to try and clone genes encoding *S. sanguis* antigens into *E. coli*. The strategy employed was to use pUC18 plasmid as the vector. Insertion of DNA into the multiple cloning site in this vector gave rise to white colonies, when the recombinant clones were grown in media containing Xgal.

Isolation of recombinant clones

S. sanguis 12 chromosomal DNA was partially digested with Pst1 restriction enzyme and used to construct recombinant clones with the plasmid vector pUC18. A total of 1700 white colonies were picked from LB agar plates containing XGal and ampicillin. Seventeen of these clones reacted in a colony immunoblot assay with antisera raised against either whole cells of *S. sanguis* 12 or *S. sanguis* 12 fibrils. An example of a colony immunoblot is shown in Fig. 12.

Fig. 12. Colony immunoblot. Colonies with inserts were replica plated, and lysed *in situ*. Lysed colonies were blotted onto nitrocellulose paper and reacted with anti-12 serum (A), *S. sanguis* 12 cells; (B), *E. coli* JM83(pUC18); and (C), positive clone (pSA2).



Expression of cloned antigens

Preliminary characterization of the antigens expressed in the recombinant clones was done by SDS-PAGE (7%) (Fig 13) and Western immunoblotting (Fig. 14). Six of the clones expressed two proteins having M_r s of 20,000 and 36,000. One of these (SA2), plus four of the remaining 11 clones were chosen for further study. *E. coli* SA2 (Fig. 13, lane 4) and *E. coli* SA3 (Fig. 13, lane 9) produced enough *S. sanguis* protein to be visualized on a Coomassie blue or silver nitrate stained gel; all other recombinant proteins were identified by immunoblotting.

Recombinant SA1 expressed a protein with a M_r of 64,000 which reacted with anti-12 serum. Recombinant SA2 produced large quantities of a 36,000 and a 20,000 M_r proteins. The 36,000 kd (*Streptococcus sanguis* adhesin B; SsaB) protein reacted with anti-12 serum. Recombinant SA3 expressed an array of high M_r proteins which reacted with anti-12 serum. Recombinant SA4 expressed proteins with M_r s of 75,000 and 68,000, both proteins reacted with anti-12 serum. Recombinant SA5 produced a 50,000 M_r protein which reacted only with the anti-fibril serum.

The length of the DNA insert of each clone was determined by digesting the appropriate plasmids with EcoR1, Pst1, BamH1, Hind111 and Sal1 restriction endonucleases. The sizes of the inserts were 3.0, 2.8, 6.6, 2.5, 1.7 kb respectively for clones SA1 to SA5 (Fig. 15). Some characteristics of these 5 recombinants are itemized in Table V.

Surface location of cloned antigens

In order to localize the cloned antigens in *S. sanguis* 12 the following experiments were done. (a) Anti-12 and anti-fibrillar sera were allowed to react with whole cells of *S. sanguis* 12 to remove antibodies which reacted with surface antigens. When the adsorbed antisera were tested on a Western blot, they did not react with any of the cloned antigens indicating that the *S. sanguis* antigens expressed in *E. coli* were probably located on the *S. sanguis* cell surface. (b) Sonicated lysates of each of the five recombinant clones

Fig. 13. SDS-PAGE analysis of recombinant clones. cells were lysed by boiling in SDS solubilization mixture and electrophoresed. The gel was silver stained. Lanes: 1, molecular weight markers as in Fig. 4; 2 to 18 represent colonies which reacted positively on a colony immunoblot assay; 19, *E. coli* JM83 (pUC 18); and 20, mutanolysin digest of *S. sanguis* 12.

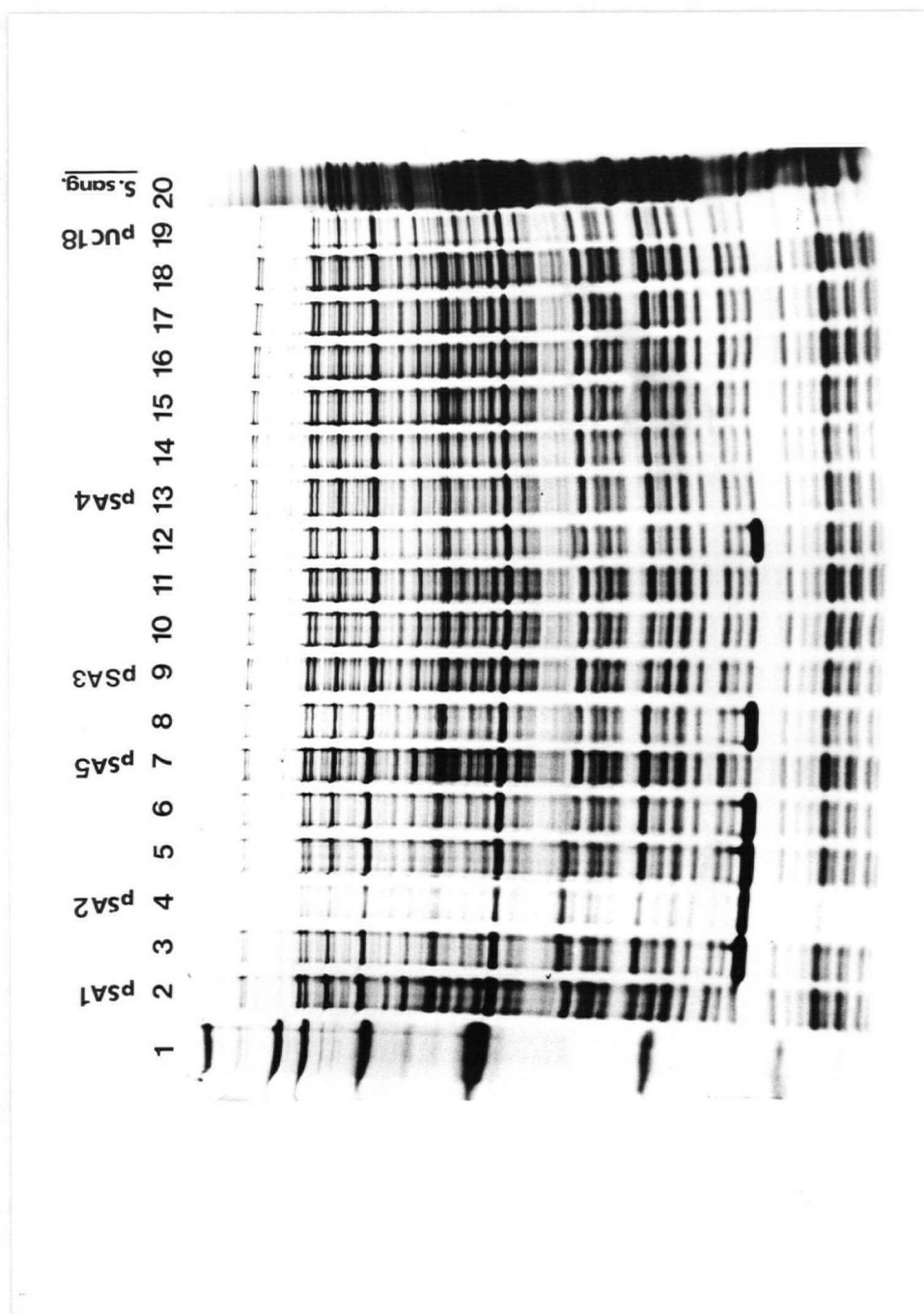


Fig. 14. Western blot analysis of recombinant clones. Recombinant clones were solubilized by boiling in SDS- β ME, and electrophoresed. Gels were blotted onto nitrocellulose and reacted with (A) anti-12 serum, and (B) anti-fibril serum. Lanes 1-6 are lysates of *E. coli* JM83 containing different plasmids: lane 1, pUC18; lane 2, pSA1; lane 3, pSA2; lane 4, pSA5; lane 5, pSA3; lane 6, pSA4. Lane 7 represent a mutanolysin digest of *S. sanguis* 12.

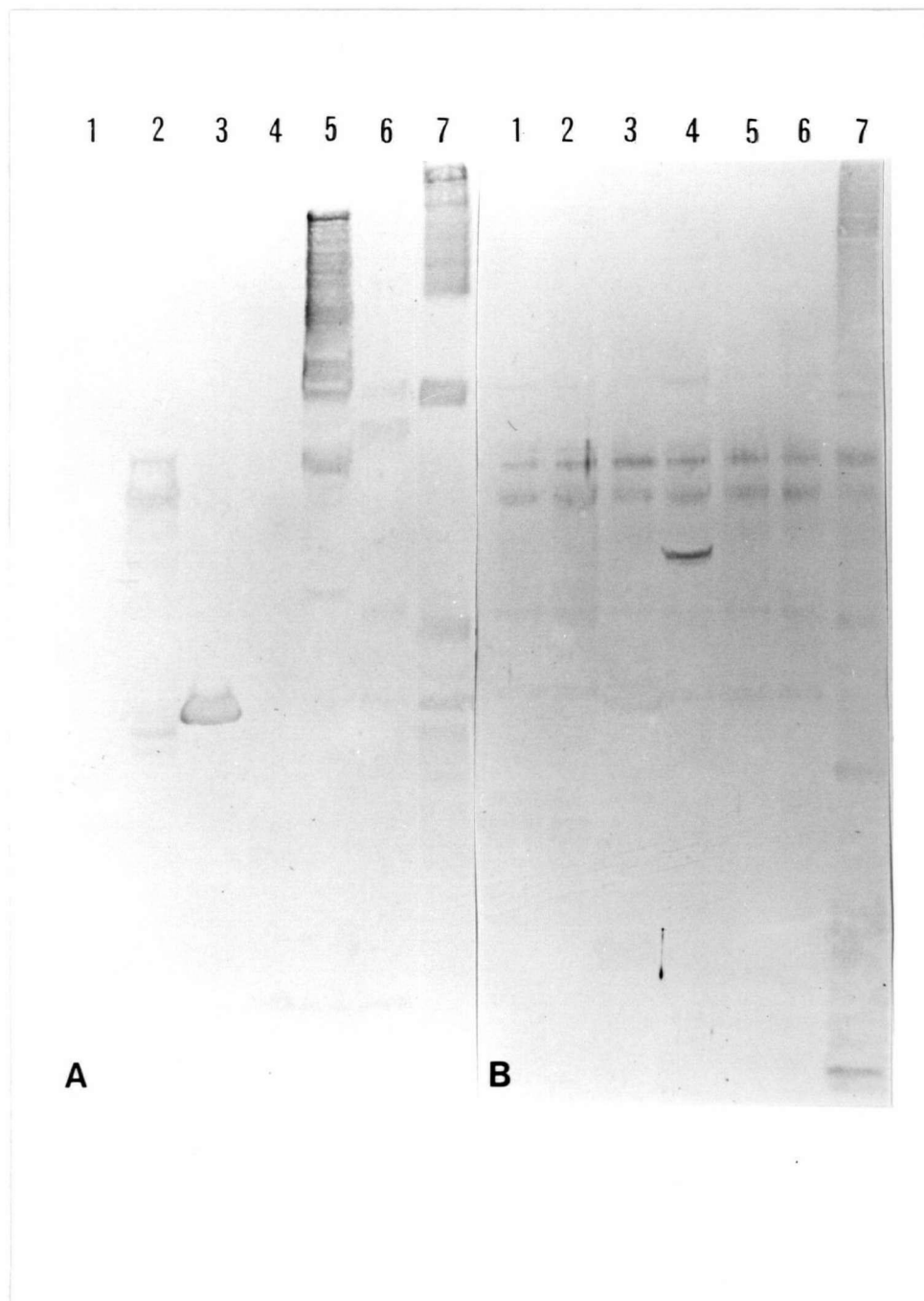


Fig. 15. Plasmid DNA analysis of recombinant clones. Plasmid DNA was digested with Pst1 restriction enzyme and analyzed by agarose gel electrophoresis. pSA3 was a partial digest with Pst 1.

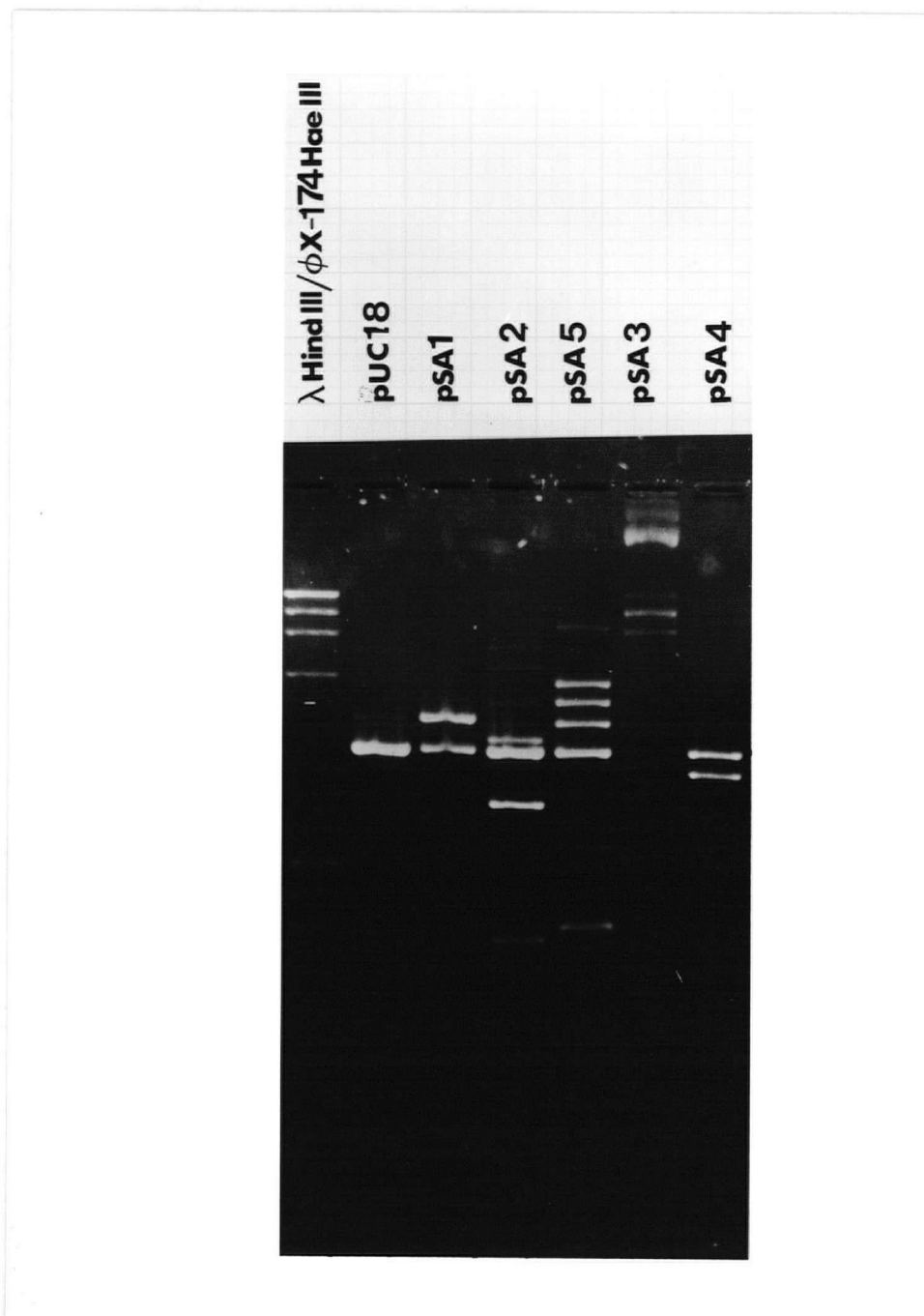


Table V. Recombinant clones

Strain	construction	Insert size (kb)	Protein (kd)	Reaction with	
				anti-12	anti-fibril
SA1	JM83(pSA1)	3.0	64	+	-
SA2	JM83(pSA2)	2.8	20 & 36	+	-
SA3	JM83(pSA3)	6.6	HM _r ^a	+	-
SA4	JM83(pSA4)	2.5	75 & 68	+	-
SA5	JM83(pSA5)	1.7	50	-	+

^a HM_r - There were number of high M_r proteins with M_r's between 200,000 and 92,500. M_r's of proteins and insert DNA were calibrated as described in Materials and Methods.

were used to raise antisera in mice. The antisera were incubated with *S. sanguis* 12, and the cells visualized by epifluorescence. Antisera raised against *E. coli* SA1 and SA2 reacted positively with *S. sanguis* 12, clearly indicating that the recombinant proteins are located on the surface. The antisera raised against recombinant lysates SA3, SA4, and SA5 did not react with *S. sanguis* 12. The lack of reactivity of the other recombinant antisera is not surprising given the small amounts of recombinant proteins expressed in these clones. The low level of antigens may not have stimulated the production of antibody directed to the recombinant proteins. In order to resolve this problem, it will be necessary to partially purify and concentrate the recombinant proteins in order to stimulate an adequate antibody response. Antisera to *E. coli* JM83(pUC18) did not react with *S. sanguis* 12 cells.

Purification of proteins from *E. coli* SA2

The strong reaction of anti-SA2 with *S. sanguis* 12 suggested the recombinant proteins expressed were present in high levels in an exposed position on the *S. sanguis* cell surface and they might be involved in adhesion. Since the two *S. sanguis* proteins in *E. coli* were expressed in large quantities, it was decided to purify these two proteins.

Lysate of *E. coli* SA2 was first chromatographed on a G-75 Sephadex column. Material absorbing at 280 nm eluted in three peaks (Fig. 16). Fractions were tested for recombinant proteins by SDS-PAGE. SsaB eluted in the void volume (peak a) together with a number of cellular contaminants. Peak b had the 20 kd protein with minor contaminants. Fractions containing the 20 kd protein from Sephadex G-75 column (peak b) were pooled and rechromatographed on a Mono Q column and eluted with a salt gradient to remove any contaminating proteins.

Fractions containing the SsaB (peak a) were pooled and applied to a DE-52 cellulose column. SsaB was eluted with 0.15 M NaCl (Fig. 17). Both SsaB and the 20 kd protein were shown to be pure by (10%) SDS-PAGE (Fig. 18).

Fig. 16. Elution profile of *E. coli* SA2 lysate chromatographed on a Sephadex G-75 column. 8 ml (3 mg/ml of protein) of *E. coli* SA2 lysate was applied to the column and eluted with the column buffer. 3 ml fractions were monitored for the presence of SsaB and the 20 kd protein by SDS-PAGE.

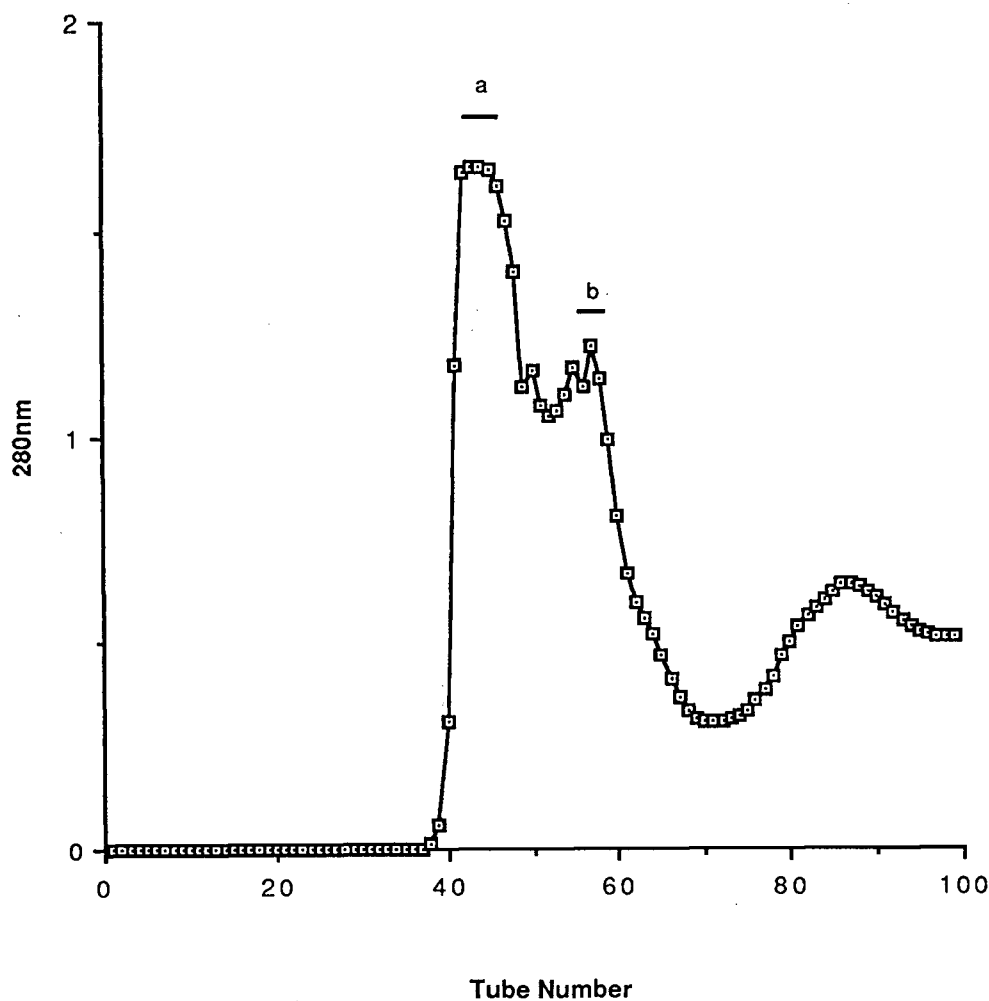


Fig. 17. Chromatography of SsaB on a DE-52 ion exchange column. Peak a of the Sephadex G-75 column was pooled and fractionated with a step gradient. Fractions were analyzed for the presence of SsaB by SDS-PAGE.

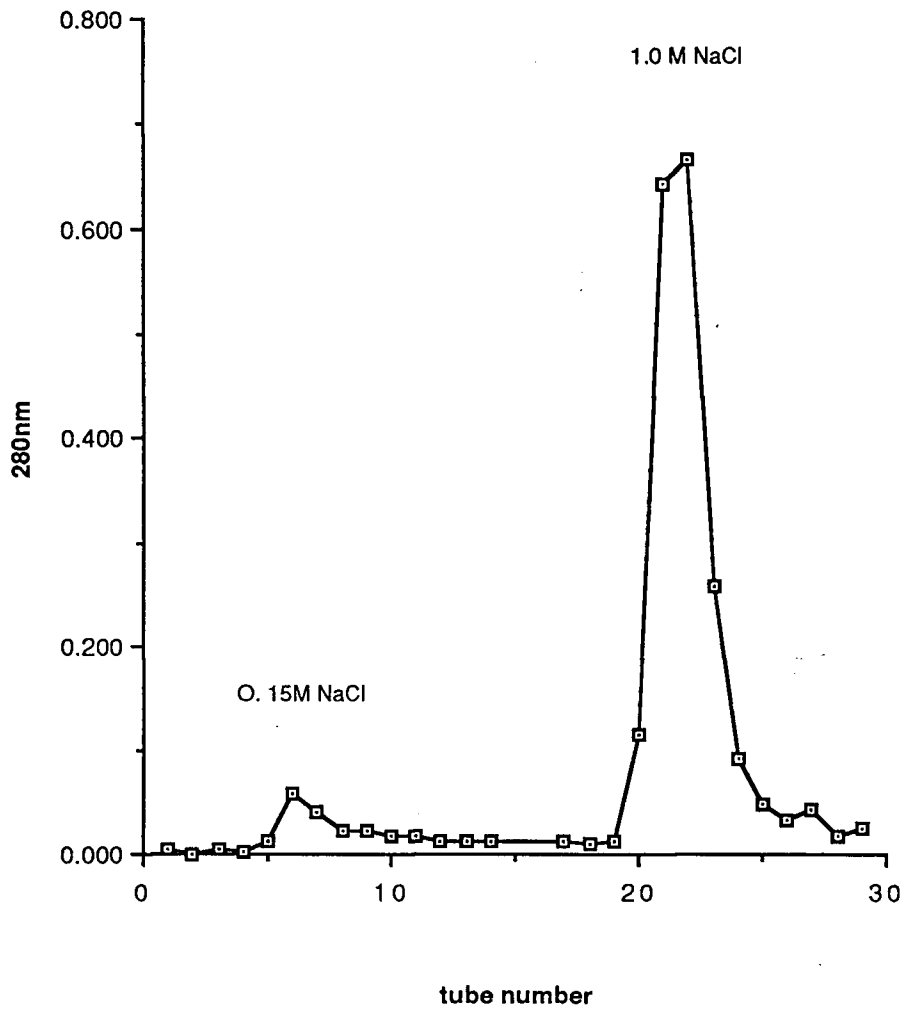
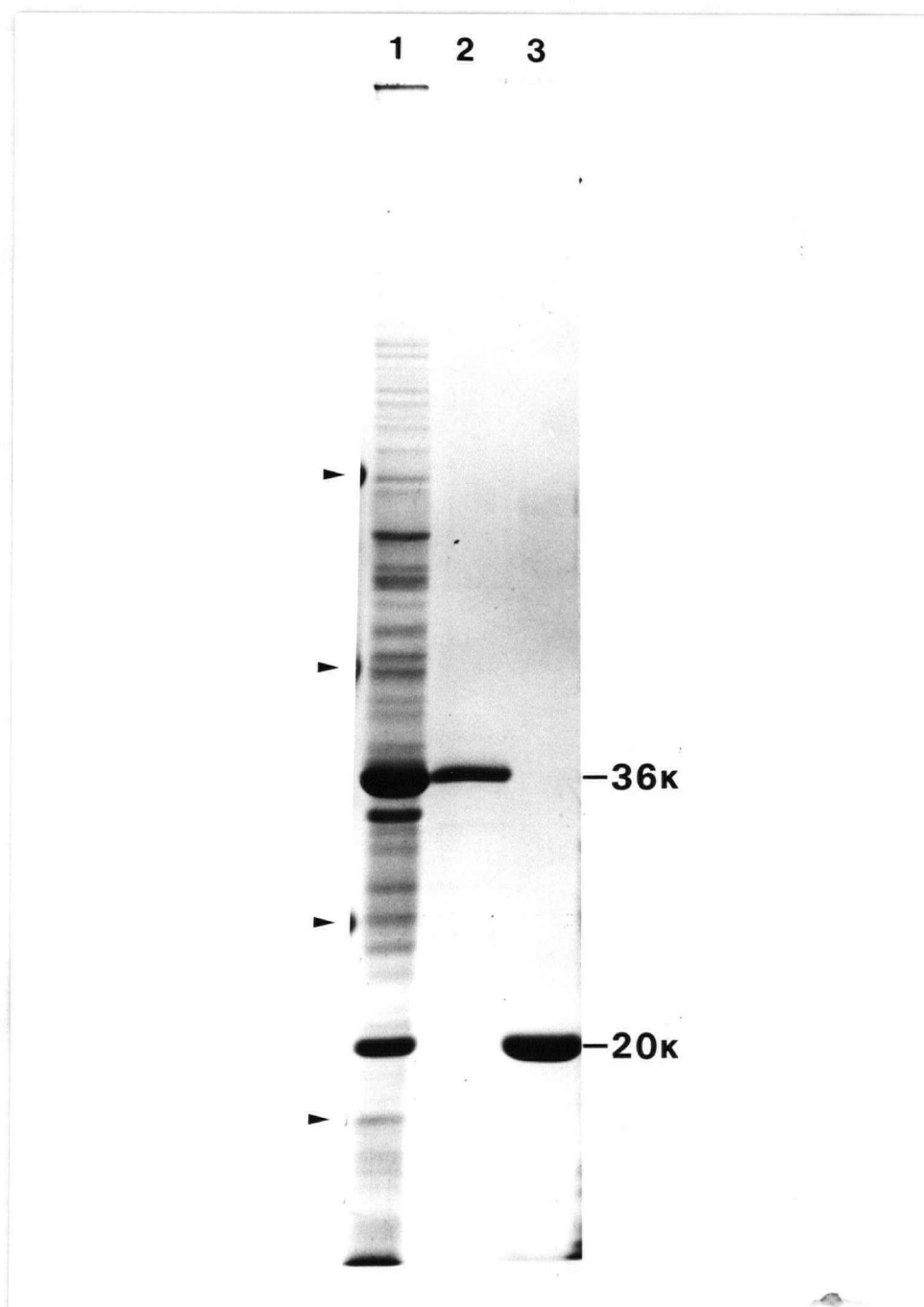


Fig. 18. SDS-PAGE analysis of purified recombinant proteins from *E. coli* SA2. Lanes: 1, Cell extract of *E. coli* SA2; 2, SsaB, 5 μ g; and 3, 20 kd protein, 5 μ g. The gel was stained with Coomassie brilliant blue. The molecular weight markers; bovine serum albumin (66,500), ovalbumin (45,000), trypsinogen (24,000), and β -lactoglobulin (18,000) are indicated by arrows.



Amino acid analysis of SsaB and the 20 kd protein are shown in Table VI. Attempts to obtain a N-terminal sequence of SsaB were not successful as the amino-terminal end was found to be modified. The N-terminal sequence of 20 kd protein is shown in Fig. 19.

Identification of *E. coli* SA2 antigens in *S. sanguis*

In order to determine whether SsaB and the 20 kd proteins were produced in *S. sanguis* in a similar form to that found in *E. coli*, antisera were raised against each of the purified proteins. These antisera were used in the following experiments.

Equal amounts of cells of *S. sanguis* strains 12, 12na and 12L were lysed with mutanolysin, and the lysates examined by Western blotting. A lysate of *E. coli* SA2, purified SsaB and the 20 kd protein, and mutanolysin digests of *S. sanguis* strains were electrophoresed, blotted onto nitrocellulose and then stained with either the anti-SsaB or anti-20 kd antisera (Fig. 20).

These experiments demonstrated that immunologically similar proteins having the same M_r are found in *E. coli* SA2 (Fig. 20, lane 1) and in mutanolysin digests of whole cells of *S. sanguis* 12, 12na and 12L (Fig. 20, lanes 4,5,6 respectively), indicating that the *S. sanguis* 12 genes for SsaB and the 20 kd protein had been cloned intact into *E. coli*. The 20 kd protein in *S. sanguis* could only be detected when the gel was overloaded with *S. sanguis* lysate.

S. sanguis 12, 12na and 12L differ in their susceptibility to lysis with mutanolysin. Strain 12L is the least sensitive followed by 12na and then 12. In the experiments shown in Fig. 20, the amounts applied to the gel were equalized to reflect the number of cells treated with mutanolysin. Thus the differences in amounts of SsaB may reflect differences in susceptibility. However, when the lysates were adjusted to equivalent levels of protein, there was no increase in the amounts of SsaB detected in Western blots of 12L. This is consistent with earlier studies (Morris *et al.*, 1985) which showed that cell walls of 12L contain very little protein when compared to the parent strain.

Table VI. Amino acid composition of the 20 kd and SsaB proteins.

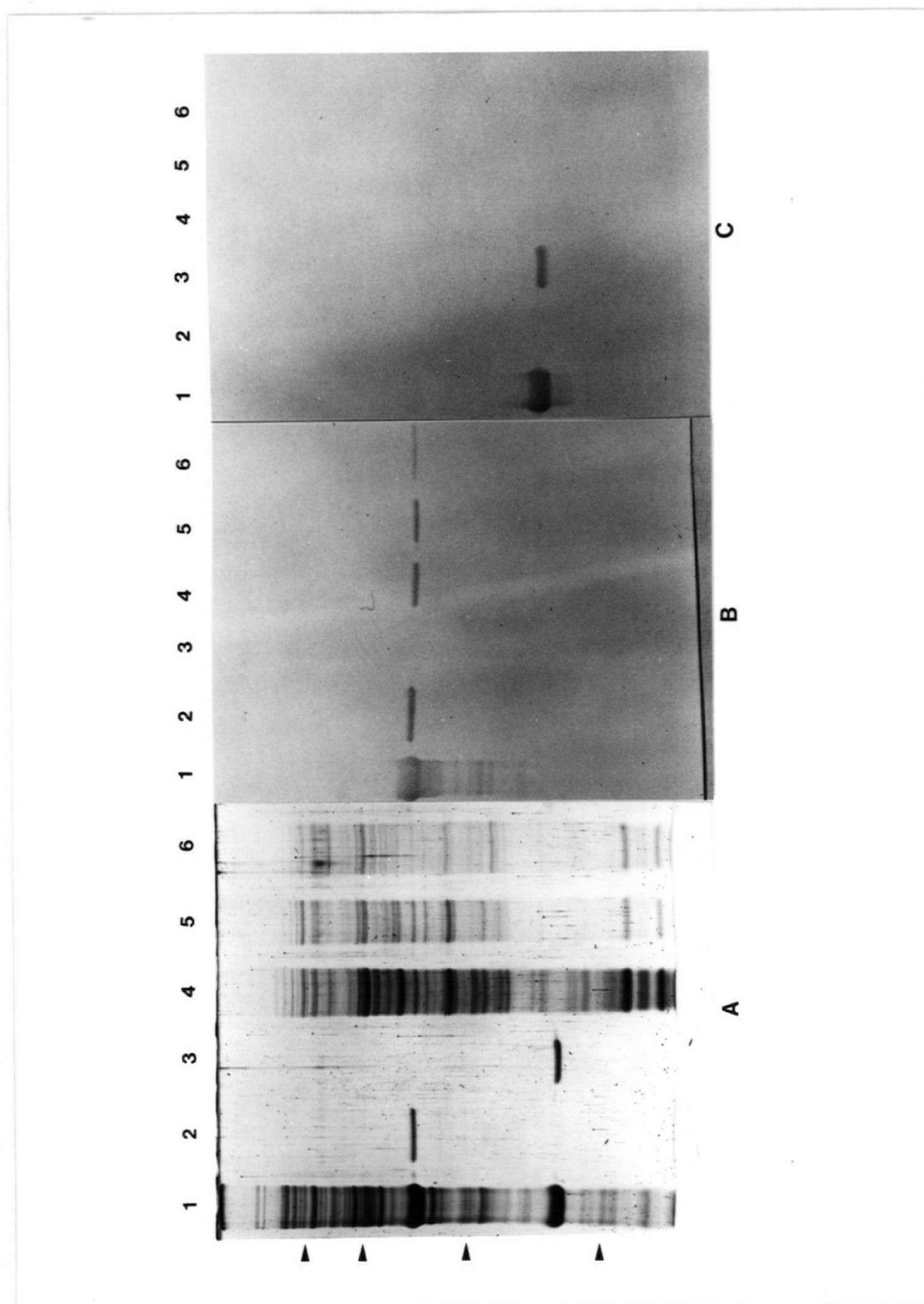
Amino acid	Residues per molecule	
	20 kd	SsaB
Asx	27	51
Thr	18	15
Ser	12	23
Glx	15	42
Pro	05	16
Gly	10	19
Ala	19	26
CysA	02	01
Val	17	20
Met	02	05
Ile	09	20
Leu	19	23
Thr	06	15
Phe	09	08
His	03	04
Lys	08	31
Arg	05	06

Basen on apparent M_r s, there are approximately 186 amino acids in the 20 kd protein, and 325 amino acids in SsaB.

Fig. 19. N-terminal amino acid sequence of the 20 kd protein.

?
Thr - Thr - Phe - Leu - Gly - Asn - Pro - Val - Thr - Phe -
Thr - Gly - Lys - Gln - Leu - Gln - Leu - Gln - Val - Gly -
Asp - Thr - Ala - ? - Asp - Phe - Ser - Leu - Thr - Ala -
Thr - Asp - Leu - Ser - Lys - Lys - Thr - ? - Ala - Asp -
Phe - Ala -

Fig. 20 Western blots of cell lysates of *E. coli* SA2 and *S. sanguis* against anti-SsaB and anti-20 kd serum. Panels (a), gel stained with Coomassie brilliant blue; (b), Western blot of the gel reacted with anti-SsaB serum; (c), Western blot of the gel reacted with anti-20 kd serum. Lanes: 1, Cell lysate of *E. coli* SA2; 2, SsaB; 3, 20 kd protein; 4,5,6 are mutanolysin digests of *S. sanguis* 12, 12na, and 12L respectively. Arrows indicate molecular weight markers as in Fig. 18.



SsaB could be extracted from *S. sanguis* 12 with 2% SDS at room temperature, more of the molecule was extracted by boiling for 5 min in SDS-BME. The amount of SsaB released by this method was less than that released by mutanolysin digestion of an equivalent amount of cells. The 20 kd protein was not released by this procedure.

Location of SsaB in *S. sanguis*

The antisera to the purified proteins were used in an immunogold labelling technique to provide additional evidence that SsaB was present on the *S. sanguis* cell surface. Cells were first incubated with anti-SsaB serum, washed and then incubated with gold beads conjugated to goat anti-rabbit serum. The anti-SsaB serum reacted strongly with *S. sanguis* 12 forming a halo around the cells (Fig. 21). The antiserum also reacted with strain 12na (Fig. 22) but not with strain 12L (Fig. 23). Non-immune serum did not react with the cells (Fig. 24) and neither did the anti-20 kd serum (Fig. 25). Thus it appears that SsaB is found in large quantities on the cell surface of *S. sanguis* 12 and to a lesser extent on 12na but not on the surface of 12L. These observations were confirmed by immunofluorescence analysis.

SsaB could be detected in Western blots of mutanolysin digests of *S. sanguis* 12L but could not be seen on immunogold labelled cells. In an attempt to gain insight into this discrepancy it was decided to cross-section the cells and look for SsaB within the cell. Previous studies by Weerkamp *et al.*, (1986) with an *S. salivarius* coaggregation deficient mutant (HB-V5) indicated that the mutation affected the transport of the adhesin rather than its synthesis. The adhesin accumulated in the cell cytoplasm and could be detected by immunological analysis of cross-sectioned cells.

Sections of *S. sanguis* 12 or 12L were prepared and processed as described in Materials and Methods. The sections were reacted with anti-SsaB serum and then incubated with gold bead conjugated to goat anti-rabbit serum (Fig. 26). Neither *S. sanguis* 12 or 12L were labelled by this procedure. The inability to label the cells may be due to: (1), loss of antigenicity of SsaB during preparation of the specimen; (2), too little SsaB to detect;

Fig. 21. Electron micrograph of *S. sanguis* 12 labelled with anti-SsaB serum. Cells were reacted with anti-SsaB serum and then incubated with gold bead conjugated goat anti-rabbit serum. Negatively stained with uranyl acetate.

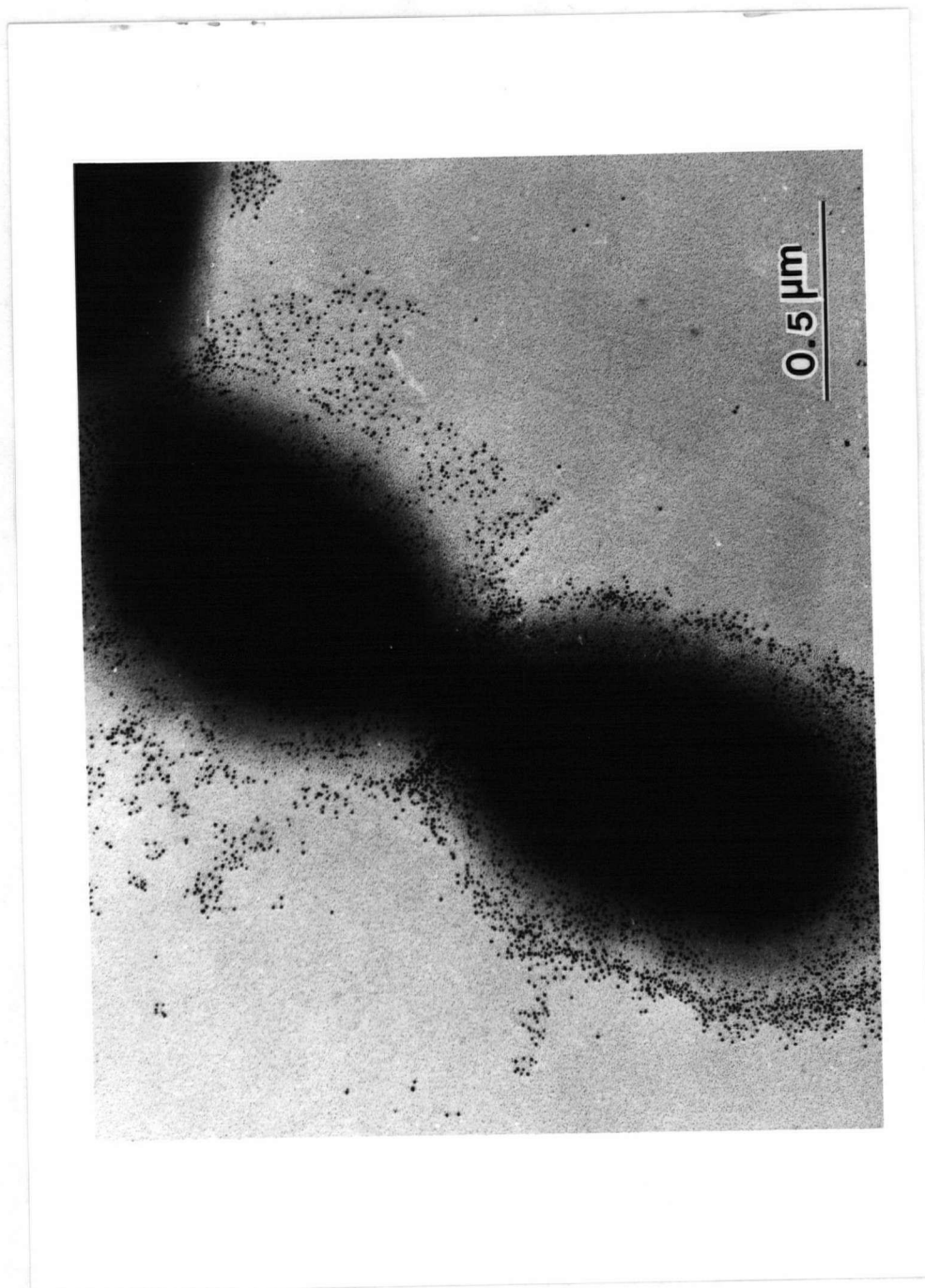


Fig. 22. Electron micrograph of *S. sanguis* 12na labelled with anti-SsaB serum. Cells were reacted with anti-SsaB serum and then incubated with gold bead conjugated goat anti-rabbit serum. Negatively stained with uranyl acetate.

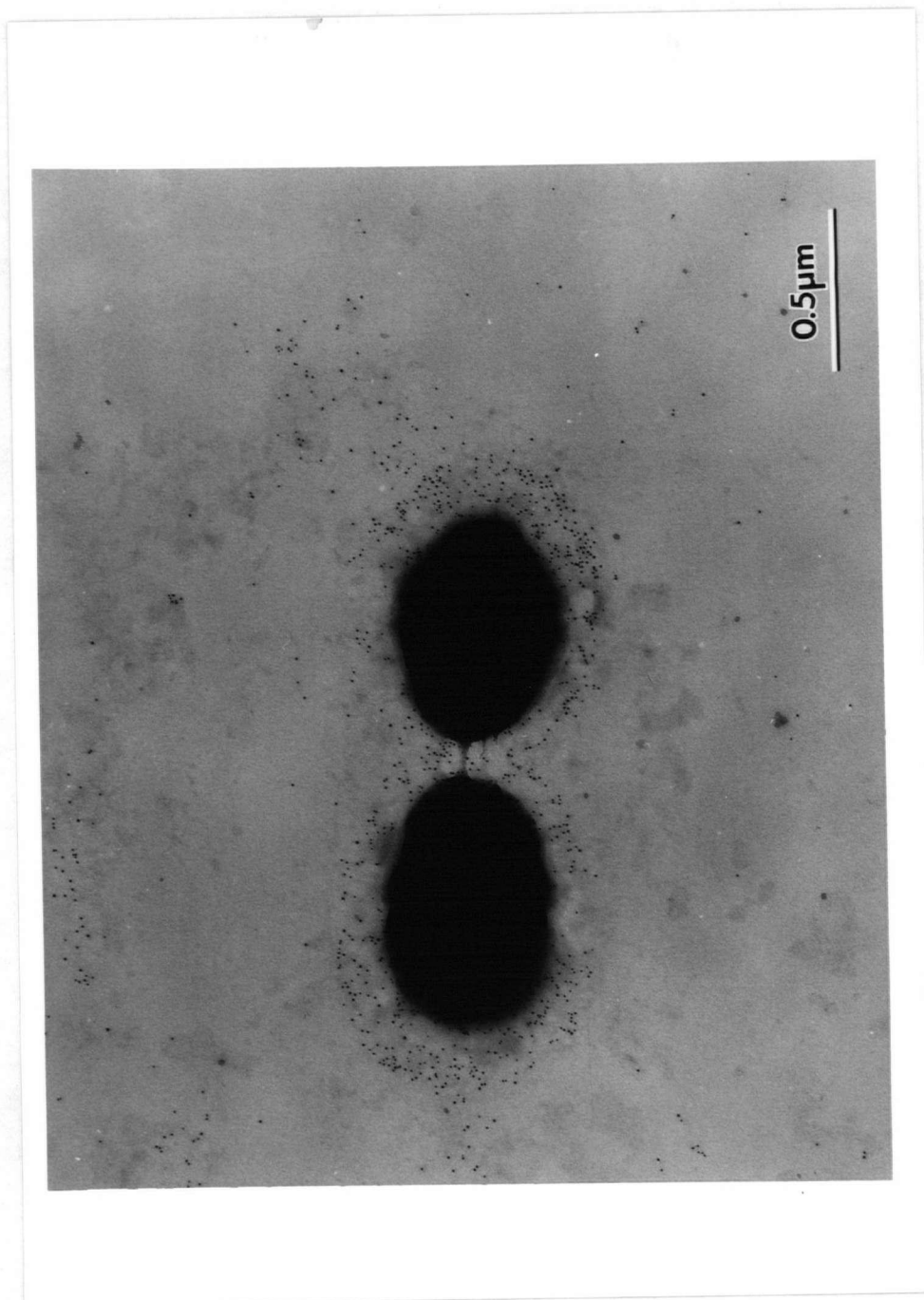


Fig. 23. Electron micrograph of *S. sanguis* 12L labelled with anti-SsaB serum. Cells were reacted with anti-SsaB serum and then incubated with gold bead conjugated goat anti-rabbit serum. Negatively stained with uranyl acetate.

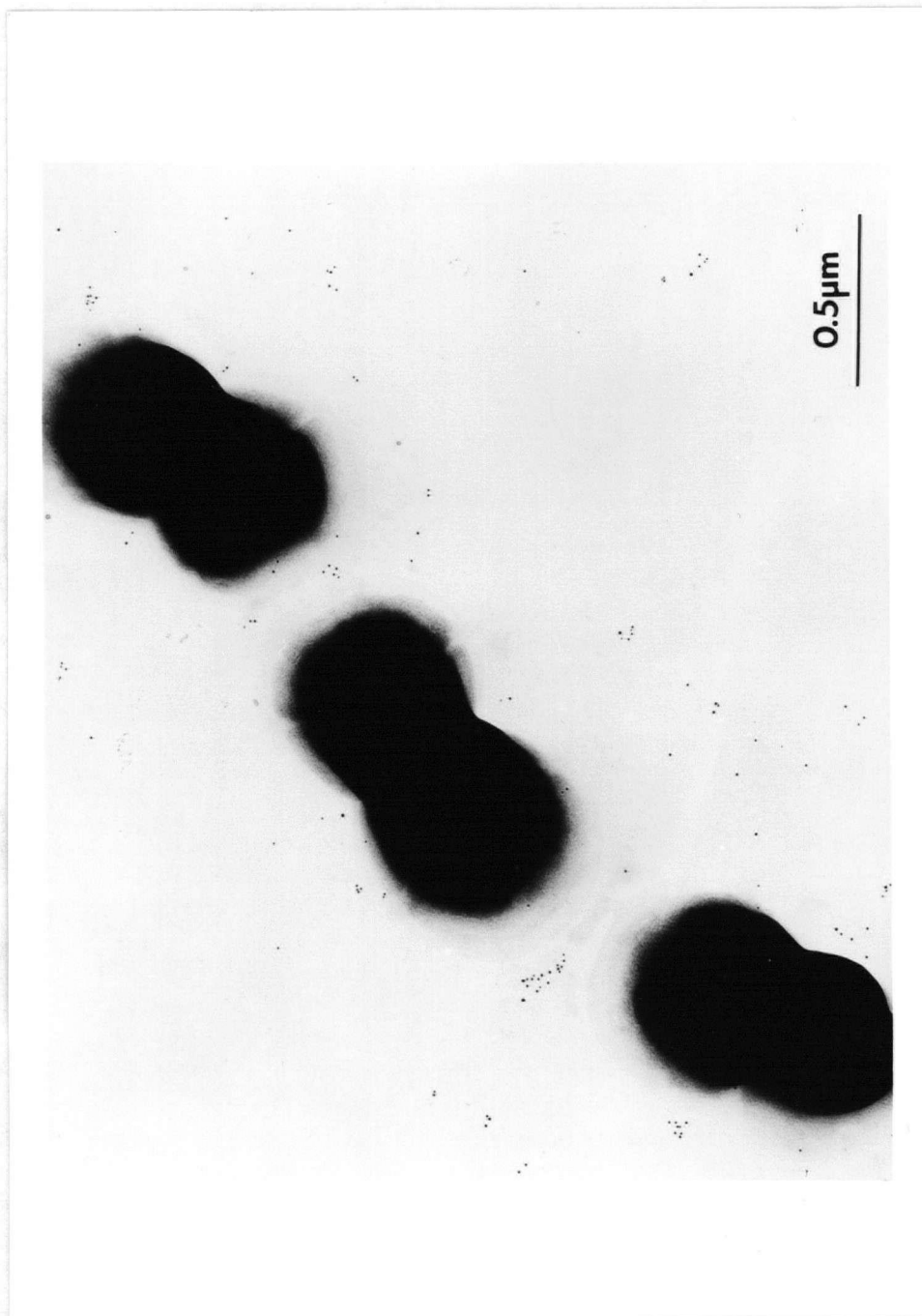


Fig. 24. Electron micrograph of *S. sanguis* 12 labelled with non-immune serum. Cells were reacted with non-immune serum and then incubated with gold bead conjugated goat anti-rabbit serum. Negatively stained with uranyl acetate.

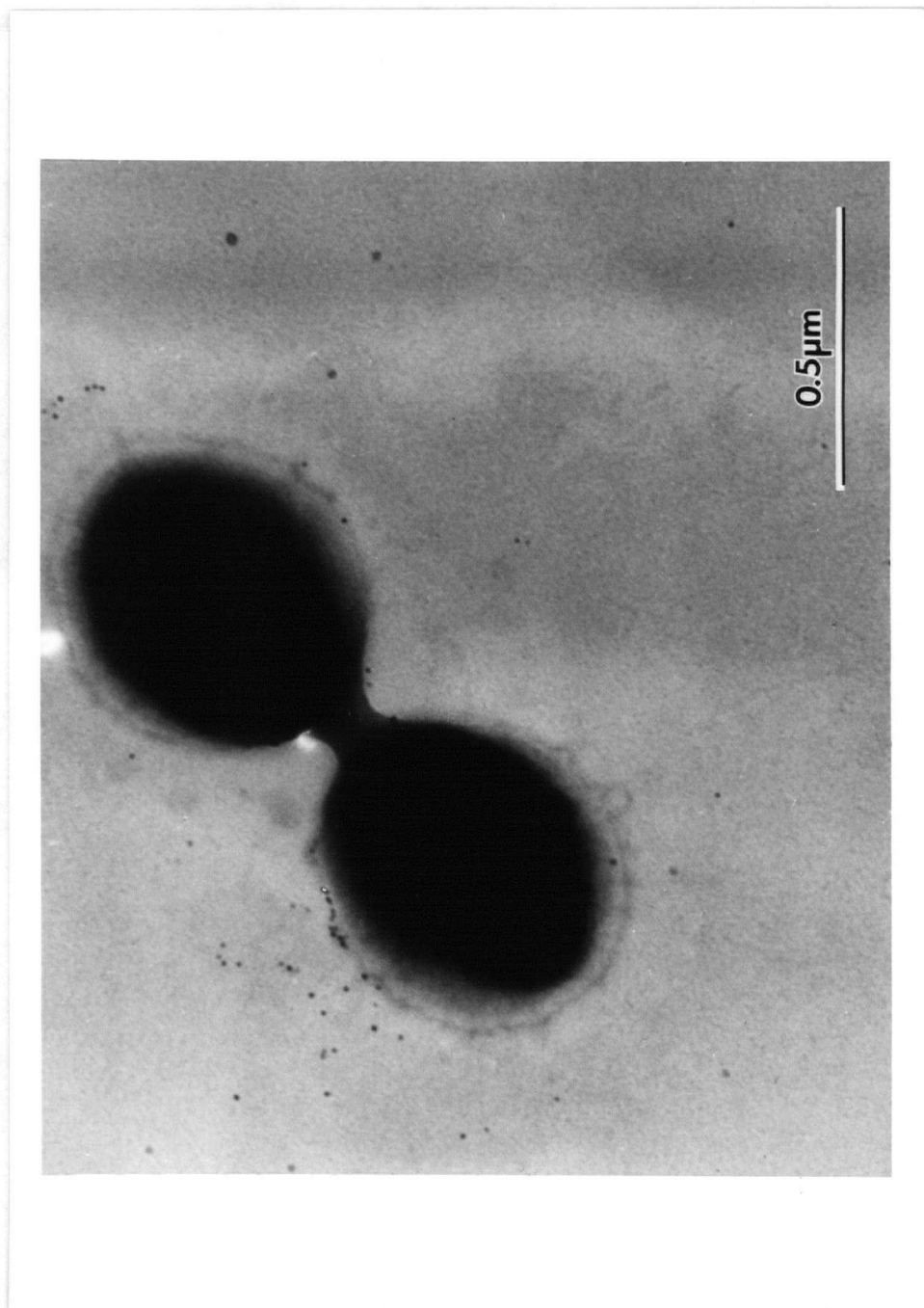


Fig. 25. Electron micrograph of *S. sanguis* 12 labelled with anti-20 kd serum. Cells were reacted with anti-20 kd serum and then incubated with gold bead conjugated goat anti-rabbit serum. Negatively stained with uranyl acetate.

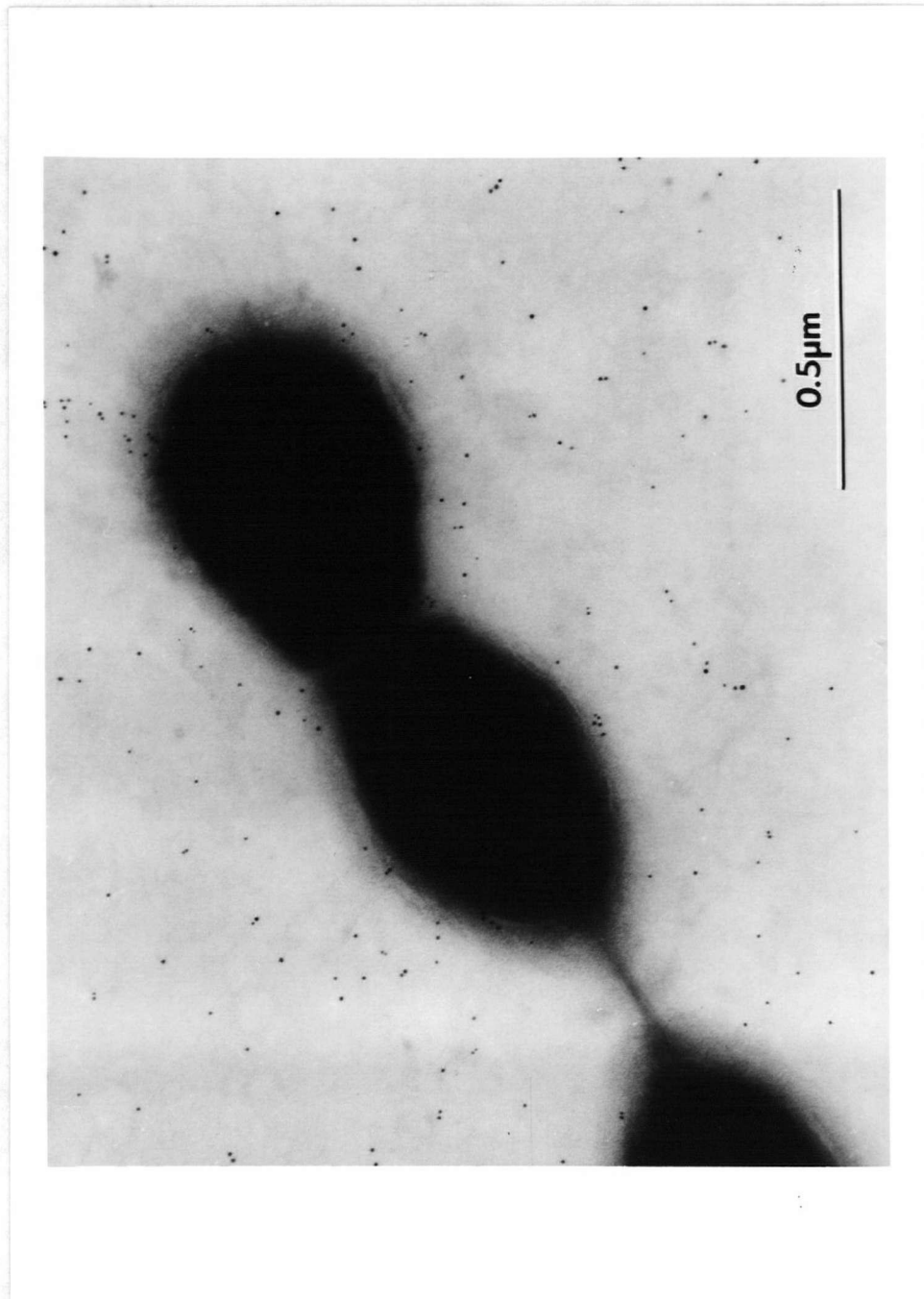
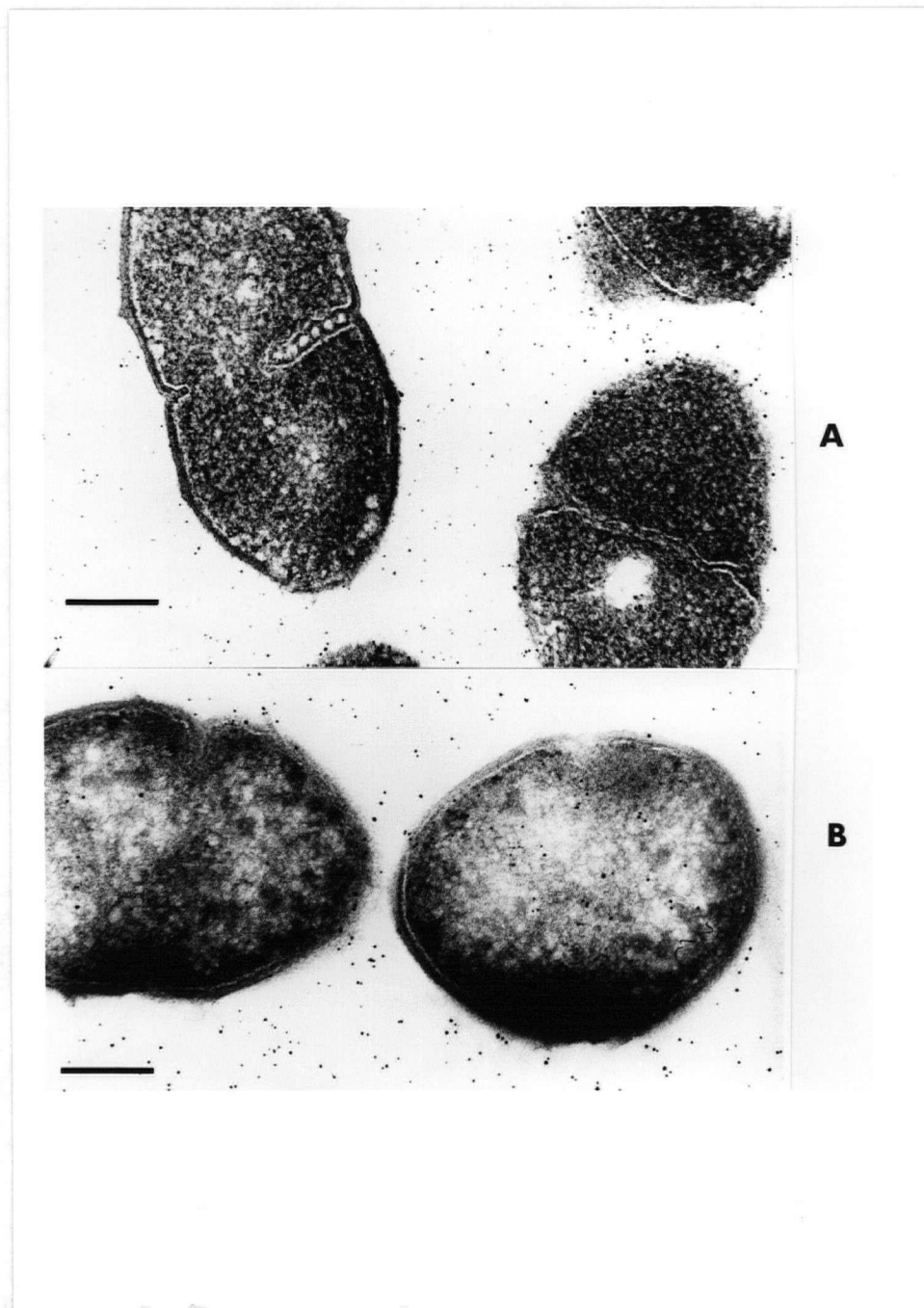


Fig. 26. Electron micrograph of cross-sectioned *S. sanguis* cells. Cross-sectioned cells were reacted with anti-SsaB serum and then incubated with gold beads conjugated to goat anti-rabbit serum. Negatively stained with phosphotungstic acid. Panels: (A), *S. sanguis* 12; and (B) *S. sanguis* 12L. Bar, 0.5 μ m.



and/or (3) no antigen in the cytoplasm. The inability to detect the SsaB on the surface of thin section of *S. sanguis* 12 favors the argument that the antigenicity of the native SsaB has been lost.

Restriction analysis of pSA2

The regions of the pSA2 plasmid expressing SsaB and the 20 kd protein were examined by restriction analysis and subcloning. Cleavage with EcoR1 indicated that the streptococcal insert was 2.8 kb in size. Deletion derivatives of pSA2 were made by endonuclease digestion, followed by either self ligation or ligation to dephosphorylated pUC18 which had been linearized with the appropriate restriction endonuclease. The physical map of pSA2 and its deletion derivatives is shown in Fig. 27. The expression of the two polypeptides was analysed by Western blotting. The results indicate that the structural genes for these two polypeptides were located within a 1.9 kb PstI or BamH1 fragment. Similar quantities of the 20 kd protein were synthesized when the 1.2 kb HindIII fragment was in either orientation suggesting that transcription was under the control of a streptococcal promoter. Both proteins were expressed in JM101 in the absence of an inducer. The amount of SsaB expressed was affected by the orientation of the insert. When the insert was in the opposite orientation, the amount of SsaB expressed was reduced substantially but the protein could be detected by Western blot. When the 2.3 kb SalI fragment was subcloned in the reverse orientation, SsaB and a truncated 20 kd protein (18 kd) were expressed at a reduced level. The 18 kd protein reacted with the anti-20 kd serum. Therefore, the rest of the SalI/PstI fragment must contain part of the region coding for the 20 kd protein as well as the streptococcal promoter.

Southern hybridization studies were done with a BamH1 digest of total *S. sanguis* DNA and pSA2 (Fig. 28). The 1.9 kb BamH1 fragment of pSA2 was used as the probe. This probe hybridized with a single band at 1.9 kb with both *S. sanguis* DNA and pSA2 DNA which had been digested with the BamH1 restriction enzyme.

Fig. 27. Partial restriction map and characteristics of deletion mutants derived from pSA2. The size of the proteins expressed by each subclone is indicated in kilodaltons and the amount expressed is indicated as follows: [++], as in parent plasmid; [+], less than in parent plasmid; and [-] not expressed. The heavy lines represents the 2.8 kb *S. sanguis* 12 DNA insert in pSA2. DNA fragments in the subclone are indicated by the medium density line. '18' represents a truncated 20 kd protein. B, BamH1; H, HinD111; P, Pst1; and S, Sal1.

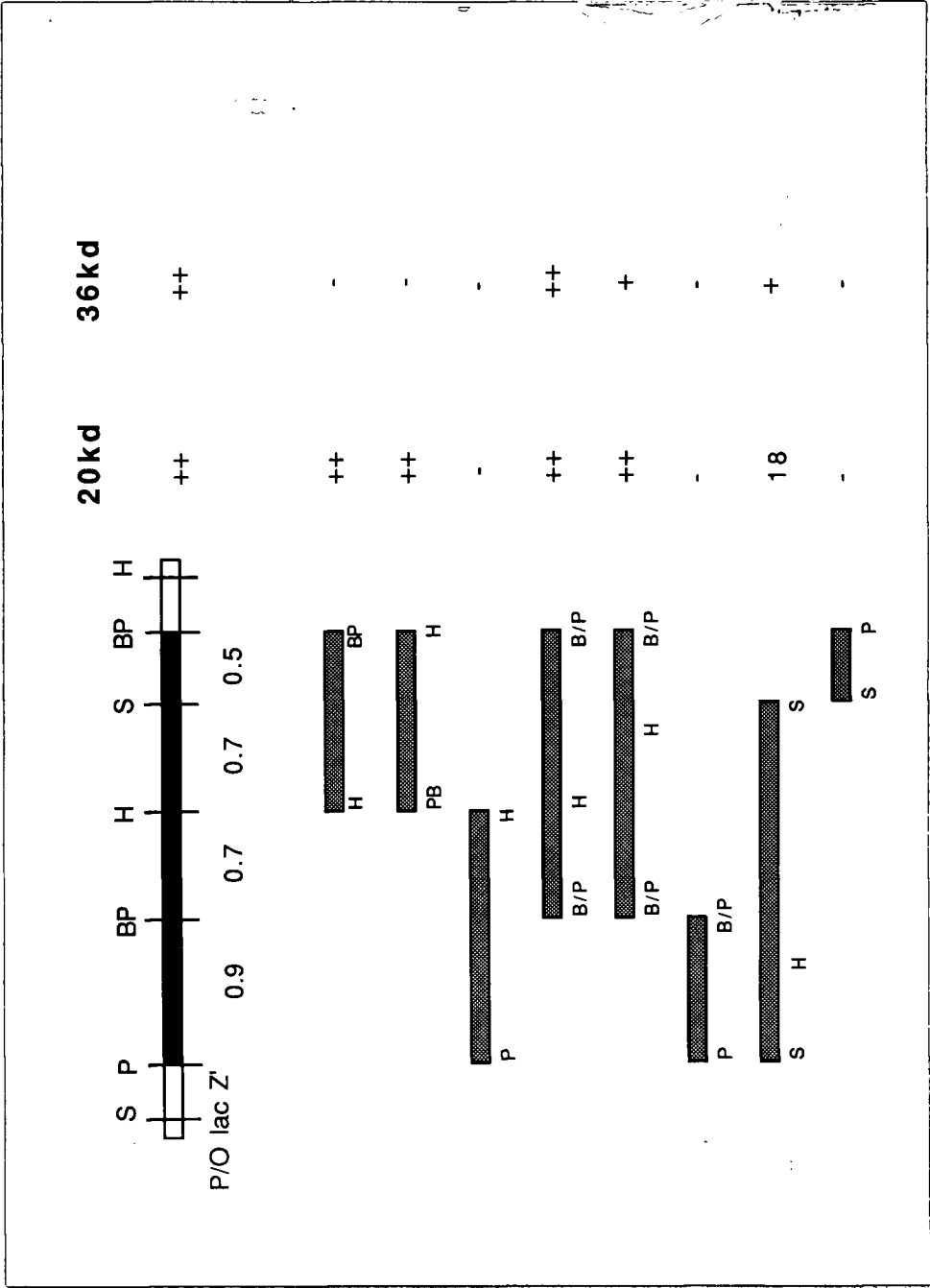
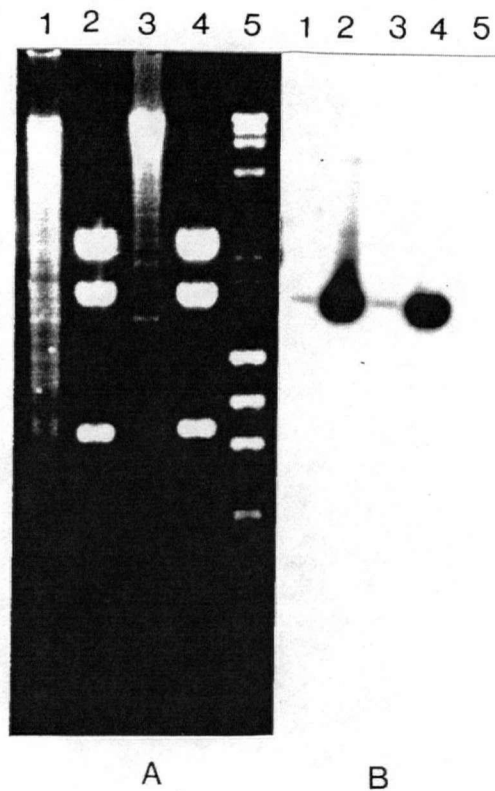


Fig. 28. Southern blot hybridization with the 1.9 kb BamH1 restriction fragment of pSA2. Panel (A) DNA fragments separated by agarose gel electrophoresis. Panel (B) autoradiogram. Lanes: 1, *S. sanguis* 12 chromosomal DNA digested with Pst1; 2, plasmid pSA2 digested with Pst1; 3, *S. sanguis* 12 chromosomal DNA digested with BamH1; 4, plasmid pSA2 digested with BamH1; and 5, molecular weight markers as in Fig. 15.



Adherence inhibition

The possibility that the SsaB protein was an adhesin mediating binding to either the pH or neuraminidase-sensitive receptor in a salivary pellicle was examined in the S-HA bead assay. The assays were done with *S. sanguis* 12 which binds to both receptors, *S. sanguis* 12na which binds only to the pH-sensitive receptor, S-HA beads which contained both receptors, and S-HA beads which had been treated to remove the pH-sensitive receptor.

SsaB inhibited binding by 26% when *S. sanguis* 12 was incubated with beads which had both receptors. Greater than 70% inhibition of binding was observed when *S. sanguis* 12na was incubated with untreated S-HA. There was no inhibition when *S. sanguis* 12 was incubated with S-HA which had been treated to remove the pH-sensitive receptor. The inhibition was dose dependent (Fig. 29). Maximum inhibition occurred when the concentration was 10 µg per ml. These results suggest that SsaB is the adhesin mediating binding to the pH-sensitive receptor. The 20 kd protein had no effect on the binding of either organism to S-HA or to pH modified S-HA.

Prevalence of SsaB in *S. sanguis*

A number of strains *S. sanguis* were screened for the presence of SsaB by Western immunoblotting of cell lysates and by immunofluorescence of whole cells with anti-SsaB serum. All strains of *S. sanguis* contained a 36,000 M_r protein which reacted with the anti-SsaB serum on Western immunoblots.

The expression of SsaB on the surfaces of these strains was tested by immunofluorescence microscopy (Table VII). All the strains reacted positively in this immunofluorescence assay, however there was considerable variation in intensity. For example, strain 12 and NY 101 showed a fluorescence level of 4, a bright halo around the cell, whereas strains 12L and ATCC 10557 had a fluorescence score of 0, and 1, respectively. This suggested that, although the SsaB was produced by all strains of *S. sanguis*,

Fig. 29. Inhibition of adherence of *S. sanguis* 12na to S-HA by SsaB. 100% adherence is the number of cells bound (6.25×10^7 cells per 5 mg of S-HA) in the absence of inhibitor.

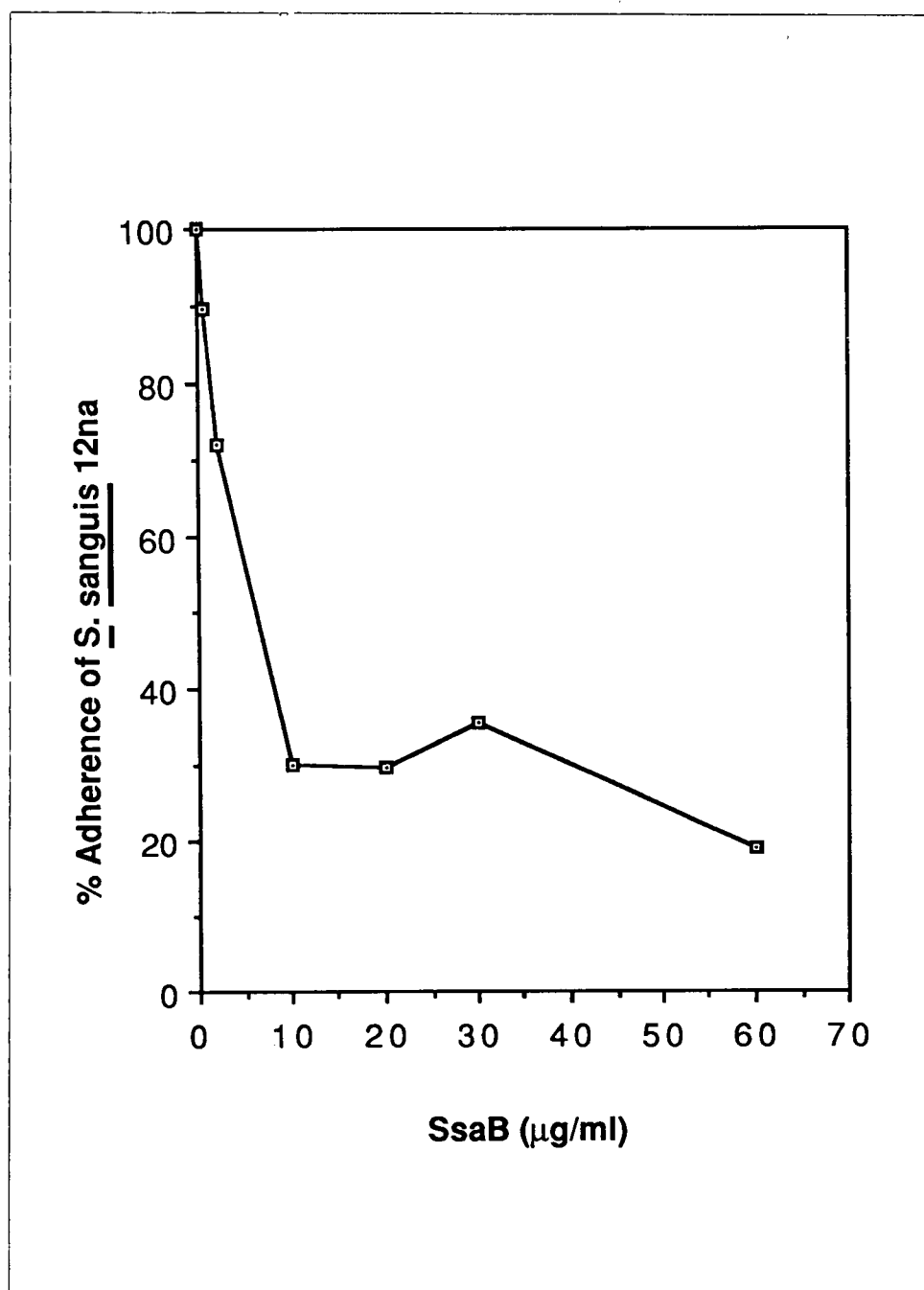


Table VII. Prevalence of SsaB in strains of oral *Streptococcus*.

Organism	Fluorescence score ^a	Western blotting ^b
<i>S. sanguis</i> 12	4	+
12na	3	+
12L	0	+
N	1	+
ATCC 10556	3	+
10557	1	+
10558	3	+
NY 101	4	+
929	1	+
2A	4	+
J1	1	+
J2	2	+
J3	1	+
J4	1	+
<i>S. mutans</i> Lk2-2	ND	-
M1751	ND	-
27352	ND	-
<i>S. salivarius</i> HB	ND	-
HBV52	ND	-
<i>S. mitis</i>	ND	-

^a4 - very strong, 3 - strong, 2 - moderate , 1 - reactive, 0 - not reactive.

^b +, present; - , not present.

ND, not done.

the level of expression or the availability of this protein on the surfaces varied. This could explain the differences in the ability of these strains to adhere to S-HA.

A number of oral streptococci were screened to determine if they contained SsaB. Mutanolysin digests or SDS- β ME extracts of whole cells of *S. mutans* (Lk2-2, M1751 and 27352), *S. salivarius* (HB and HB-V5) and *S. mitis* did not react with anti-SsaB serum on Western immunoblots suggesting that SsaB was specific to *S. sanguis*.

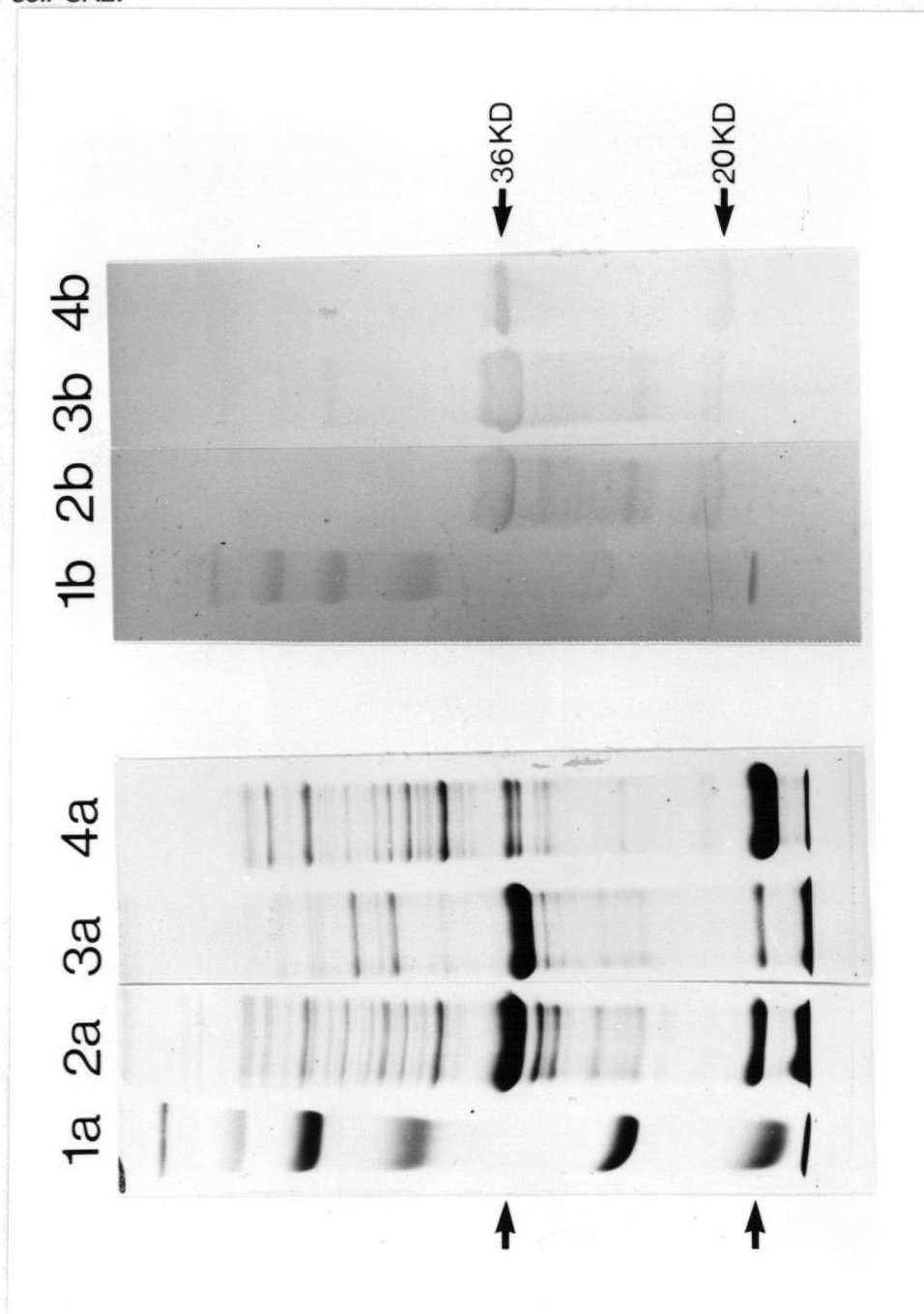
Localization of SsaB in *E. coli* SA2

Since SsaB was expressed on the surfaces of *S. sanguis* 12, it was decided to examine whether this protein was transported to the *E. coli* SA2 periplasm. Localization of recombinant proteins in *E. coli* SA2 was done by fractionating the cells using the chloroform shock technique (Ames *et al.*, 1984). The periplasmic fraction and cytoplasmic fraction were examined for the presence of the marker enzymes β -lactamase and glucose-6-phosphate dehydrogenase respectively. The periplasmic fraction contained 88% of the total β -lactamase and had no detectable glucose-6-phosphate dehydrogenase. The cytoplasmic fraction contained 10% of the total β -lactamase and 95% of the total glucose-6-phosphate activities. These fractions were analyzed for the presence of 20 kd and SsaB proteins by SDS-PAGE (12%) and Western blots (Fig. 30). Most of the 20 kd protein expressed in *E. coli* SA2 was found in the periplasmic fraction (Fig. 30, lane 4a). This is surprising given the fact that it is the 20 kd protein not SsaB which is present on the surface of *S. sanguis* 12. The majority of SsaB was found in the cytoplasmic fraction and only a small amount in the periplasm (Fig. 30, lanes 3a, and 3b). The cytoplasmic fraction contained a higher molecular weight protein which reacted with anti-SsaB serum (Fig. 30, lane 3b). Immunofluorescence analysis with anti-SsaB and anti-20 kd sera revealed that the proteins were not expressed on the surface *E. coli* SA2.

Organization of SsaB on *S. sanguis* 12

In order to try and gain an insight into the configuration of SsaB on *S. sanguis* 12, chemical

Fig. 30. Localization of recombinant proteins in *E. coli* SA2. Periplasmic and cytoplasmic fractions were analyzed by SDS-PAGE and Western blotting. Left hand panel: (a) gel stained with Coomassie brilliant blue; right hand panel (b) Western blot of the gel reacted with the mixture of anti-SsaB and anti-20 kd serum. Lanes: 1, molecular weight markers as in Fig. 18; 2, cell lysate of *E. coli* SA2; 3, cytoplasmic fraction of *E. coli* SA2; and 4, periplasmic fraction of *E. coli* SA2.



cross-linking experiments were done. Whole cells of *S. sanguis* 12 were chemically cross-linked with DSP or DTBP, and then digested with mutanolysin. The digests were solubilized with 2% SDS and analyzed by two dimensional gel electrophoresis. The first dimension contained SDS and the second dimension SDS with β -mercaptoethanol to reduce S-S cross-links. The gels were run in duplicate, one gel was silver stained (Fig. 31) and the other Western blotted (Fig. 32) with anti-SsaB serum. Optimum concentrations for cross-linking with DSP and DTBP were determined by mixing *S. sanguis* 12 with increasing amounts of the cross-linkers. The optimal concentration was determined to be the point at which most of the SsaB appeared to be cross-linked as measured by its disappearance on Western blotting with anti-SsaB serum. The optimum concentration was 150 μ g of DSP and 100 μ g of DTBP when mixed with *S. sanguis* 12 cells ($OD_{660}=1$). Unfortunately SsaB appeared to irreversibly lose antigenicity following reaction with the cross-linkers. Cross-linked material could not be detected in one dimensional Western blots stained with anti-SsaB. Even dot blots of cross-linked material lost their ability to react with anti-SsaB. Only a small proportion of the reactivity with anti-SsaB serum returned following reduction with β -mercaptoethanol as measured in dot blots or following electrophoresis. This means that the small amount of SsaB observed in Fig. 32 might represent a larger amount of cross-linked SsaB. Both DSP and DTBP gave similar results. Chemical cross-linking of SsaB in the whole cells led to the formation of higher M_r components (66,200 and 86,000) which comigrated with SsaB in the second dimension and reacted with anti-SsaB serum (Fig. 31, and Fig. 32). Whether these HM_r components represent true dimers or trimers of SsaB is not clear. However the evidence suggests that this is the case because other constituents having the appropriate M_r were not observed either above or below the cross-linked SsaB (first two arrows to the left) molecule in the second dimension. In addition there appeared to be internal cross-linking which made the molecule smaller. This may be seen in Fig. 31 as the spot appearing to the right of the diagonal (indicated by the small arrow to the right).

Fig. 31. Two dimensional SDS-PAGE analysis of chemically cross-linked *S. sanguis* 12. Cells were cross-linked with DTBP (100 μ g) for 1h. Cells were digested with mutanolysin for 18 h and the lysate was incubated with 2% SDS for 30 min at 37°C. Samples were then electrophoresed in a 10% SDS-PAGE gel. Strips containing the samples were cut out and incubated with 10% β ME for 15 min at room temperature and then sealed onto a second dimension 10% SDS-PAGE gel and electrophoresed. The gel was silver stained. Large arrows indicate molecular weight markers as in Fig. 18. Small arrows indicate cross-linked SsaB.

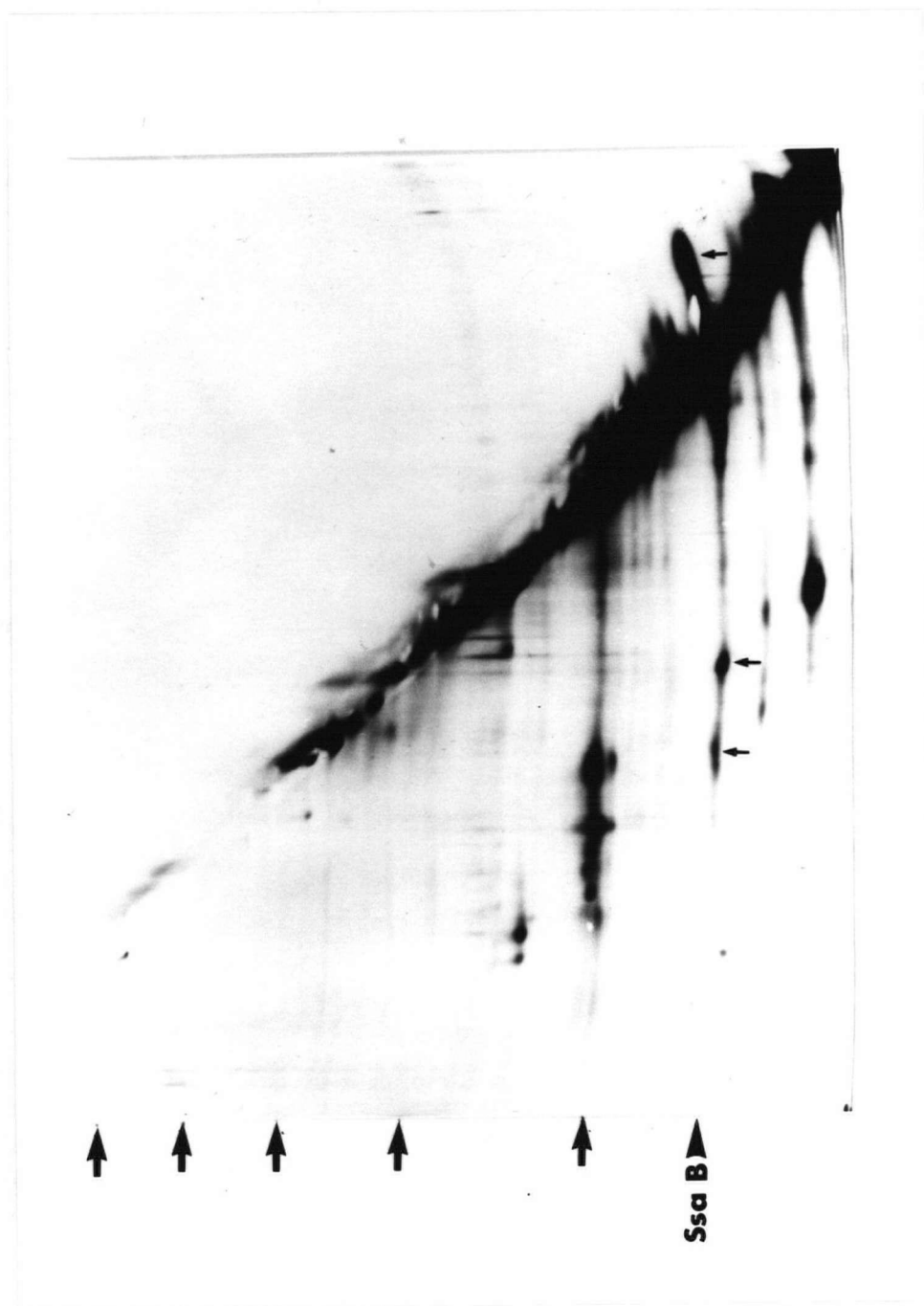
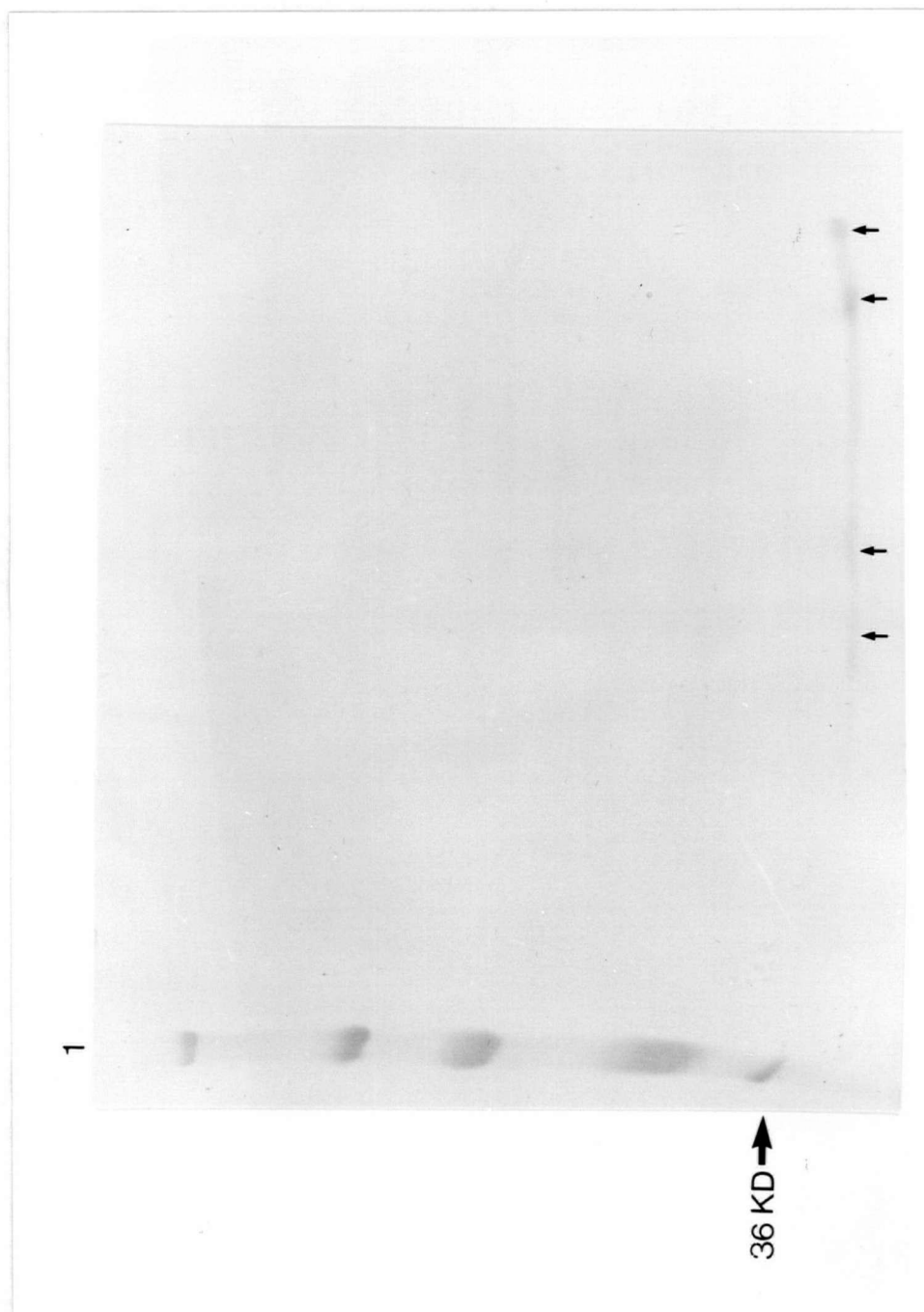


Fig. 32. Western blot of chemically cross-linked *S. sanguis* 12. Representative gel is shown in Fig. 31. Lane 1 contains molecular weight markers as in Fig. 18 and purified SsaB as indicated by the large arrow. Small arrows represent cross-linked SsaB.



DISCUSSION

Fimbriae have been reported to be involved in adhesion of some strains of *S. sanguis* to S-HA. Gibbons *et al.*, (1983b) found that non-fimbriated mutants of *S. sanguis* FC-1 were non-adherent and hydrophilic, while Fachon-Kalweit *et al.* (1985) were able to inhibit binding to S-HA with antibody raised against purified fimbriae from *S. sanguis* FW213. In the studies reported here, *S. sanguis* 12 has been shown to have long fibrils and densely packed short fibrils (Fig. 2). These structures have consistent lengths but variable width and therefore have been referred to as fibrils as suggested by Handley *et al.*, (1985).

The technique of freezing followed by homogenization has provided a means of releasing fibrils from the surface of *S. sanguis* 12. The technique resembles that of Nagata *et al.*, (1983) who repeatedly froze and thawed *S. sanguis* ATCC 10557 in order to obtain a galactose binding lectin .

Loss of the long fibrils from *S. sanguis* 12 did not change the organism's hydrophobicity suggesting that other constituents are responsible for this property. On the other hand these long fibrils would appear to be important in salivary aggregation as there was a decrease in aggregation with the loss of fibrils. This is in contrast with the observation of Rosan *et al.*, (1982), that salivary aggregation and adherence is mediated by two different factors. Our finding suggests that both adherence and aggregation are mediated by long fibrils on *S. sanguis*.

The observation that the fibrillar preparation contained only a single protein of M_r greater than 300,000 (LFP) when analysed by SDS-PAGE represents the first positive identification of a fibrillar molecule from *S. sanguis*. The M_r of the fibrillar protein is in the same range as the fibrillar antigens b and c of *S. salivarius* HB, which have been identified as the *Veillonella*-binding protein and host attachment factor, respectively (Weerkamp *et al.*, 1981, and 1982). The LFP also resembles the *S. salivarius* antigens in containing low levels of nonpolar amino acids, and as in the case of *S. salivarius* antigen c, a high percentage of carbohydrate.

Immunogold electron microscopy with antibody raised against the protein confirmed that the molecule was associated with fibrils present on whole cells of strain 12 and with fibrils isolated from the organism. This conclusion was substantiated by the observation that a monoclonal antibody 32.33 reacted with both the LFP and the fibrils.

The fibrillar material appeared to be a carbohydrate-protein complex as the band stained positively with both periodate-Schiff reagent and Coomassie brilliant blue. The loss of the periodate-Schiff reactive material following proteolytic digestion provides additional evidence that carbohydrate is associated with the protein. Treatments with lysozyme and mutanolysin did not alter the M_r of the LFP suggesting that large pieces of peptidoglycan are not associated with the preparation. In this respect *S. sanguis* 12 was similar to the other streptococcal fibrils which have been characterized. Fimbriae isolated from *S. sanguis* FW213 were found to contain both carbohydrate and protein (Fives-Taylor *et al.*, 1987), but these workers were unable to dissociate the fimbriae into subunits. The *S. salivarius* HB fibrillar adhesins were found to contain carbohydrates (Weerkamp *et al.*, 1981, and 1982).

The question of whether the long fibrils are composed of subunits remains unresolved. Attempts were made to dissociate LFP into subunits by methods which usually break down fimbriae of Gram-negative microorganisms. Fimbrial proteins from *E. coli* are insoluble in their native form, but may be disaggregated by guanidine hydrochloride or HCl. However, if the LFP described here consists of subunits, then it seems likely that they are held together by covalent bonds, since none of the treatments (urea, guanidine, EDTA, HCl, or sodium thiocyanate) produced subunits (McMichael *et al.*, 1979; Eshdatt *et al.*, 1981). Fibrillar proteins from the Gram-positive organisms *A. viscosus* (Wheeler *et al.*, 1980) and *S. salivarius* (Weerkamp *et al.*, 1981) have also proved resistant to dissociation into subunits, although this cannot be taken as a general rule for all Gram-positive organisms because *C. renale* fimbriae are easily disaggregated (Kumazawa *et al.*, 1972). Recently *A. viscosus* T14V type 1 (Yeung *et al.*, 1987) and type 2 (Donkersloot *et al.*, 1985) fimbriae have been cloned into *E. coli* and their subunit size has been determined to be 65

kd and 59 kd respectively.

One indication that the *S. sanguis* 12 long fibrils are made up of repeating subunits comes from the experiments in which fibrils incubated with subtilisin for 18 h (Fig. 11). Under these conditions a series of lower M_r proteins were formed. Each protein differed by approximately 45,000 daltons. The regular differences in M_r suggests that this may be a repetitive structure which is cleaved by subtilisin at specific points. The reactivity of these proteins with anti-LFP and anti-fibril antibody demonstrates that at minimum they are immunologically related.

The reason why some of the fibrils can be dissociated by SDS whereas some are resistant is not known. The relationship between LFP and the repetitive structures released by subtilisin remains to be elucidated.

The observation that adherence of *S. sanguis* 12 to S-HA is sensitive to anti-fibril antibody suggests that the fibril is one of the the S-HA adhesins. The long fibrils function as an adhesin mediating attachment to the neuraminidase-sensitive receptor on S-HA. This conclusion is supported by the following findings. (1) Adherence inhibition was demonstrated only when pH modified S-HA was used as the binding substratum. The pH modified S-HA has only the neuraminidase-sensitive receptor. (2) The binding of strain 12na (which lacks the adhesin binding to the neuraminidase sensitive receptor) was not affected by anti-fibril antibody. (3) Strain 12na was shown by electron microscopy to lack long polar fibrils (Morris *et al.*, 1985). (4) Neither LFP nor fibrils were isolated from strain 12na.

The inability of anti-LFP antibody to inhibit binding could be due to loss of active-site antigenic determinants caused by denaturation with SDS as anti-LFP antibody was raised against LFP protein excised from a SDS-PAGE gel. Such a protein would probably not retain its tertiary structure and may have lost the epitopes required to induce formation of antibodies blocking adherence. Alternatively, the fibrils may contain more than one type of subunit and the binding site may be on a subunit distinct from the LFP. Recent studies with Gram-negative fimbriae indicate that the fimbrial adhesin and the fimbrillin subunit are

encoded by different genes (Clegg *et al.*, 1987). The finding that Mab 32.33 bound only to the terminus of fibrils supports the concept of a unique terminal subunit. Alternatively, the Mab may recognize a terminal epitope which is hidden by opposing subunits and therefore this epitope is only available at the terminus of the fibrils.

Although adherence inhibition could be demonstrated with the fibrils, further characterization of LFP was discontinued because of difficulties in obtaining sufficient quantities of material and the difficulty of working with a large insoluble molecule which did not dissociate into subunits. In order to circumvent these problems and obtain preparations devoid of carbohydrate, it was decided to use recombinant DNA techniques to identify gene/s encoding the adhesin mediating attachment of *S. sanguis* 12 and 12na to S-HA via the pH sensitive receptor.

Genes coding for a number of *S. sanguis* surface antigens were cloned into *E. coli* JM83. The clones were judged to be unique by restriction enzyme analysis and SDS-PAGE analysis of the expressed genes. With one exception the *S. sanguis* genes were expressed at relatively low levels. Clone SA2 overexpressed two proteins of M_r 20,000 and 36,000. The orientation of the insert did not affect the expression of the 20 kd protein which indicates that the insert contained a streptococcal promoter that was recognized by *E. coli*. Expression of the 36 kd protein was reduced but not eliminated when the insert was cloned in the reverse orientation. Data from subcloning indicate that the promoter is likely to be on the Sal 1/Pst 1 0.5 kb fragment of pSA2.

Clone SA5 produced a 50 kd protein that reacted with the anti-fibril serum but not with anti-12 serum on immunoblots. The insert size was barely enough to code for this protein. The lack of reactivity with anti-12 serum suggests that this may be a truncated protein which does not contain epitopes exposed on the *S. sanguis* cell surface. The other four clones, which reacted with anti-12 serum could represent different antigens present on the surface of this organism.

One of the problems of cloning into plasmid vectors is that inserts must be relatively small

and thus there is an increased likelihood of obtaining incomplete genes coding for truncated proteins. This is not the case for SsaB and the 20 kd protein because both proteins could be detected in mutanolysin digests of *S. sanguis*. This demonstrates that the gene is expressed in *S. sanguis* and that the complete gene has been cloned.

The protein has been given the designation SsaB (*S. sanguis* adhesin B). We have chosen this terminology with the view that a standard system should be developed to name the specific protein adhesins as they are identified. In this system the fimbrial antigen cloned by Fives-Taylor *et al.*, (1987) from *S. sanguis* FW213 would be designated SsaA.

The SsaB protein is found on the surface of *S. sanguis* and therefore must contain the necessary signal sequences to affect its transport through the cytoplasmic membrane and subsequent incorporation into the surface of the cell wall. In an attempt to determine if the protein was exported to the periplasm of *E. coli*, the organism was fractionated into the periplasmic and cytoplasmic constituents. Interestingly the 20 kd protein which could not be found on the cell surface or the culture supernatant of *S. sanguis* 12, was present in the periplasm of *E. coli* indicating that it contained signal sequences recognized by *E. coli*. On the other hand the majority of SsaB was found in the cytoplasm and presumably lacked the necessary sequences for its transport to the periplasm.

Although a function cannot be attributed to the 20 kd protein, the presence of this protein in the periplasm, and its close association with the *SsaB* gene in the chromosome suggests it may be involved in the export and/or assembly of SsaB on the *S. sanguis* cell surface. It is possible the genes encoding for SsaB and the 20 kd proteins could be arranged in a operon, like the fimbrial operons in *E. coli* (Normark *et al.*, 1983; Orndorff *et al.*, 1984; Klemm *et al.*, 1985;).

Immunogold bead labelling with antibody raised against SsaB showed that the protein was present on the cell surfaces of both *S. sanguis* 12 and 12na but was not detectable on the non-adherent hydrophilic variant 12L. This is in keeping with previous work which showed that the cell walls of 12L contained very little protein which could be released by

mutanolysin digestion (Morris *et al.*, 1985) and very little in the way of surface structures that are visible by electron microscopy. The small amount of SsaB in 12L that was detected by immunoblotting may indicate that the protein is retained in the cell in a manner similar to that observed in *S. salivarius* HB-7 and HB-V5 (Weerkamp *et al.*, 1986) or it simply could be that the cell surface contains low levels of the protein which are not detected by the immunogold procedure. Attempts to locate SsaB within the 12L cell by thin sectioning followed by immunogold labelling were not successful.

The immunogold staining pattern suggested that SsaB was not a subunit of the *S. sanguis* 12 long fibrils (Morris *et al.*, 1985). This is supported by the observation that anti-serum raised against the whole fibrils did not react with this protein. Thus it is clearly distinct from the *S. sanguis* FW213 fimbrial adhesin cloned by Fives-Taylor *et al.* (1987). Indeed the DNA insert containing the FW213 fimbrial gene did not hybridize with the *S. sanguis* 12 genome (personal communication-P. Fives-Taylor). Unlike *S. sanguis* FW213 fimbriae, SsaB could be found in all *S. sanguis* strains suggests that *S. sanguis* FW213 probably belongs to a distinct serotype group. While it is not possible to relate SsaB to a specific structural unit, it is apparent that there is a concentration of this molecule at a point removed from the cell wall. This results in a halo when the concentration is at its highest. It is possible that this corresponds to the tips of the more densely packed short fibrils or "fuzzy coat" described originally by Gibbons *et al.*, (1972).

Localization of SsaB on the cell surface suggested that this molecule might play a role in adherence to S-HA. This possibility was proven correct when it was shown that as little as 10 µg of the molecule was sufficient to inhibit binding. The finding that the adherence of *S. sanguis* 12na was strongly inhibited whereas the binding of the strain 12 was only weakly inhibited provides good evidence in support of the two site binding model. *S. sanguis* 12na has only one adhesin to bind to S-HA. When it is blocked with SsaB there is no adhesion. But strain 12 has two adhesins to bind to S-HA. Therefore when one of the adhesins is blocked, it is still able to bind to S-HA.

The possibility that the SsaB could exist as a high M_r polymer was explored because SsaB purified from *E. coli* by gel filtration and ion exchange chromatography eluted in the void

volume indicating that in the recombinant it exists in a polymeric form. Incubation with SDS at room temperature reduced it to a monomer. It is not known whether the protein produced in *E. coli* is an artifact resulting from overproduction in a foreign host or whether it represents a specific polymeric association. It is interesting to note that SsaB released by mutanolysin digestion of *S. sanguis* 12 also exists in a polymeric form which elutes in the void volume of a Sephadex G-75 column.

Further evidence that SsaB exists in a higher M_r form was provided by chemical cross-linking studies. These studies suggested that SsaB is able to form cross-linked higher M_r complexes on the surface of *S. sanguis* 12. These data suggest that SsaB molecules on the surfaces of *S. sanguis* 12 are closely associated, and one could speculate that multimers of this protein could form the short fibrils or the "fuzzy coat" seen on bacterial surface. One of the difficulties experienced in these experiments was the loss of antigenicity of cross-linked SsaB molecules. Only a small percentage was detected by Western blots following the reduction of the cross-linked SsaB under conditions which should have yielded monomers. The studies with cross-linking agents are inconclusive, and in order to verify our observations, cross-linking agents with different length of spacer arms and with different reactive groups must be used to determine whether SsaB exists in a polymeric form.

Anti-SsaB antibody was used as a probe to confirm the presence of SsaB in different strains of *S. sanguis*. When mutanolysin digests of various strains were Western blotted and reacted with anti-SsaB serum, all strains were found to contain a 36,000 M_r protein. When this probe was used as an immunofluorescence marker all of the strains reacted positively but there was variation in the intensity of fluorescence. This suggests that the extent of SsaB expressed is different or that there is a difference in the availability to react with anti-SsaB antibody.

It was surprising to find that the ATCC strains 10556, 10557 and 10558 representing all biotypes and serotypes expressed SsaB as strain ATCC 10557 is considered by some as belonging to the *S. mitis* group (Rosan, 1978). *S. mitis* isolated in our laboratory did not

contain any SsaB as determined by Western blot analysis. This throws more confusion on the problem of the proper classification of ATCC 10557. Given the results reported here it would appear that it should be assigned as *S. sanguis*. None of the other oral streptococci (*S. mutans*, *S. salivarius* and *S. mitis*) tested reacted positively with anti-SsaB serum indicating this protein is specific for *S. sanguis*.

The study described here underscores the complexity and specificity of the reactions involved in the attachment of *S. sanguis* to S-HA. The isolation of two different adhesins each recognizing a unique S-HA receptor provides the first direct proof for the two site binding model illustrating the binding of *S. sanguis* 12 to S-HA (Morris *et al.*, 1984). The adhesin recognizing the neuraminidase sensitive-receptor is associated with the long fibrillar like structures. The second adhesin recognizing the acid labile receptor is a 36,000 M_r protein. Studies should be directed to; (1) the identification of the genes responsible the synthesis of fibrils; and (2) the organization of these adhesins on the cell surface of *S. sanguis* 12.

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