The Role of the Streptococcus mutans P1 Protein C-terminus in Surface Localization and Cellular Location of the Protein Anchor

by

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THE ROLE OF THE STREPTOCOCCUS MUTANS P1 PROTEIN C-TERMINUS IN SURFACE LOCALIZATION AND CELLULAR LOCATION OF THE PROTEIN ANCHOR

BY

MARY K. HOMONYLO MCGAVIN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

To my parents,
the late Michael Homonylo MD, FRCS(C),
and Muriel Homonylo BA,
who taught me the value of an education.

and

to my husband Martin BSc, PhD, for his continued support and understanding.

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Abstract

Streptococcus mutans is the most significant aetiological organism causing human dental caries. In common with other infections, the successful initiation of caries is governed by the ability of *S. mutans* to adhere to host tissues. The major surface protein P1 of *Streptococcus mutans* has been shown to function as an adhesin, promoting the attachment of the organism to a salivary glycoprotein, which coats tooth surfaces.

The P1 protein adhesin shares a number of common features with other surface proteins of Gram-positive bacteria, which are believed to reflect a common mechanism of protein retention and cell surface localization. These conserved features are found within the C-terminal region of the protein, and consist of (i) a hydrophilic cell wall-spanning domain, (ii) a hexapeptide consensus sequence, LPXTGX, which may function as a sorting sequence for cell surface localization, (iii) a hydrophobic, membrane-spanning domain and (iv) a short C-terminal hydrophilic sequence enriched in charged amino acids. Although many cell surface proteins of Gram-positive bacteria share these features, few have been studied in detail and little is known of the contribution of individual C-terminal domains to the anchoring process in the cell wall, or the precise subcellular location of the protein anchor. Furthermore, some strains of *S. mutans* are unable to retain P1 on the cell surface even though they possess all the C-terminal domains implicated in protein anchoring.

This study describes the outcome of three specific objectives which were to investigate (1) the potential existence of a common mechanism for surface protein anchoring among Gram-positive cocci, (2) the role of the

cell wall-spanning domain in retaining P1 on the cell, and (3) the nature and precise subcellular location of the P1 anchoring structure.

The first objective was accomplished by using a genetic approach in which the full length and truncated *spaP* gene constructs encoding P1 protein were expressed on a shuttle vector and transformed into a P1-negative mutant *Streptococcus mutans* SM3352, *Streptococcus gordonii* DL-1 and *Enterococcus faecalis* UV202. The transformed cells, when assayed for expression of the recombinant P1(rP1) protein with specific antisera in ELISA assay, all demonstrated rP1 predominantly on the cell surface. Western immunoblotting and fluorescent microscopy also demonstrated the rP1 on the surface of the transformed cells. Deletion of sequences encoding the entire C-terminus resulted in liberation of P1 into the supernatant. These results suggested that the C-terminus was critical for cell association of the P1 protein and that the P1 anchor of *S. mutans* could also function as an anchor in related Gram-positive bacteria. The common nature of the anchor of surface proteins was also shown by using the C-terminus of the staphylococcal fibronectin-binding protein. A fusion protein, constructed by ligating the DNA encoding the C-terminus of the *Staphylococcus aureus* fibronectin-binding protein with the DNA encoding the N-terminal P1 protein, was transformed into *S. mutans* SM3352 and *S. gordonii* DL-1. This chimeric protein was also found to be surface-associated in both streptococcal strains, indicating that the C-terminus from a staphylococcal protein could also function as an anchor in streptococcal species. These results further support a common mechanism for surface protein anchoring among Gram-positive cocci.

The second objective was also approached by use of a truncated *spaP* gene. The *spaP* gene was truncated at the C-terminal end of the wall-

spanning domain, excluding the LPXTGX consensus sequence, and transformed via a shuttle vector into *S. mutans* SM3352 and *S. gordonii* DL-1. The P1 protein coded by the truncated *spaP* gene had only the hydrophilic wall-spanning domain at the C-terminus and was 'partially' cell associated. That is, during subcellular fractionation, the truncated P1 protein remained associated with purified cell walls, but could be removed from the walls by boiling in SDS-mercaptoethanol. In contrast, the native P1 was not completely removed from isolated walls by a similar SDS treatment. Therefore, the complete wall-spanning domain may function in the retention of P1 via intercalation with the peptidoglycan.

The nature and location of the P1 anchor was assessed by sub-fractionation of *S. mutans* into cytoplasmic membrane and cell wall fractions. Each fraction was assayed for the presence of P1 using monoclonal antibodies and antisera specifically generated against the P1 anchor. Western blotting experiments indicated P1 was wall associated. Extraction of the cell wall with hot trichloroacetic acid removed the wall carbohydrate from the peptidoglycan. Western immunoblotting demonstrated the P1 anchor was retained in the carbohydrate-free peptidoglycan fraction. A P1 non-retainer strain, which carries the full length P1 protein but sheds the protein into the culture supernatant, was used to compare with the P1 retainer strain which does not lose P1. Unlike the retainer strain, while P1 could be demonstrated in the wall fraction of the non-retainer strain, it was readily removed by boiling in SDS-mercaptoethanol. Amino acid analysis indicated no obvious peptidoglycan structural differences, which could account for the differences in the retainer and non-retainer phenotypes. We speculate that the differences observed in the retainer and non-retainer strains was due to

a defect in the mechanism involved in protein anchoring in the latter organism. Further examination of the retainer and non-retainer strains may lead to a better understanding of the mechanisms responsible for the anchoring of the surface protein of Gram-positive bacteria.

Chapter 1

Literature Review

Introduction

Streptococcus mutans, commonly found in the oral flora of humans, is a significant aetiological agent in dental caries (93). Among its surface proteins, it expresses P1 which acts as an adhesin promoting the attachment of the organism to a salivary glycoprotein which coats oral surfaces, including the enamel of teeth (88, 82, 120). P1 has also been described as antigen I/II (140), IF (68) , PAc (121), SR (1, 116), PAg (119) and antigen B (139). This adhesin is important in mediating oral colonization by *S. mutans* and can, therefore, be considered a virulence factor in caries. Significantly, P1 shares structural sequences with cell-surface proteins of other Gram-positive bacteria. This is the case with a motif within the C-terminal portion of the protein, which is associated with localizing or anchoring these proteins into the bacterial cell wall. The presence of these common structural motifs in the C-terminus of Gram-positive surface proteins has resulted in the proposal that a common mechanism for cell surface localization and assembly exists among Gram-positive bacterial species. This thesis investigates this possibility of a common mechanism for surface protein assembly and the association of the C-terminal protein anchor with the Gram-positive cell surface using the *S. mutans* P1 as a model.

The review of the literature will initially give a brief discussion of the ecological and environmental aspects of *S. mutans* colonization of the oral cavity, including the biological function of P1 as an adhesin in mediating colonization events. P1 is only one example of many cell-surface proteins of Gram-positive bacteria which mediate adhesion and contribute to virulence, consequently, the functions of other adhesins will also be

considered. The common structural features of Gram-positive adhesins will be described by using the *S. pyogenes* M protein as an example. Because the *S. aureus* protein A adhesin is cross-linked to the peptidoglycan, the structure of the peptidoglycan of Gram-positive walls will be reviewed. Also, the structural motifs involved in the cell-surface localization and anchoring will be discussed, in addition to the current hypothesis regarding the anchoring mechanism. The relevance of this study to the development of live oral recombinant vaccines will be briefly considered.

The Influence of Bacterial Adhesion on Oral Ecology

The oral environment

The oral cavity provides a complex environment that includes several distinct habitats for microbial communities, such as mucosal surfaces represented by the lips, cheek, palate, tongue and gingivae, and the non-shedding surfaces of the teeth (9, 10). During development from infant to adult, the surfaces for colonization in the mouth change from soft mucosa to include the enamel of the teeth (9). These morphological changes generate new microbial habitats formed by physical interaction between the erupted teeth and the mucosa. Other factors, such as diet, salivary flow, host immunity, antibiotics and dental treatment continually influence the ecological conditions of the oral cavity. Therefore, in order to survive in the mouth oral bacteria must be able to remain on surfaces and avoid or adapt to the changing physical and biochemical features of the environment (9, 10).

A thin film of saliva normally flows over the tooth surface (26, 165). Salivary components, generally glycoproteins, deposit from this film and adhere to the tooth surface, becoming the acquired pellicle. Generally bacteria do not adhere directly to the enamel surface of teeth, but to the acquired pellicle or to already adherent bacteria via their surface adhesins, (47). Teeth also provide a number of different physical environments due to their anatomy (i.e., pits and fissures in enamel) promoting colonization by specific populations of bacteria (9). On some areas of the tooth surface, particularly the occlusal pits and fissures, bacteria become sequestered and are, therefore, protected from adverse environmental conditions.

The acquired pellicle provides an initial base for microbial adhesion to the tooth surface during the development of dental plaque. Dental plaque forms as a layer on the enamel and is comprised of complex layers and microcolonies of bacteria within an extracellular matrix of macromolecules of host- and bacterial origin (9, 47). Dental plaque is an essential factor in the aetiology of caries, since changes in the microenvironment of plaque can influence the bacterial composition and contribute to the pathogenic potential of plaque. Mucosal surfaces present a more challenging environment to bacteria than tooth surfaces since mucosal cells are continually sloughed off and swallowed, removing the adherent organisms. However, renewed mucosal surfaces are readily re-colonized by the microorganisms present in the saliva (47).

Saliva contains a number of components influencing bacterial colonization, such as ions important in maintaining oral pH and various anti-bacterial factors, including lysozyme, lactoferrin, and immunoglobulins (49). Salivary glycoproteins are also important because they can promote bacterial aggregation leading to their physical removal

from the oral cavity or, conversely, promote adhesion of bacteria to oral surfaces. Components of saliva and gingival crevicular fluid can also provide a nutrient source for resident microorganisms (9). Variations in composition, particularly in the volume and flow rate of saliva, can effect the nature of the oral flora (49).

A very important habitat for oral bacteria is the gingival crevice region which forms at the junction between the teeth and gingivae. This crevice is bathed by gingival crevicular fluid (GCF), a serum transudate that plays an important role in host defense by providing a means for serum immunoglobulins, complement factors, and leukocytes to enter the oral cavity (47, 49). Although GCF can play a role in removing non-adherent organisms, it can also provide a rich nutrient source for microbes which colonize the sub-gingival area and may be associated with periodontal disease (49).

Acquisition of the oral flora

The complexity and dynamic environment of the oral cavity influences the colonization patterns of oral bacteria. Colonization describes a series of stages involved in the development of a microbial flora that is influenced by factors governing accessibility to the host, adherence and growth of bacteria (9). Colonization of the oral cavity begins shortly after birth and is initiated by bacteria transmitted from mother to child and through the ingestion of fluids (10, 49). Acquisition of organisms from the birth canal seems of minor consequence, since these organisms do not become established in the infant's mouth (18). This process exemplifies the selectivity of the oral environment, since only a limited number of bacterial species colonize a newborn (47). The first organisms to become established

are referred to as pioneer species and are predominantly streptococci, particularly *S. salivarius*, *S. mitis* and *S. oralis* (10, 49). When more than one bacterial population colonizes a particular habitat, a bacterial community is established and each of the colonizing populations serves a particular function within the community. As the infant grows older, the oral flora becomes more complex and may include anaerobic organisms such as *Veillonella* and *Prevotella*. (10)

The process of change and growth among the initially-colonizing bacterial populations is referred to as primary succession (9). The outcome of succession is influenced by both external (allogenic) and internal (autogenic) environmental changes, the latter result from the metabolic and other activities of the community itself (9). For the first few months of life, succession occurs in communities on mucosal surfaces since such surfaces provide a suitable habitat for *S. sanguis, S. mitis, Actinomyces naeslundii, Rothia dentocariosa, Haemophilus, Veillonella, Neisseria* and *Prevotella* (10). The tongue is thought of as the predominant reservoir for *Streptococcus* species (99). Significant allogenic changes in the oral environment occur as a result of tooth eruption, leading to the establishment of new microorganisms including *S. mutans, S. sobrinus, S. sanguis* and *Actinomyces* species (10). This is an example of secondary succession, where growth and re-development occur within the disrupted habitat until the community is in a stable relationship with the environment (9). Such a community is termed a climax community. These oral climax microbial communities remain in balance with the environment of their habitat and are relatively stable with respect to the proportion of bacterial genera and species.

Bacterial adherence and plaque development

The composition of microbial communitites has an influence on the development of cariogenic plaque. Caries lesions result from the demineralization of tooth enamel, caused by the accumulation of end-products from microbial metabolism of carbohydrate creating localized acidic environments (161). Therefore, the development of dental plaque and succession of bacteria on tooth surfaces are important since the flora within the plaque can influence the development of caries. Plaque development starts with the adherence of microorganisms to the acquired pellicle. Adherence requires specific mechanisms in contrast to physical retention, which is the sequestering of the organism in a protective habitat. With oral bacteria, this specificity is provided by cell-surface adhesins. Adherence can also involve non-specific associations with the tooth surface via van der Waal's attractive forces, surface hydrophobicity and electrostatic repulsion, although these non-specific factors produce weak attraction relative to adhesins (47, 49). Specific bacterial adhesins bind in a stereochemically specific manner to host receptors of the acquired pellicle leading to firm surface attachment (47). Aggregation among different bacteria (co-aggregation) also promotes their establishment by specific mechanisms and can facilitate the entrance of a new organism into the plaque community (83).

In order for a micoorganism to become established within the plaque, it must be able to grow in the presence of other organisms. This may require adaptation of their physiology to changes occurring within the environment (17, 19). For example, under conditions of very high carbohydrate concentrations, *S. mutans* produces large amounts of acid (161). Strains of this species protect themselves from substrate killing by

employing a mechanism, the lactate gate, for the release of large amounts of acid in the form of lactate, formate, acetate and ethanol (17, 19). *S. mutans* is also capable of adapting to growth in acidic environments, which can suppress or eliminate other organisms (19). These adaptative mechanisms provide *S. mutans* with a competitive advantage over other saccharolytic plaque bacteria. Population shifts in the plaque due to high carbohydrate and concomitant acid production lead to increases in aciduric bacteria promoting the development of caries lesions (161). The successful competition by non-pathogenic commensal organisms within the oral habitat is beneficial to the host and results in a dental plaque community associated with health. Competition for initial colonization sites on the tooth enamel pellicle can influence the composition of plaque and this composition is also influenced by the tissue tropism specified by the interaction of surface adhesins with host cellular components.

The physico-chemical mechanism of adhesion

Adhesion has been described in terms of a physico-chemical mechanism by using the Derjaguin, Landau, Verwey, Overbeek (DLVO) theory. For specific adhesion to occur, a microorganism and a host surface must come into relatively close contact. In the case of plaque formation, the microorganism and the acquired pellicle are both negatively charged and, therefore, repulsive forces have to be overcome before adhesion can take place (99). As a bacterium approaches a surface, both specific and non-specific interactions will occur that will govern whether attachment and colonization is successful. The interactions of an inert particle and a substratum have been described in physico-chemical terms by the DLVO theory of particle deposition (155). The theory states that the total

interactive energy of adhesion of a particle is the sum of the van der Waals attractive energy and the generally repulsive, electrostatic energy. However, the complexity of biological systems generally precludes the strict application of mathematical models.

Aqueous solutions allow for the adsorption of ions from the aqueous environment and these charges are balanced by counterions creating an electrical double layer surrounding the particle. As the particle approaches a surface, a weak van der Waals attraction is induced by the fluctuating dipoles within the approaching molecules. However, a repulsive force will also be encountered due to the overlap of the electrical double layers. The magnitude of the repulsion will be governed by the ionic strength, dielectric constant of the suspending medium, and by the charge of the outer layers of the particle and on the surface to be colonized.

A net attraction can occur at two separation distances. These are at the primary minimum where the separation distance is very small, and the secondary minimum where the separation distance is approximately 10-20nm. These two distances are separated by a repulsive maximum. Organisms can remain in equilibrium at the secondary minimum and this may result in reversible adhesion. In time, the adhesion may become irreversible because of the short range specific interaction of bacterial adhesins, such as P1, with ligands in the acquired pellicle. For this to occur, hydrophobic forces, resulting in the removal of water from between the two surfaces is essential for allowing the two surfaces to come within close enough proximity for involvement of short range interactions.

Once adherent, microorganism will secure its attachment by the liberation of extracellular products, co-aggregation with other adherent organisms, and multiplication to produce confluent growth. The process of

attachment, growth, removal and reattachment is a dynamic and continuous process in microbial plaque.

Adhesins of Actinomyces and oral streptococci

Specific adhesins of two genera found in the oral cavity, *Actinomyces* and *Streptococcus* have been extensively characterized. Adhesion mediated by fibrils and fimbriae (reviewed in 7, 47) help to locate bacteria on epithelial cells, tooth pellicle, and also mediate adhesion between bacterial cells of different species. The latter phenomenon is referred to as coaggregation (83). Fibrils are distinguished from fimbriae by their ability to clump together and in having no measurable width. Bacteria can posses both structures in different arrangements on the cell surface. The expression of these structures can influence the ability of the organism to colonize oral surfaces.

Human strains of *Actinomyces naeslundii* (genospecies 1) possess only Type 2 fimbriae and *Actinomyces viscosus* (now *A. naeslundii* (genospecies 2) have two distinct types of fimbriae, Type 1 and 2, that are involved in adhesion. Type 1 *A. naeslundii* fimbriae bind to proline-rich proteins (PRP), in the aquired pellicle, but do not bind the soluble PRP in saliva (51, 115). The salivary PRP are unique phosphoproteins which become adsorbed to the tooth surface and are components of the acquired pellicle. This difference in binding of Type 1 fimbriae to surface-bound, but not soluble PRP, has lead to the idea of cryptitopes (47). Cryptitopes are binding domains that remain hidden until the protein undergoes a conformational change after absorption to a surface, exposing the binding domain. This conformational change from a soluble to an adsorbed form is

believed to occur when PRP binds to tooth enamel. Conformational changes may also occur through enzymatic modifications.

A. naeslundii Type 2 fimbriae are involved in galactosyl binding to mammalian cells via glycoproteins or glycolipids (20). Initially, these cellular glycoproteins require treatment with neuraminidase to remove the terminal sialic acid residue and expose the galactosyl residue. This is an example of how enzymatic modification exposes a cryptitope that can interact with the bacterial adhesin. The Type 2 fimbriae also mediate lactose-inhibitable coaggregation with *S. sanguis, S. mitis* and *Streptococcus morbillorun* (83). Analysis of mutants defective in fimbriae expression showed that Type 1 fimbriae were more efficient at binding bacteria to the tooth surface, while Type 2 fimbriae were important in coaggregation reactions with other bacteria (21). The function of fimbriae with respect to oral colonization has been assessed by using monoclonal antibodies (20, 22). Type 2 fimbriae were responsible for binding *Actinomyces* to epithelial cells and to plaque streptococci, while the Type 1 fimbriae were involved in binding to the tooth surface.

Streptococcus salivarius, an early colonizer of oral mucosal surfaces of infants, can be grouped into the Lancefield K+ or K- serotypes (7). The K+ strains possess fibrils, while the K- strains possess fimbriae (56). The K+ strains have 3 classes of adhesins. The first class, designated host attachment factor (HAF) or Antigen C, is involved in adherence to host tissues, salivary aggregation reactions, haemagglutination and adhesion to buccal epithelium (166, 167). The second class is Veillonella-binding protein (VBP) or Antigen B involved in co-aggregation with *Veillonella* (166). The third type of adhesin is responsible for co-aggregation with *Fusobacterium nucleatum*. The K- strains do not haemagglutinate,

agglutinate with saliva, or adhere well to buccal epithelia cells (166). It is thought that the K+ strains are able to colonize the oral cavity either by the HAF or by attachment to previously-colonized *Veillonella*. *S. salivarius* provides a good example of an organism expressing more than one adhesin, a factor promoting colonization into the oral ecosystem (7).

Streptococcus sanguis, a pioneer plaque organism that rapidly colonizes tooth surfaces, has a high affinity for neuraminidase-sensitive receptors found in the salivary pellicle (10, 50). Strains of *S. sanguis* with peritrichous fibrils also coaggregate with *A. viscosus* and *A. naeslundii* and demonstrate superior adhesion to saliva-coated hydroxyapatite (HA), in comparison with strains with tufted fibrils (171). Strains possessing both peritrichous fibrils and fimbriae demonstrate the greatest adhesive capabilities. Fachon-Kalweit *et al.* (32) were able to inhibit adhesion of *S. sanguis* to saliva-coated HA using antisera generated against the fimbriae.

The fibrils of *S. sanguis* (strain 12) are associated with a 36 kDa protein encoded by the *ssaB* gene, which mediates adhesion to saliva-coated hydroxapatite (HA), via a pH-sensitive host receptor (46). The SsaB protein was shown, by immunogold labelling with anti-SsaB antisera to localize to the tips of densely packed short fibrils (109). These same antibodies were able to inhibit adhesion of a non-aggregating variant strain (12na) to saliva-coated HA. However, the anti-SsaB antisera did not completely block the adherence of the wild-type *S. sanguis* strain 12, providing evidence in support of a two-site binding model (110). This model describes two types of salivary receptors for bacterial adhesins, one sensitive to neuraminidase, and a second type inactivated by prolonged incubation at 37°C. Strain 12 binds to both receptors, while strain 12na binds only to the second type. In

addition to the short fibrils, long fibrils on *S. sanguis* also recognize the neuraminidase-sensitive receptors in salivary pellicle (109), a reaction which can be inhibited with antibodies generated against the long fibrils.

An *S. sanguis* adhesin, formerly designated SSP-5 (28), when expressed in *Enterococcus faecalis*, confers both salivary-agglutinin binding and salivary agglutination abilities to the *Enterococcus* (27). This 205 kDa protein bound salivary agglutinin and was inhibited by sialic acid--containing sugars (30). Thus, SSP-5 is the adhesin responsible for binding *S. sanguis* to the neuraminidase-sensitive receptor found in the salivary pellicle. Some strains of *S. sanguis* have recently been reclassified as *Streptococcus gordonii* M5, and consequently adhesin SSP-5 has been renamed SspB (29). Strain *S. gordonii* DL-1 has been shown to possess an adhesin designated SspA involved in co-aggregation with *Actinomyces naeslundii* and adherence to salivary-agglutinin glycoprotein (72). Recent genetic analysis revealed that the two genes encoding SspA and SspB are tandemly arranged on the *S. gordonii* chromosome in both the M5 and DL-1 strains (29). Two additional high molecular weight (approx. 260 kDa) surface proteins, designated CshA and CshB, have also been identified in *S. gordonii* and implicated in co-aggregation reactions and colonization of the oral cavity (104). These latter high molecular weight proteins are encoded by genes at separate chromosomal loci and are not tandemly arranged.

Streptococcus mutans also has surface structures which have been implicated in adhesion to tooth surfaces. In addition to protein P1, *S. mutans* generates glucan-binding lectins and glucosyltransferases, that bind the glucans synthesized from sucrose (55) and, as a consequence, *S. mutans* is able to adhere to plaque through co-aggregation with plaque

glucans, enhancing the ability to colonize and multiply within the plaque environment. Detailed description of the role of P1 in adhesion and aggregation is presented on page 17.

Other *S. mutans* surface proteins that may participate in adherence include fibrils and a recently designated protein referred to as the wall-associated protein (WapA) (35, 139), also referred to as antigen A (139) or antigen III (141). The gene encoding this protein, *wapA*, has been cloned and sequenced (35) and predicts a protein of a M_r of 45,000. However, the native protein isolated from the culture supernatant is only a 29 kDa proteolytic cleavage product. WapA is believed to play a role in the colonization of tooth surfaces (131).

The Role of Surface Proteins in Pathogenesis

There are excellent examples in the literature of the mechanism of host colonization, including specific adherence and evasion of the immune response. In most cases these functions depend on cell surface proteins. Although there are considerable data on the role of Gram-positive cell-surface proteins in adhesion to host tissues, except for *S. pyogenes*, less is known of antigenic variation. In the following section, different aspects of cell surface proteins in pathogenicity and virulence will be considered. Although *S. mutans* surface proteins and adherence form the topic for many studies, a role for P1 in antigenic variation and avoidance of the host immune system has not been explored.

In order to determine the role(s) played by surface proteins in infectious diseases, it is important to recognize the characteristics of pathogenic organisms that contribute to the aetiology of infection. A

bacterial pathogen colonizes a host in order to propagate and eventually be transmitted to a new host (36, 44). Falkow (34) has described the attributes of the bacterial pathogen as follows: (a) able to gain entry into a specific host species, (b) able to find a unique niche within the host, (c) able to evade, circumvent or exploit the host's innate defense mechanisms, (d) able to multiply, (e) able to exit the host and be transmitted to a susceptible host; and (f) able to cope with the host's adaptive immune system. The roles of surface proteins, including *S. mutans* P1 protein, in promoting the pathogenesis of an organism will be considered under the following headings.

Adherence of Gram-positive cocci to host tissues

Attachment to host tissue components is important in the early stages of the infectious process and may target the organism to specific host cell populations and to a particular host species. The expression of a particular mosaic of surface proteins may, therefore, govern colonization patterns. This is believed the case for the group A streptococci, where M protein binds IgG3 (135), factor H (the C3b convertase regulatory protein (67, 71), human serum albumin (2) and fibrinogen (170). Protein F (57, 157), a fibronectin binding protein, and M protein are regulated by the environmental levels of O_2 and CO_2 (117). Expression of M protein is up-regulated by CO_2 while that of protein F is down-regulated. Each of these proteins has been demonstrated to facilitate adhesion to different cultured skin cell types, suggesting their expression can influence the colonization outcome (12, 69, 118, 164). In cases where there is abundant fibronectin, such as, at wound sites, it might be expected that expression of protein F would be up-regulated. In addition, the immunoglobulin-binding proteins

(IGPs) expressed by streptococci may influence the bacterium's ability to colonize the pharyngeal epithelium since this tissue is coated with the normal flora, saliva and secretions containing fibronectin and immunoglobulins (23). The ability to adhere to these macromolecules may enable the organism to resist being removed by mechanical means, although this hypothesis has not been experimentally tested (23).

The collagen receptor of *Staphylococcus aureus* is another example where a particular surface protein is associated with a specific tissue tropism. Some *S. aureus* cells possess the *cna* gene encoding the collagen binding protein (Cna), and are common among isolates from septic arthritis and osteomyelitis (126, 127), but rare among isolates from soft tissue infections. This suggests that the expression of this particular surface protein is directly responsible for the ability to localize and cause diseases of the joints and has conferred a particular tissue tropism to these isolates. More recently, the presence of a collagen-binding adhesin has been described for the mutans group of streptococci and this protein may contribute to adhesion to the root surfaces of teeth (154). A collagen-binding adhesin of *S. pyogenes* has also been identified (163), although at the present time, it is not known if the streptococcal adhesins will possess a common ligand- binding motif as demonstrated among FnBP's of *S. aureus* and other *Streptococcus sp.* (102). The *Staphylococcus aureus* fibronectin-binding protein has a conserved arrangement of amino acids that function as the ligand-binding domain close to the carboxy-terminal portion of the protein (102). A similar arrangement of amino acids has been identified in fibronectin-binding proteins of other organisms, including *Streptococcus dysgalactiae* (102) and *Streptococcus pyogenes* (108), suggesting conservation of important protein structures (133). Whether duplication of

this ligand binding domain in *Staphylococcus* and *Streptococcus* is the result of intergenic recombination is unknown, although a recombination mechanism is suspected.

Adherence and aggregation of S. mutans involving P1 protein

The ability to adhere to host surfaces is fundamental to colonization and to the specificity of tissue tropism associated with a particular pathogen. Streptococci in the oral cavity have evolved specialized protein structures that are responsible for tissue tropism. *S. mutans* is associated specifically with adherence to the salivary pellicle covering the tooth surface. *S. mutans* cannot be isolated consistently from the oral cavity of infants until after the teeth have emerged (10, 47). This association with tooth surfaces has been attributed to the specific interaction of the major surface protein P1 and salivary agglutinins present in the pellicle (11).

Initial studies consistent with the concept that P1 is an adhesin, demonstrated that variants of *S. mutans* deficient of P1 exhibited decreased binding to saliva-coated hydroxyapatite (HA) and decreased cell-surface hydrophobicity (82, 100). Similarly, mutant strains of *S. mutans* serotype c produced by insertional inactivation of the P1 gene also showed decreased binding to saliva-coated HA and decreased surface hydrophobicity (88). The components in saliva promoting the adhesion of *S. mutans* to saliva-coated HA have been characterized as a high molecular weight glycoprotein, salivary agglutinin (31), as well as the proline-rich proteins, although the latter show weak specific activity (47). Salivary agglutinin, purified by affinity chromatography using monoclonal antibodies, is a high molecular weight (Mr 300,000) glycoprotein promoting both adhesion and aggregation of *S. mutans* (31). The adherence of *S. mutans* to saliva-coated HA (82),

salivary agglutinin-coated HA (15, 30, 88) and agglutinin-mediated aggregation (15, 30, 82, 88) is calcium dependent. Although both adhesion and aggregation are observed, these two interactions may involve different portions of the P1 molecule (15). Other salivary molecules to which *S. mutans* has been shown to bind include secretory immunoglobulin A (138), proline-rich polypeptides (48) and fibronectin (4).

In vitro and in vivo bacterial adhesion experiments

It is difficult to obtain substantial evidence to show a correlation between specific surface protein expression and virulence *in vivo*. This problem stems from a dependence on animal models that emulate the natural disease and their use in evaluating genetically manipulated pathogens. However, good animal models that can emulate the human disease do not always exist, as is the case for group A streptococcal infections. To get around this problem, *in vitro* bactericidal tests were developed to determine the effect of M protein on opsonization (146). Caparon *et al.* (16) used tonsillar and buccal epithelial cells to assess the role of M proteins in adhesion. In their study, an M protein isogenic mutant was constructed in which the *emm* locus was replaced by an antibiotic marker and tested for *in vitro* adhesion. The results suggested that M protein was not involved in adhesion, but may contribute to coaggregation and the formation of microcolonies on tonsillar epithelial cells. Hollingshead *et al.* (65) demonstrated that M protein had no effect on the initial colonization of pharyngeal mucosa in a rat model, but implicated M protein in the persistence of infection.

The association between the expression of immunoglobulin G (IgG)-binding proteins by group A streptococci and virulence was examined

by Raeder and Boyle (132) in a mouse skin air sac model. Group A strains isolated from skin sites displayed a higher frequency of IgG-binding activity than strains isolated from the nasopharynx. Expression of IgG-binding proteins was correlated with the ability to establish a lethal skin infection. However, isogenic mutants for the IgG-binding protein(s) have not been tested in this animal model. The complexity and number of surface proteins coded by the M-supergene family and the diversity of their binding specificities will make assessment of the contribution of individual members to group A streptococcal pathogenesis difficult.

Protein A-deficient mutants of *Staphylococcus aureus* generated by allelic replacement were slightly less virulent in animal models of peritonitis and subcutaneous infections (124), but no differences in virulence were observed between the wild-type and the mutant in the mouse mastitis model. Allelic-replacement mutagenesis was used to demonstrate the association of the *S. aureus* collagen adhesin with the development of septic arthritis when isogenic *S. aureus* strains were injected into mice (126). Transposon mutagenesis of *S. aureus* generated low fibronectin-binding mutants, resulting in 250-fold fewer organisms associated with the heart when tested for attachment to traumatized heart valves in a rat endocarditis model (84). Site-specific mutagenesis of the fibrinogen-binding protein or clumping factor of *S. aureus* produced mutants defective in the ability to cause endocarditis in rats with catheter-induced aortic vegetations (108). These mutants produced approximately 50% less endocarditis than the wild-type parent strain. However, parent and mutant strains were found in equal numbers in the spleen suggesting that the lower infectivity of the mutants was due to their inability to colonize damaged valves and not due to resistance to host defenses.

Insertional mutagenesis of both *S. aureus* fibronectin-binding proteins genes, *fnbA* and *fnbB*, produced strains completely defective in adhesion to polymethylmethacrylate coverslips coated with fibronectin *in vitro* (52). Adherence capabilities of this double mutant were assayed in rat traumatized aortic and pulmonary heart valves and indicated no differences in adhesion between the wild-type and mutant strain (41). The results obtained from the insertion mutant and those obtained from the transposon mutant were inconsistent. These two studies used mutants generated by two different methods and not mutants generated by allelic replacement creating difficulty when analyzing *in vivo* experments since in either case, the mutants could have reverted to wild-type, because the gene had not been deleted from the chromosome (43).

The role of bacterial surface proteins in colonization of oral surfaces has been investigated by using primarily *in vitro* adhesion and aggregation assays. Insertional inactivation of the 3' of the *S. gordonii* gene, *cshA* encoding a 290 kDa surface protein (CshA), resulted in the generation of a mutant that secreted a 260 kDa truncated protein devoid of a C-terminus. This mutant exhibited reduced cell-surface hydrophobicity and was impaired in the ability to coaggregate with *Actinomyces naeslundii* (103). McNab *et al.* (104) identified a second *S. gordonii* polypeptide, designated CshB. Insertional mutagenesis of both the *cshA* and *cshB* genes was conducted to investigate the role of these proteins in the colonization of the murine oral cavity. Cell-surface hydrophobicity and co-aggregation with *A. naeslundii* were decreased in the double-mutant strain. Oral colonization of *Streptococcus* - and *Lactobacillus*-free mice by *S. gordonii* found 90% of the mice colonized by the wild-type strain. In contrast, the *cshA* mutant and the *cshB* mutant colonized only 1 in 7 and 1 in 6 mice

respectively, suggesting a role for the wild-type polypeptides in oral colonization.

Two additional polypeptides have been identified in *S. gordonii* encoded by tandem genes, *sspA* and *sspB* (formerly *ssp-5*) (29). Insertional inactivation of *sspA* reduced binding to salivary-agglutinin glycoprotein and *A. naeslundii* (73). When both genes were insertionally inactivated there was a further decrease in adhesion to salivary agglutinin and to *A. naeslundii*. However, the double mutant retained 50% of its ability to bind salivary agglutinin and was still capable of binding to saliva-coated hydroxyapatite. The authors suggest that *S. gordonii* may express multiple adhesins capable of binding to salivary agglutinin, although the role of these proteins in *in vivo* colonization has not been addressed.

The role of *S. mutans* proteins in disease has been studied by using mutants constructed by allelic exchange and by testing these mutants in either *in vitro* adhesion assays or in a rodent caries animal model (97). Insoluble glucan synthesis and fructan synthesis are important for virulence, probably by promoting adherence of the bacteria to teeth. Insertional inactivation of the *S. mutans* wall-associated protein A gene (wapA) decreased sucrose-dependent adherence to glass and the ability of cells to self-aggregate. The wall-associated protein may be important for colonization of tooth surfaces *in vivo*, although animal colonization studies have not been conducted (131).

Adhesion of *S. mutans* to the high-molecular-weight salivary agglutinin is mediated by surface protein P1. An isogenic mutant in *spaP*, the gene encoding P1, results in decreased hydrophobicity and decreased adhesion to salivary agglutinin-coated HA (88). However, in a study conducted by Bowen *et al.* (11) both the mutant *S. mutans* 834 and the wild-

type *S. mutans* were shown to adhere to glucan-coated surfaces providing evidence for at least two mechanisms of colonization. When the mutant and wild-type *S. mutans* were tested for their ability to colonize teeth and cause caries in a rat model system, both strains were able to induce smooth-surface caries in intact and desalivated rats fed a high sucrose diet. The authors suggested that sucrose helps to promote colonization by providing an ideal tooth surface. It was later discovered that the isogenic mutant used in this study was capable of expressing the amino-terminal 612 amino acids of P1 (14). This N-terminal segment of P1 has since been implicated in the adhesion to salivary agglutinin (24), which could explain why no differences in adhesion and cariogenicity were observed between the mutant and wild-type strains in this animal model study.

The assessment of individual surface proteins as bacterial virulence factors is difficult due to the requirement of a suitable animal model. The number of individual surface proteins identified and implicated in adhesion events continues to expand. The identification of surface proteins with multiple binding activities, as observed in the M supergene family, also makes assessment of the role of these proteins in pathogenesis difficult. It is apparent from these numerous studies, that adhesion and pathogenesis are multifactorial events that can involve several surface proteins.

Antigenic variation

The ability to change surface antigenic proteins to avoid immunological detection and elimination has been well documented for a number of pathogenic bacteria. Examples include the variable major protein (VMP) of *Borrelia* species (5), the pili of *Neisseria gonorrhoeae* (106)

and the *S. pyogenes* M protein (13, 37). The ability to change immunodominant surface antigens through gene splicing events is termed antigenic variation. The gene-splicing events involved in antigenic variation allow for economic usage of the chromosome, while generating the greatest possible diversity, reminiscent of the diversification of the eukaryotic antibody repetoire (12). The ability to change the antigenicity of surface proteins is important because it allows the pathogen to escape immunoglobulin-specific clearance, providing more opportunity to colonize and multiply within the host. This prolongs the time available for transmission of the pathogen to a new host and is, therefore, an important virulence factor.

During relapsing fever, the Borrelia VMP undergoes structural alterations which arise from silent gene rearrangements (5). Using this mechanism for antigenic variation, *Borrelia* is capable of evading antibody clearance from the blood. *Neisseria* also demonstrate antigenic variation in the pili displayed on their surface (53, 106). In this case, the sequence variation within the expressed pilin gene is generated by intragenic recombination where there is transposition of one or more minicassettes from the silent gene to the expressed gene.

S. pyogenes has developed mechanisms to change the antigenic structure of the antiphagocytic M protein. The repetitive sequences within the *emm* locus can undergo intragenic recombination and/or nucleotide substitutions leading to over 80 distinct M serotypes identified to date (79). The greatest degree of diversity is associated with the N-terminus of the protein, which is the recognition site for opsonic antibodies (37, 58). Opsonic antibodies generated against the N-terminus result in selection of organisms expressing new variants of the M molecule. This opsonogenic

epitope requires only a few changes in amino acid composition to escape immune recognition and, therefore, to nullify clearance of the organism (12). Antigenic variation of M protein may also contribute to the existence of the carrier state in which people with no overt disease continue to harbor and shed the organism. These organisms, having successfully escaped immune recognition and colonized the pharynx, increase the probability of transmission to uninfected individuals because they have effectively extended their survival time within the host (34). Antigenic variation can also contribute to re-infection of the same host with organisms expressing M proteins with novel opsonic epitopes not previously encountered by the host immune system.

Antigenic variation resulting from recombination of DNA sequences can also give rise to surface proteins with unique functions or binding specificities. The *emm* supergene family (Table 1.1) is an example of functional diversity created by recombination events. This supergene family encodes the immunoglobulin-binding proteins (IGPs) and the M and M-like proteins of group A streptococci that share a number of similarities at the genetic level (reviewed in 12, 23, 79, 169). These proteins are variants of a common ancestral protein and may have resulted from intergenic recombination and gene duplication events (23, 60). The M-like proteins encoded by the *emmL* genes share M protein structural characteristics, but there is no evidence that these proteins confer resistance to phagocytosis. The genes for the immunoglobulin-binding proteins (IGPs) include: *fcrA*, which binds IgG1, IgG2, and IgG4 (59); the *enn* gene, which binds IgG3 (13) or IgA (91), and the *emmL* genes, which also bind immunolglobulins and serum components. These genes and the *emm* gene reside in a

Table 1.1

The S. pyogenes M Protein Supergene Family

Protein	Putative ligand	Reference
M protein	antiphagocytic binds factor H, fibrinogen IgG3, albumin	2, 37, 67, 71, 170
Immunoglobulin Binding Proteins		
M-like proteins		
EmmL55	binds IgG1, IgG2, IgG3, IgG4, fibrinogen, albumin	13
EmmL3	binds fibronectin, fibrinogen, albumin	
EmmL18	binds fibrinogen, albumin	
EmmL2	binds IgG1, IgG2, IgG3, IgG4	
EmmL64/14	binds IgG1, IgG2, IgG3	
FcRA	binds IgG1, IgG2, IgG4 fibrinogen	59
Enn	binds IgA or IgG3	13, 91, 130
Protein H	binds IgG1, IgG2, IgG3, IgG4	2
Sir	binds IgG3 and IgA	152
Arp	binds IgG3 and IgA	70

coordinately-regulated locus in *S. pyogenes*, termed the *Vir* regulon (129). The *Vir* gene cluster demonstrates strain to strain variation with respect to the serum opacity factor phenotype and the type and number of IGP genes. However, the *Vir* regulon is consistently flanked by a positive regulator (the *virR* gene or *mry* (128, 129) at the 5' end and a C5a peptidase gene (*scpA*) at the 3' end (150). The IGPs (see Table 1.1) can include the Enn protein (70, 130), which binds IgG3 or IgA; the FcRA protein (59, 152), which binds IgGs (13); protein H (2) and the antiphagocytic M protein (37). All of these proteins can be expressed on the cell surface, although generally not simultaneously.

The identification in *S. gordonii* of tandem genes encoding the surface proteins SspA and SspB also suggests the involvement of gene duplication events reminiscent of the M protein supergene family (29). The possibility that these proteins in *S. gordonii* may have different ligand-binding properties has yet to be investigated.

The proteins in the M-family gene cluster have similar structural elements, including the alpha-helical coiled-coil conformation and conserved C-terminal cell-anchoring domains (12, 79). Analysis of the DNA sequence encoding these proteins reveals considerable homology in the C-terminal two thirds of the protein while the amino-terminal regions are divergent (130, 150). These proteins bind soluble immune system components and interfere with normal complement-dependent opsonophagocytosis but demonstrate functional and antigenic heterogeneity. Boyle (12) proposed a model for generation of functional diversity through recombination events involving module shuffling. Each functional unit was regarded as a module and homologous recombination at genetic hot spots generated diversity. This would result in an array of

functionally diverse surface proteins with affinity for various host ligands (Table 1.1). The antigenic and functionally-diverse array of proteins expressed on the surface of the organism may enhance the ability to evade opsonization by specific antibodies by changing the type and distribution of surface proteins expressed during the course of infection. In addition to the other functions attributed to these surface proteins, the diversity and the regulation of their expression during the course of infection could provide a significant advantage to the pathogen by allowing selective evasion of antibody-mediated clearance.

Antigenic variation in P1 and P1-like proteins

The observation of variable numbers of tandem repeats and the identification of a variable region in which amino acid substitutions are clustered within the P1-like protein structures suggest the potential for antigenic variation (8, 85, 158). These same phenomena have been observed among the *S. pyogenes* M proteins and the immunoglobulin-binding proteins demonstrating antigenic variation (12). In addition, the anti-P1 monoclonal antibody cross-reactivity analysis of streptococcal strains identified overlapping, but not identical MAb subsets, suggesting antigenic differences among P1-like proteins (14). However, no immunological differences were observed among P1 proteins expressed by *S. mutans* serotypes c, e and f strains, although restriction length polymorphisms exist within their variable domains (8, 14).

Although it is apparent that the structure and mechanism of adherence of various cell-surface proteins, including *S. mutans* P1, have been identified, there is little information on whether these molecules

mediate antigenic variation or other structural alterations associated with functional changes.

Molecular mimicry of host proteins

Although bacterial surface proteins are known to interact with host tissues to facilitate colonization, these proteins may also be involved in evasion of the host response. Bacteria 'cloaked' with host protein may not be recognized as foreign and surface protein interactions may also sequester bacteria into host cells where they are protected.

The ability to bind host serum proteins may provide a pathogenic organism with the means to shield itself from the immune system. If a bacterium displays host proteins on its cell surface, the immune system recognizes the cell as non-foreign and no specific antibody response is mediated. There are several surface proteins, including the *S. aureus* protein A and the *S. pyogenes* M-like proteins, which bind the Fc region of immunoglobulins effectively coating themselves with immunoglobulins. This renders the immunoglobulin Fc domains inaccessable for complement deposition and subsequent phagocytosis. The immunolglobulin $F(ab)_2$ antigen-binding domains are also unable to engage their specific antigen, interupting normal antibody-mediated immune clearance of the infecting organism. Microorganisms which cover themselves with complement components or fibronectin also avoid phagocytosis by allowing the organism to escape immune detection (reviewed in 12, 23, 34, 36, 63). Alternatively, if bacteria masked with serum proteins encounter activated plasma cells, the immune system may generate autoimmune antibodies recognizing the plasma proteins presented by the bacterium (23, 94). The ability to coat themselves with host

proteins is considered a function of the surface proteins of the infecting microorganism.

In addition to providing the bacterium with a disguise, extracellular matrix (ECM) molecules can also be used for attachment. The binding of ECM, such as fibronectin, can be used to facilitate the attachment of bacteria to endothelial cells by exploiting fibronectin-endothelium integrin interactions (63). In some cases, the pathogen can express an adhesin which truly mimics the integrin and is used to promote the interaction of the bacterium with endothelial cells (159). Pathogenic organisms can also express adhesins with the appropriate integrin-binding motif, the Arg-Gly-Asp, leading to uptake of the pathogen by macrophages (134). This interaction can lead to internalization of the pathogen into the cell sequestering the pathogen from immune surveillance. Intracellular pathogens may replicate unincumbered within the cell and, in some cases, are transported to new sites within the host (34, 36, 63).

Two or more bacterial surface-protein adhesins can act together to stimulate a cascade of events involved in the process of leukocyte transmigration across endothelium, as is the case of *Bordetella pertussis* (63, 159). Secondary effects of adhesins include the ability to induce cytokine responses and either reduce or induce inflammation of host tissues. Cytokine release and inflammation would enhance endothelial permeability and leukocyte recruitment, which may promote the invasive potential of the pathogen.

Normal host immune responses may also be circumvented by bacterial surface proteins sharing homology with proteins involved in the immune response. One example is the *S. aureus* surface protein Map (Major Histocompatabilty Complex (MHC) class II analogous protein),

which shares homology with the MHC class II peptide binding domain (66, 75). This protein, released from the cell surface under acidic conditions, could possibly interupt normal antigen processing by competing with the host MHC class II binding domain. Although this hypothesis has not been tested experimentally, the inhibition of antigen processing may lead to an absence of, or a poor, humoral (and anamnestic) response. Under these circumstances, the organism would escape immune detection by specific antibodies and have more opportunity to establish itself within the host. In addition, the absence of an anamnestic response would allow the same strain to re-colonize the same host, as has been observed in *S. aureus* infections.

Environmental or sensory signaling

In addition to their functioning in adhesion, aggregation, and immune evasion, surface proteins may also play a role in sensing the external environment. To date, there has been no glycosyl-phosphatidylinositol (GPI)-like receptor found in prokaryotic cells emulating the same structure in eukaryotes. Nevertheless, this does not preclude the possibility of the existence of a sensory system. Boyle (12) has suggested that the phenotype for adherence and colonization may be very different from that required for invasion. Therefore, surface proteins which signal or sense environmental changes provide a mechanism of determining the appropriate activation or suppression of selected genes. This type of sensory mechanism would be required for bacteria to switch from an adhesion phenotype to an invasion phenotype given the appropriate conditions.

A hypothetical model for sensory transduction via the *S. pyogenes* immunoglobulin-binding proteins (IGPs) has been proposed by Cleary and Retnoningrum (23). The model predicts that interaction of surface proteins with plasma proteins would induce a conformational change in the C-terminal domain of the Ig-binding protein. The conformational change would then render a second component susceptible to phosphorylation and result in the activation of DNA-binding proteins capable of regulating the expression of other genes. The second component is necessary since the cytoplasmic tail of surface proteins is too short to function as a kinase and/or may be proteolytically cleaved from the protein upon insertion into the peptidoglycan. There is no evidence to support this model. A sensory system would have implications in pathogenesis, because surface proteins could distinguish specific tissues or extracellular matrix components and regulate the expression of other potential virulence factors. Differential expression of surface proteins on streptococci has been noted for the M and M-like protein family of which immunolglobulin-binding proteins are a member. There is, however, no definitive evidence that surface proteins can function in a signaling pathway capable of sensing the external environment (12).

Because of the potential role of the C-terminus in cell signalling, there has been increasing interest in understanding the association of surface proteins with the cell. Initially, experiments involving M protein suggested a transmembrane location of the hydrophobic domain leaving the charged tail on the cytoplasmic face of the membrane for signal transmission once the ligand binding domain(s) was engaged. However, recent studies on the anchoring of protein A suggest this hypothesis does not apply for *S. aureus*. If surface proteins play a role in signalling then it

may not be by a common mechanism. The possibility that the C-terminal anchoring of surface proteins to the cell wall may relate to environmental sensing makes the site and anchoring mechanism of considerable interest.

Structure of Cell Surface Proteins

The molecular characterization of a number of Gram-positive surface proteins has identified several common structural features. Historically, one of the most extensively studied surface proteins is the *S. pyogenes* M protein. Many of the common features of Gram-positive adhesins can be illustrated by examination of the biology and structure of M protein at the molecular level. Early studies by Lancefield indicated that M protein was an important antiphagocytic virulence factor (67, 71) and immunity to *S. pyogenes* infection was due to type-specific anti-M antibodies (37). By using type-specific antisera, over 80 different serotypes of M protein have been identified (79). Thin section electron micrographs of cells reacted with ferritin-labeled M-specific antisera demonstrated the localization of M protein as a fuzzy coat on the cell surface (153). Isolation of M protein from cells with a bacteriophage-associated lysin and subsequent purification indicated that the native M protein had a molecular size of 58 kDa (40). Structural analysis identified an alpha-helical, coiled-coil structure with a repeating seven-residue periodicity of nonpolar amino acids characteristic of other coiled-coil proteins such as tropomyosin in eukaryotic cells (98). Each M protein is a dimeric molecule which extends 50-60 nm from the cell surface (37, 153).

Genetic organization of the emm gene

Several of the characteristics of surface proteins have been defined by analysis of the DNA and amino acid sequence data obtained from the gene encoding the *S. pyogenes* M6 protein, designated *emm*-6 (Fig. 1.1) (64). One of the most striking common features is the existence of tandemly repeated amino acid sequences. A distinct repetitive amino acid sequence (a repeat or block) is designated by a letter and the individual copy of the repeat is designated by a number (A1, A2, B1, B2, ect) (79). The M6 protein has four identified tandem repeats: A, consisting of 14 amino acids; B, consisting of 25 amino acids; C, consisting of 42 residues, and D, consisting of 7 residues. The number of times each block of amino acids is repeated also varies from 5 times for A and B, 3 for C, and 4 for D (64). The number of distinct repetitive amino acid blocks and the number of times each block is repeated varies among M protein types. Tandem repeats have been observed in a large number of surface proteins from Gram-positive bacteria and these can vary in length from two residues (101) to over 100 residues (79). Generally, the internal repetitive blocks are highly conserved, while the external tandem repeats, flanked by a unique M sequence, are more degenerate, suggesting the internal repeats have accumulated fewer divergent point mutations (79). The C-terminus of the M protein (Fig.1.1) is characterized by a hydrophilic 44 amino acid wall-spanning domain, the LPXTGX consensus sequence consisting of LPSTGE, the hydrophobic 19 amino acid membrane-spanning domain followed by the KRKEEN charged tail (64). The role of these domains in the localization and anchoring of M protein is addressed in detail beginning on page 52.

The presence of repetitive amino acid sequences within the M protein molecules suggests that they evolved through slippage during

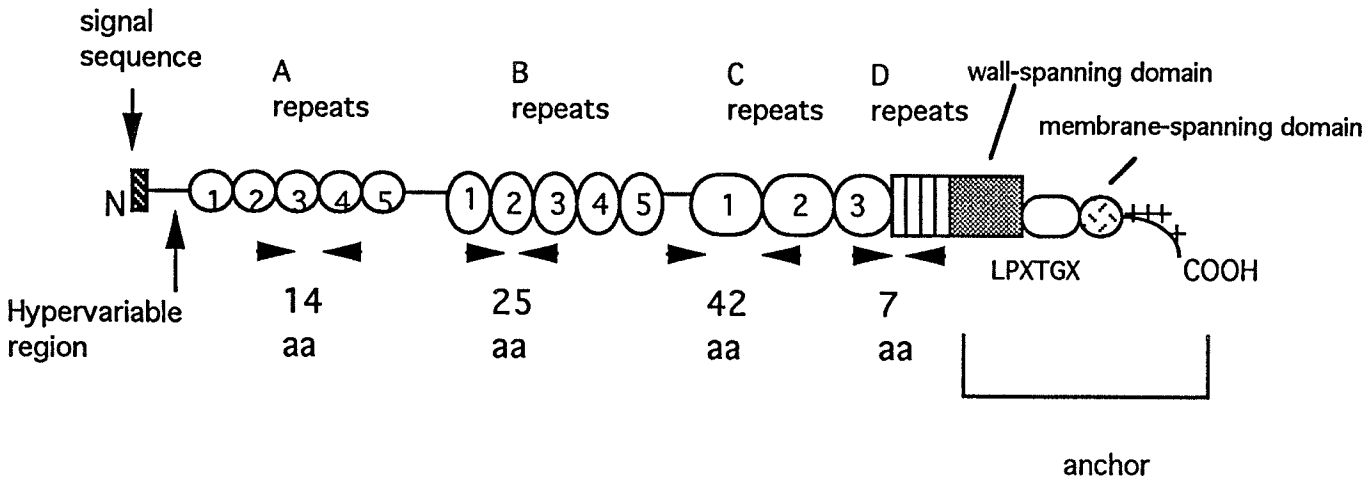

Figure 1.1. Molecular structure of the *S. pyogenes* M protein.

replication or through intergenic recombination events leading to the addition or deletion of copies (12, 23, 37, 79, 130). Recombination events can provide a means of creating further sequence diversity and are thought to contribute to the antigenic variation observed among the extensive number of serologically-defined M proteins (74). Recombination is also believed to be involved in the evolution of the *emm*-like gene family observed among *Streptococcus* species (12, 23). This gene family has been defined on the basis of homology to the *S. pyogenes emm* gene and also by chromosomal location downstream of the regulator designated *virR* (or *mry*) (128, 129). The appearance of these *emm*-like genes may be the result of intergenic recombination or gene duplication events. There are other examples of surface proteins that are encoded by two different genes, such as, the fibronectin binding proteins of *Streptococcus dysgalactia* and *S. aureus*, (2, 76, 92). In *S. aureus*, the two tandem gene copies are also believed to have risen from gene duplication events

Structure of Streptococcus mutans surface protein P1 and P1-like proteins

S. mutans possess a major surface protein (M_r 185,000 - 205,000) with several of the characteristic structural motifs described for M protein. This protein was first described by Russell and Lehner (140) as a trypsin-sensitive polypeptide cleavable into two distinct antigens, designated initially as surface antigen (SA) I/II (140) and later as P1 (42). Electron micrographs of *S. mutans,* immuno-gold labelled for P1 protein, show peritrichous fibrils surrounding the cell (55). This is analogous to the "fuzzy coat" description of the *S. pyogenes* M protein (37, 153). However, there are strains of *S. mutans*, referred to as non-retainers, in which intact antigen P1 is found predominantly in the culture supernatant,

consequently, these strains lack the layer of fibrils surrounding the cell surface (3). The reason for the liberation of antigen P1 from the cell surface of non-retainer strains is unknown.

Genetic organization of the spaP gene

The gene encoding the *S. mutans* serotype c P1 adhesin, designated *spaP*, was cloned and sequenced from strain NG5 by Lee *et al.* 1988 and the analogous gene has also been cloned and sequenced from *S. cricetus* and *S. downeii* (116, 120, 121). A number of the common features first identified in the *emm* gene for the *S. pyogenes* M protein are also present in the *spaP* gene (reviewed in 8). The entire *spaP* gene is encoded by 4,782 base pairs (bp) and the deduced amino acid structure identified an amino-terminal leader sequence of 38 residues, which is removed during post-translational processing of the protein (80). Tandem repetitive sequences also occur within the *spaP* gene (see Fig. 1.2). The amino terminal third of the protein is comprised of an alanine-rich region (A-domain) made up of three 82 residue tandem repeats (80). The A-domain contains a high proportion of alanine residues arranged in a similar heptad periodicity, as would be predicted for alpha-helical coiled-coil proteins (8). This structure is commonly found within surface proteins of the oral streptococci and is similar to those described for *S. pyogenes* M protein and the PspA protein of *S. pneumoniae* (8). Within the central portion of the molecule is a cluster of 144 residues designated as the variable domain (V-domain), followed by a proline-rich region (P-domain) comprised of three 39 residue tandem repeats. The carboxy terminus of the P1 protein shares homology with several surface proteins including M protein and is characterized by a hydrophilic wall-spanning domain and an LPXTGX motif, consisting of

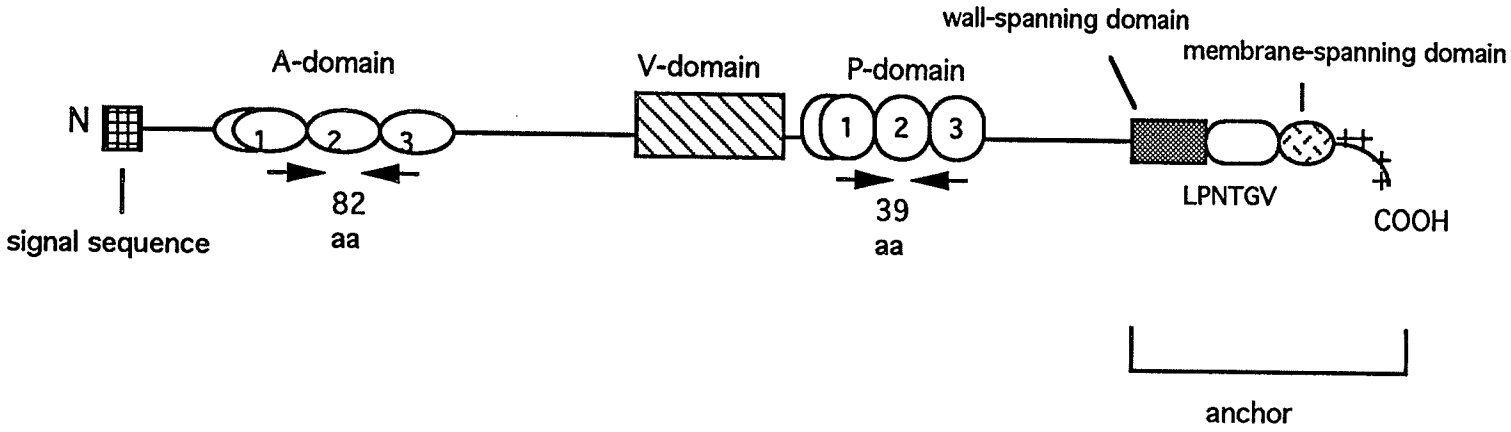

Figure 1.2. Molecular structure of the *S. mutans* P1 protein.

LPNTGV (80). This is followed by the 20 hydrophobic amino acid membrane-spanning domain and a charged tail consisting of KAKKD (80). The significance of the C-terminal protein domains in protein localization and anchoring has been discussed in detail beginning on page 59.

Conservation of the gene encoding antigen P1 and P1-like proteins in oral streptococci

P1-like surface proteins have been identified for a number of the oral streptococci suggesting that a family of streptococcal surface proteins exists (96, 29). Southern hybridization studies using *spaP* DNA sequences identified highly homologous regions in *S. mutans* serotypes c, and e, *S. downeii* (serotype h), *S. sobrinus* (serotypes d/g), *S. cricetus* (serotype a) and non-mutans alpha-haemolytic streptococci, suggesting that some regions of *spaP* are highly conserved (85, 87, 96). The SpaA protein of *S. sobrinus* (serotype g) strain 6715 was 66 % homologous and the *S gordonii* SspB protein 56% homologous to *S. mutans* P1 based on deduced amino acid sequences (85). In addition to the homology found in these P1-like proteins, many of the amino acid substitutions were shown to be conserved (85). The N-terminal signal sequences were identified, but varied in length from 38 residues for P1, SspA, SspB, to 50 residues in SpaA (80, 85). The alanine repetitive or A-domain defined for P1 was also evident in SspB, SspA and SpaA. The proline-rich repeats (P-domain) were also present, but consisted of only two 39 residue repeats in SpaA as opposed to the three repeats observed in other surface proteins (85). However, there was some strain to strain variation observed in *S. sobrinus* for the number of tandem repeats. This phenomenon has also been reported among *S. pyogenes* M-types and streptococcal immunolglobulin-binding proteins (12). Most of the

substitutions in the primary amino acid sequence among P1-like proteins were in the variable region (V-domain) rather than scattered throughout the protein (8). This substitution is reminiscent of the hyper-variable domain in the extreme N-terminus of M protein (Fig. 1.1), which defines each *S. pyogenes* M-type (37). The carboxy terminus is the most highly conserved sequence amongst all the streptococcal P1-like proteins (29). The four characteristic domains can be readily identifed and include the wall-spanning domain, the LPXTGX motif, the membrane-spanning domain and the charged tail (29).

Antigenic relationships among the P1-like proteins

In addition to the structural relationship observed for P1-like proteins, there is also an immunological relationship (8). This antigenic relationship was demonstrated when monospecific antisera (6) or monoclonal antibodies (MAbs) (3, 14) were used to investigate surface proteins from *S. mutans* serotypes c and e, *S. downeii*, *S. sobrinus* and S. *cricetus*. Western immunoblot and whole cell ELISA (enzyme-linked immunosorbant assay) analysis with a panel of anti-P1 monoclonal antibodies against a number of streptococci identified overlapping, but not identical, subsets of cross-reactive MAbs with *S. sobrinus* and *S. gordonii* (14).

There have been previous reports of cross-reactive antibodies recognizing both streptococcal surface proteins and human myocardial tissues (25, 162). The alpha-helical, coiled-coil structure of some streptococcal proteins resembles that of myosin and these α-helical proteins were, therefore, suspected to be responsible for antigenic cross-reactions. However, further investigation using monoclonal antibodies raised against

P1 has not confirmed this cross-reactivity (3, 6). Original heart tissue preparations were believed to be contaminated with IgG and the suspected cross-reactivity may have been due to IgG epitopes shared with P1 (8). The P1 domain responsible for the IgG cross reactivity was identified by Moisset *et al.* (107) and since this P1 region is immunogenic, it could lead to the synthesis in humans of natural anti-IgG antibodies.

Mapping of the P1 ligand binding domains

The mapping of the aggregation- and the adhesion-binding domains for P1 protein has led to discrepancies in the literature due to differences in experimental approach, making comparison of the results difficult. As a consequence, a brief summary of these experiments is included and the putative binding domains are represented in Fig. 1.3. Initial experiments by Brady *et al.* (15) tested the ability of a number of strains of mutans streptococci to adhere to salivary agglutinin-coated HA and to aggregate in the presence of fluid phase agglutinin. The results demonstrated that organisms which expressed an immunologically indistinguishable P1-like protein, including *S. mutans* serotype c and e, *S. cricetus* and *S. downeii* were able to adhere. On the other hand, *S. rattus* (serotype b) strains were non-adherent and did not react with anti-P1 monoclonal antibodies, and there is no direct evidence for the expression of a P1-related protein on the surface of *S. rattus*. The non-adherent *S. mutans* strains included the non-retainer strains that do not retain P1 on their cell surface and a P1-truncated mutant 834, expressing only the N-terminal portion of P1. Of the four *S. sobrinus* strains reacting with anti-SpaA antisera, only two were adherent. The reason for the difference in adhesion among these four strains is unknown. Aggregation of mutans streptococci by salivary

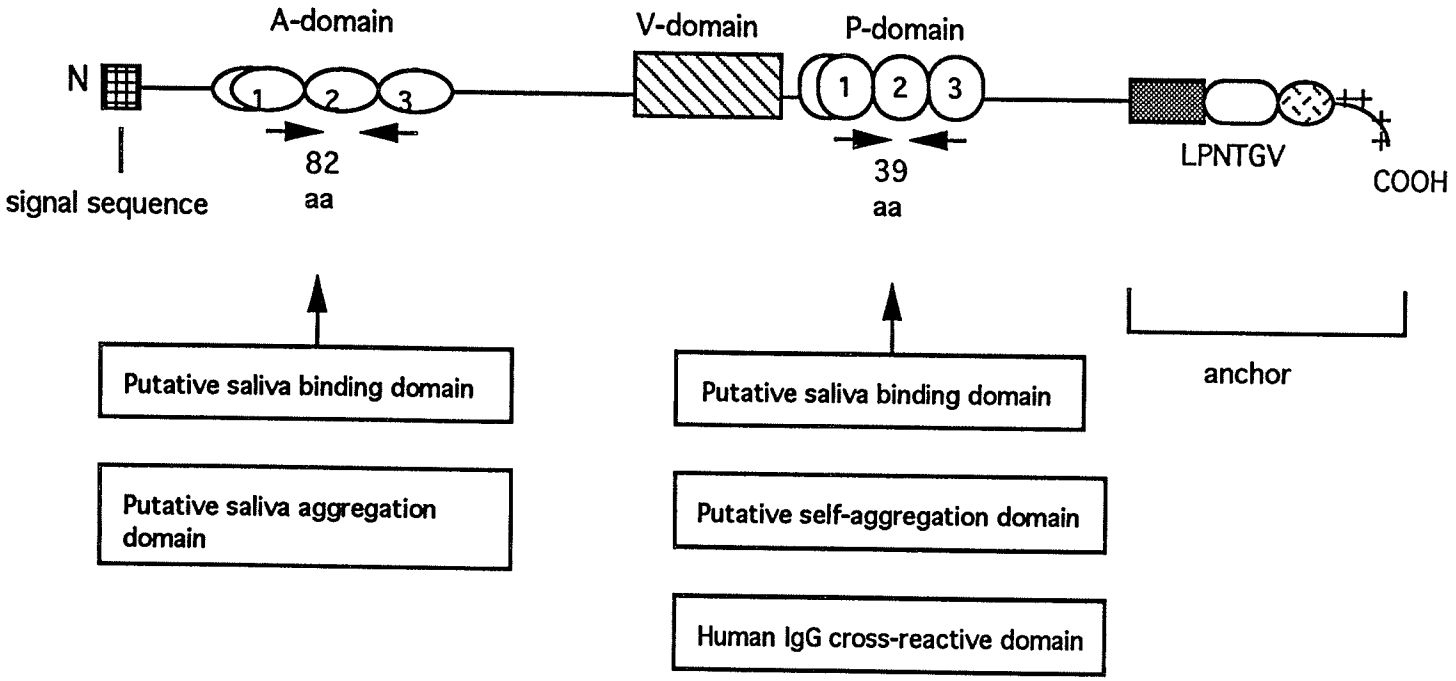

Figure 1.3. Molecular structure of the *S. mutans* P1 protein: Putative binding domains.

agglutinin also correlated with the expression of P1 and P1-like surface molecules. However, except for the *S. mutans* P1 non-retainer strains, all mutans strains (adherent and non-adherent to HA) aggregated in the presence of salivary agglutinin. Inhibition studies with monoclonal antibodies determined that some, but not all, of the monoclonal antibodies could interfere with *S. mutans* adherence to salivary agglutinin immobilized on HA and a different subset of monoclonal antibodies could inhibit aggregation in the presence of fluid-phase agglutinin.

These results indicate that some specificity is involved with the P1-salivary agglutinin interactions and that different P1 domains may be involved in adhesion versus cell aggregation. It is also interesting that the monoclonal antibodies could not be mapped to discrete regions of the linear P1 molecule suggesting that some epitopes may be dependent on tertiary structure or modification of the protein (15). This may also be an indication of the possible existence of cryptitopes within the P1 molecule. The results of Brady *et al.* (15) suggest that the N-terminal A-domain of P1 protein (Fig. 1.2) may be involved in agglutinin-mediated aggregation of streptococci. The functional domain involved in adherence to agglutinin-coated HA is believed to map to a location central to the P1 molecule, possibly closer to the C-terminus. This observation was based on the behaviour of the P1 mutant 834, which expresses a truncated P1 molecule including the A-domain (amino acids (aa) 1-612). This mutant was impaired in adherence to agglutinin-coated HA, but was still capable of agglutinin-mediated aggregation. Further experiments by Crowely *et al.* (24) demonstrated the ability of the A-domain to interact directly with salivary agglutinin in Western blot assays by a calcium-dependent interaction. The A-region, expressed as a maltose binding fusion protein, inhibited both adherence to

agglutinin-coated-HA and aggregation in the presence of fluid phase agglutinin in a concentration-dependant manner. Soluble recombinant A-domain polypeptide was also able to inhibit adherence to salivary agglutinin. However, based on the adherence and aggregation data obtained from the P1 mutant 834, other P1 domains not expressed in 834 may be involved in adherence with salivary agglutinin.

Nakai *et al.* (113) used recombinant truncated P1 (or PAc) polypeptides generated by PCR technology to determine their binding interaction with whole saliva. The results of their sandwich assay with biotinylated P1 fragments suggested that amino acid residues 39 to 864, corresponding to the A-domain of the P1 molecule from *S. mutans* serotype c, played an important role in binding to salivary components, while shorter polypeptides within the same stretch of amino acids exhibited no binding activity. An even larger peptide, including amino acid residues 39-1000, exhibited enhanced binding activity suggesting the possibility of additional binding sites. However, these differences may be due to tertiary structural differences since larger polypeptides may allow correct folding of the protein and, therefore, confer the correct conformation for binding. In addition, these polypeptides were expressed as β-galactosidase fusions, which may influence the folding of the protein and the resultant three-dimensional conformation. Fragments corresponding to the P-domain (Fig. 1.2) were able to bind directly to the P1 molecule suggesting that the P-domain of P1 contributes to self-aggregation of P1 protein. Whole saliva also inhibited the self-binding of these fragments to P1 indicating the P-domain may also possess a domain for saliva binding.

Munro *et al.* (111) used an independently-generated panel of anti-P1 monoclonal antibodies and polypeptides to determine the binding specificity

of P1 to saliva-coated HA. Previous *in vivo* studies in non-human primates had demonstrated that direct application of two monoclonal antibodies (Guy's 1 and 13) conferred prolonged protection against re-colonization by *S. mutans* (95). It was, therefore, possible that either of the monoclonal antibodies may block adhesion of P1 by recognizing epitopes within the binding domain. The recombinant polypeptides were assayed for adhesion to saliva-coated HA and for recognition by the panel of monoclonal antibodies. Both Guy's 1 and 13 monoclonal antibodies recognized a recombinant polypeptide representing residues 816 to 1213 corresponding to the P-domain of P1 (111). The three tandem repeats of proline residues are believed to form a regular structure forming a functional ligand binding site. However, monoclonal antibody Guy's 1 recognized an epitope within an additional polypeptide encompassing residues 475 to 824. Unlike the results obtained previously by Crowley *et al.* (24), the polypeptide fragment corresponding to the A-domain (residues 39 to 481) did not inhibit adhesion. The authors suggested this difference may have been due to masked binding sites or sites destroyed by proteolysis (111). A second possibility is that two distinct binding sites, within the A- and P- domains, were involved in adhesion and aggregation.

A recent study by Moisset *et al.* (107) using recombinant P1 polypeptides expressed in *E. coli* and synthetic peptides determined that the salivary glycoprotein-binding domain in *S. downeii* P1 (or SR) surface protein. Their results indicated that salivary glycoprotein exhibited concentration-dependent binding to full-length P1 and to the N-terminal 613 amino acid residues suggesting the existence of a specific binding site within the A-domain of the molecule. However, there was no binding to a synthetic 17 amino acid peptide representing the A-domain, presumably

because the peptide represented a structure too short to permit binding. When assayed by competition ELISA, the same peptide was able to block binding of salivary glycoprotein to full-length P1. These results suggest that the A-domain of the P1 molecule is important in binding to salivary glycoprotein, confirming previous results reported by Brady *et al.* (15) and Crowley *et al.* (24). In this same study, the human immunoglobulin G (hIgG) cross-reacting domain of P1 was identified with antisera generated against synthetic peptides and anti-hIgG antisera. ELISA assay results, with various antisera established the hIgG cross-reactive domain was localized to a C-terminal region of the P1 molecule defined by residues 948 to 1028. DNA hybridization with PCR generated probes corresponding to residues 973 to 1087 (within the P-domain) hybridized with all mutans streptococci, except *S. rattus* and 11 non-mutans streptococcal strains, indicating conservation of the 3' termini of the *spaP* gene confirming previous work by Ma *et al.* (96). The results obtained by Moisset *et al.* (107) confirmed the presence of a predicted IgG-cross reacting domain in P1. In addition, the presence of anti-P1 antibodies in sera from rheumatic disease patients was observed and the authors suggested that immunization with P1 may enhance the generation of anti-hIgG antibodies leading to autoimmune disease.

Collectively, these studies suggest that P1 probably possesses multiple adhesive functions leading to various biological interactions. The inability of the monoclonal antibodies to map to discrete regions of the linear P1 molecule has led to the suggestion that some epitopes may be exposed only when an appropriate protein conformation is generated (8, 47). More information concerning the tertiary structure of P1 and P1-like

protein molecules may help to define more clearly the important protein domains involved in adhesion and aggregation.

The Structure of Gram-positive Cell Walls

The site for anchoring surface protein A in *S. aureus* has been identified as the peptidoglycan. If a similar anchoring mechanism operates in oral streptococci, including *S. mutans*, it is pertinent to consider the detailed structure of the peptidoglycan of Gram-positive bacteria since the bacterial cell wall provides the support and anchoring site for surface proteins. The structure, function, biosynthesis and assembly of the Gram-positive cell wall has been extensively reviewed (136, 137, 142a, 147). Generally, the cell wall of Gram-positive organisms is an amorphous layer of approximately 20-50 nm in thickness, immediately adjacent to the cytoplasmic membrane, 40% of which is comprised of peptidoglycan. In addition to peptidoglycan, the cell wall contains one or more accessory polymers, including teichoic acid (TA), teichuronic acid (TUA), or polysaccharide. These polymers are linked to the cell wall either directly or indirectly by phosophodiester bonds to the C6 of N-acetyl muramic acid within the glycan backbone. Generally, the cell wall is described as being composed of insoluble peptidoglycan and covalently-linked polymers and does not include non-covalent linked structures. Lipoteichoic acid (LTA) is linked to the cytoplasmic membrane, but can also appear secreted in both an acylated and deacylated form, leaving behind the fatty acid component in the membrane. Almost half of the *S. mutans* cell wall is comprised of rhamnose-containing group polysaccharides which may also contain glucose (142b, 148, 168b). The

neutral polysaccharides contain rhamnose and galactose in *S. rattus*, and rhamnose, galactose and glucose in *S. sobrinus* (142b, 162b). *S. rattus* strains also contain a glycerophosphate-containing polymer (glyceroteichoic acid) in their walls (162b). The rhamnose polysaccharide of *S. rattus* is associated with the wall teichoic acid, possibly through a covalent linkage (148).

The function of the cell wall is to protect the cell from internal and external changes in osmotic pressure and to maintain the shape of the organism (147). It also functions as a molecular sieve by prohibiting large macromolecules from reaching the membrane. Other molecules, such as DNA and proteins, have to be able to penetrate the cell wall and enter the cell membrane. The negative charge associated with the cell wall attracts divalent cations facilitating their transport into the cell. Divalent cations may also function in adherence to substrates (including host cells) and in aggregation reactions (147).

The Gram-positive peptidoglycan

The chemical structure of peptidoglycan is consistent within a given organism and has been useful as a taxonomic tool in the classification of bacteria (reviewed in 142a). Peptidoglycan is a heteropolymer made up of glycan strands cross-linked through short peptides. The glycan strand consists of alternating β–1,4- linked N-acetyl glucosamine and N-acetyl muramic acid residues (137, 142a). Most of the variation in the glycan chain occurs from acetylation or phosphorylation of the muramyl 6-hydroxyl groups. The stem peptide or peptide subunit is linked to the carboxyl group of the N-acetyl muramic acid and typically contains alternating amino acids of both L- and D- configurations. This peptide moiety is usually comprised of L-ala - D-glu - X - D-ala, where X is an L-

diamino acid (Fig. 1.4). Other variations exist, including substitution at the glutamic acid α-carboxyl group or the addition of D-alanine at the terminus of the stem peptide subunit. The greatest variation is seen at position three, which may include L-lysine and less frequently L-ornithine or meso-diaminopimelic acid, as well as several other diamino acids. The stem peptides are then cross-linked from the amino group of the L-diamino acid to the D-alanine at position 4 of an adjacent stem peptide. Some of the D-alanine residues are not cross-linked, due to either D-ala carboxypeptidase activity or through substitution by another D-ala. Stem peptides consisting of three, four and five amino acids have been identified. N-acetyl muramic acid, N-acetyl glucosamine, the stem peptide and the peptide cross-bridge comprise the basic repetitive structure of peptidoglycan, termed the disaccharide-peptide structural repeating unit (DSP) (147) (see Fig. 1.4).

The cross-linking between stem peptides can be direct or via an interpeptide cross-bridge with the latter demonstrating the greatest variation in chemical composition and mode of cross-linking among peptidoglycans (137, 142a). The mode of cross-linking has been divided into two groups, A and B with group A cross-linking extending from the amino group of a diamino acid at position 3 to the carboxyl group of D-alanine at position 4 of the adjacent stem peptide. Group B peptidoglycans are rare and the cross-linking extends from the carboxyl group of the D-glutamic acid at position 2 to the terminal D-alanine at position 4. Group B peptidoglycans always contain an L-diamino acid in the interpeptide bridge to link the two carboxyl groups. In addition to the classification of peptidoglycan based on the linkage position, peptidoglycans can be subdivided based on the composition of the cross-bridge. There are four

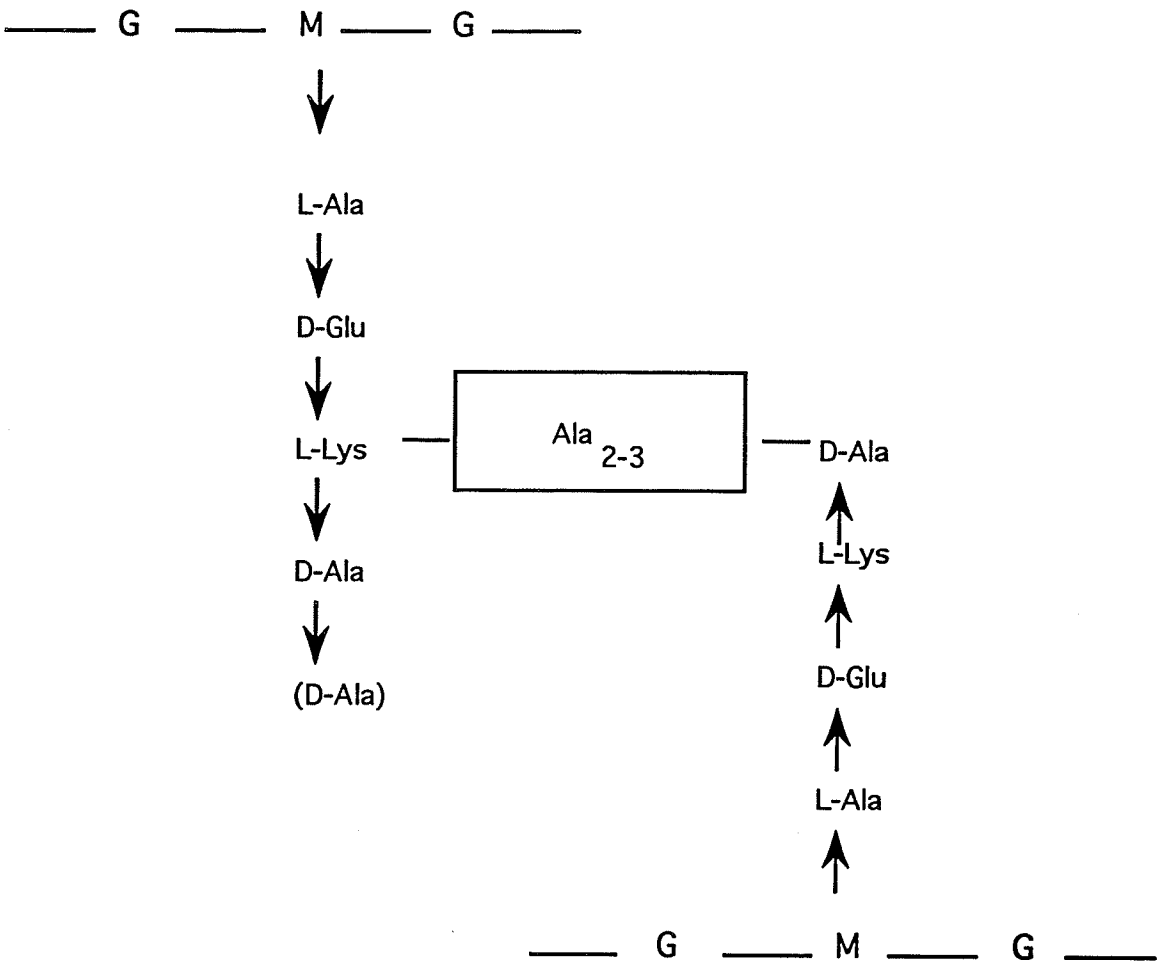

Figure 1.4. Primary repeating structure of the *S. mutans* peptidoglycan.
Abbreviations: G, N-acetyl glucosamine, M, N-acetyl muramic acid.

subdivisions for the group A peptidoglycans designated numerically as follows: (1) represents a direct cross-link with no interpeptide bridge, (2) is a cross-link comprised of polymerized peptide subunits (stem peptide), (3) is a cross-linkage via interpeptide bridges consisting of monocarboxylic L-amino acids or glycine or both, and (4) where interpeptide cross bridges contain a dicarboxylic amino acid (142a). The amino acid in position 3 of the stem peptide for Group A peptidoglycans is designated either α for L-Lysine; β for L-ornithine and γ for m-diaminopimelic acid (Dpm). Hence, the designation A3α indicates a peptidoglycan that is cross-linked via an interpeptide cross-bridge of monocarboxylic L-amino acids (and/or glycine) from the position three L-lysine to the position four amino acid of the adjacent stem peptide.

Analysis of streptococcal peptidoglycans has revealed 12 different types, the majority of which belong to the A3α type (142a). There are also examples of streptococcal peptidoglycans of the A4α type with dicarboxylic amino acids found in the cross-bridge and A1α with direct cross-links. There is a large group of streptococci having cross-bridges consisting of one to four L-alanine residues belonging to Lancefield groups A, C, D, E, F, G, H, K, L, M, P, and U (142a). One of the alanine residues can be replaced by L-Ser or Gly in fewer species, which include *S. agalactiae* (group B). The addition of L-Thr to the interpeptide bridge occurs via the ε-amino group of L-Lysine with Gly, L-Ala or L-Ala/L-Ser in the complete inter-peptide bridge (142a). Other variations include interpeptide bridges containing D-Asp found in *S. faecium*, *Streptococcus* sp. (group Q), *S. lactis* and *S. cremoris* (group N) and direct cross-linking, which occurs in the *S. viridans* I, II and IV groups and in the streptococcal groups K, O and R (142a). The peptidoglycan cross-bridge of *S. cricetus* and *S. sobrinus*

peptidoglycan consists of L-Lys-L-Thr-L-Ala (142b). *S. mutans* peptidoglycan is of the A3α type and the cross-bridge consist of L-Lys- Ala $_{2-3}$ (Fig. 1.4)(142b).

Peptidoglycan assembly

The precise mechanism for assembly of peptidoglycan (PG) is unknown, although the chain elongation model, for which indirect evidence exists, has been proposed (136). The biosynthesis of peptidoglycan precursors occurs in the cytoplasm where UDP-N-acetyl muramyl-pentapeptide is transferred to a lipid carrier, undecaprenylpyrophosphate, at the cytoplasmic membrane. The N-acetyl glucosamine and the peptide cross-bridges are added, forming the basic repeating structural unit (DSP). Polymerization of the repeating units occurs and the growing polymer is elongated proximally by a headward elongation mechanism (136, 137, 147). The precise location of polymerization is unknown, but is thought to occur on or near the cytoplasmic face of the membrane (147). A putative polymerase/export system, comprised of a transmembrane protein complex, forms a channel in the membrane through which the growing glycan chain would be extruded. An intermediate in peptidoglycan assembly has been identified consisting of uncross-linked glycan chains of 12 DSP units linked to a lipid carrier (112). This length of chain would be sufficient to span and feed through the membrane connected to events inside and outside the membrane. The DSP12 intermediate is believed to be incorporated into the longer glycan chains of the external wall by a transpeptidation reaction.

The insertion of new glycan chains into pre-existing PG is thought to occur via transpeptidation reactions where the terminal D-alanine, from either the new or pre-existing units is lost and a peptide bond is formed

either directly or through a cross-bridge peptide (136, 137, 147). Newly synthesized PG can also be incorporated by the transglycosylation reaction resulting in the formation of a β-glycosidic linkage between the disaccharide unit and the glycan chain of the pre-existing peptidoglycan (147). The polymerization of uncross-linked PG by transglycoslyation occurs prior to the formation of peptide bonds via the transpeptidation reaction (137). There is evidence to suggest that the cross-linking appears to be a random process (147).

There are few data concerning the precise location on peptidoglycan chains where non-PG polymers are attached. How ancillary polymers, such as teichoic acids, polysaccharides and proteins, are inserted into the cell wall is unknown (136). Munson and Glaser postulate that assembly of ancillary polymers occurs extracellularly from intermediates (112). However, the lack of information on cell wall assembly remains a fundamental problem in supporting any current model (136).

Localization and Anchoring Signals for Gram-positive Cell Surface Proteins

Surface protein localization signals (reviewed in 79) were first described for M protein (64, 38, 39), and similar signal mechanisms have been extensively studied for the *S. aureus* protein A (160, 144). Generally, surface proteins are synthesized initially as precursors with N-terminal signal sequences that direct translocation of the protein across the cytoplasmic membrane (79). Once translocation has occurred, a unique mechanism is believed to be involved in the localization of the protein within the Gram-positive cell wall. This mechanism, which has been postulated

for *S. aureus* only, involves a putative enzymatic activity which covalently links the protein to the peptidoglycan (114).

Significantly, a large number of Gram-positive surface proteins including the *S. mutans* P1 possess common features within the C-terminus (38, 39, 145) (Fig. 1.5). The frequent observation of a common amino acid sequence suggests that a selective pressure exists for the evolutionary conservation of these C-terminal structures (38). The highly conserved C-terminal structures of numerous cell-surface proteins are characterized by distinct domains and will collectively be referred to as the 'cell anchor' (37, 64). Distal to the extreme C-terminus is a hydrophilic domain, characterized by a high proportion of polar amino acids including proline, serine or threonine, in addition to glycine. This hydrophilic domain is referred to as the wall-associated or wall-spanning domain, and in the case of M protein and *S. aureus* protein A (123, 144), is believed to span the peptidoglycan layer of the cell wall (Fig. 1.5 i). The number of amino acid residues comprising the wall-spanning domain varies from 15 to more than 84 (38, 39). Adjacent to the C-terminus of the wall-spanning domain is a hexapeptide signature sequence for this group of surface proteins, defined by (LPXTGX) (Fig. 1.5 ii). This sequence functions in anchoring the protein to either the cell wall or the membrane, and also provides important recognition signals in the process of final localization of the protein on the cell surface (38). C-terminal to the hexapeptide consensus sequence is a membrane-spanning domain (Fig. 1.5 iii), comprised of approximately 15-22 hydrophobic amino acids (39). These hydrophobic amino acids form an α-helical structure and allow insertion of the protein into the cytoplasmic membrane. The membrane-spanning domain is followed by 5-12 charged residues comprising the final protein

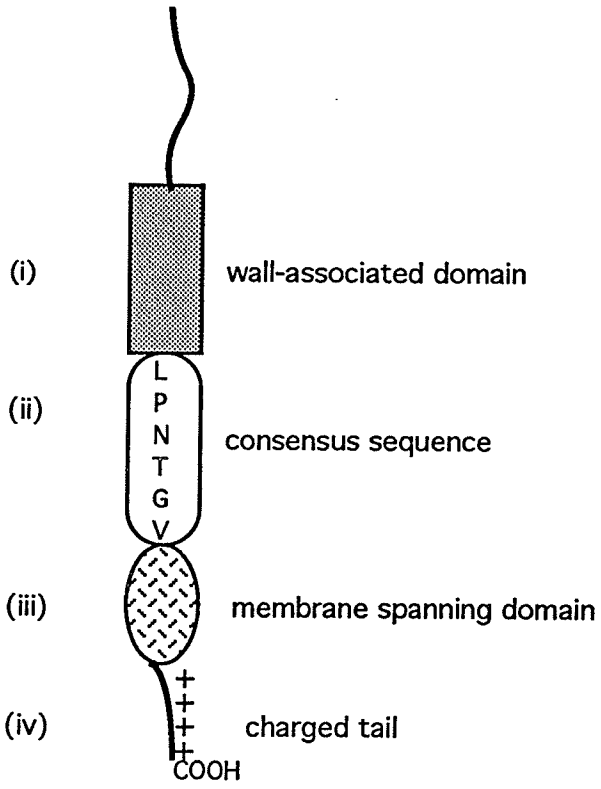

Figure 1.5. The carboxy-terminus of the P1 protein molecule.

domain, the charged tail (Fig. 1.5 iv) located at the extreme C-terminus, and is believed to function as a stop-transfer signal during the translocation of the protein through the membrane (37). The hexapeptide consensus sequence (Fig. 1.5 ii) may represent a signal for a thiol-dependent membrane anchor-cleaving enzyme (MACE), which post-translationally cleaves the C-terminus of the protein and subsequently attaches the protein to a putative membrane anchor (122).

Previously in the literature the membrane-spanning domain and charged tail have been referred to as the membrane-anchor, implying that the protein is anchored to the cytoplasmic membrane (64, 79). However, this is not the case for *S. aureus* protein A, which appears to be covalently cross-linked to the cell wall peptidoglycan (143, 151). The precise subcellular location of the protein anchors for the majority of Gram-positive cell-surface proteins has not been determined. The sub-cellular localization of the *S. mutans* P1 protein is addressed in the current study.

The role of the individual cell anchor domains has been analyzed in *S. aureus* through mutagenesis of protein A (144). These studies confirmed, through the use of deletion protein A mutants and alkaline phosphatase-protein A fusions, the requirement for the LPXTGX motif, the membrane-spanning domain and the charged tail for the correct sorting of the protein into the cell wall (144). Deletion of the charged tail and successive deletion of the cell anchor domains (including the membrane-spanning domain and the LPXTGX motif) caused secretion of the protein into the extracellular environment. The deletion of the LPXTGX motif alone resulted in miss-sorting of the phosphatase-protein A fusion, suggesting an important role of this motif in the localization of proteins to their final subcellular site (144). Further analysis by site-directed

mutagenesis of the charged tail indicated that the terminal leucine residue could be deleted with no effect on protein sorting, while deletion of the penultimate glutamic acid caused secretion of the protein. Similarly, interchanging the terminal leucine residue for cysteine also resulted in secretion rather than anchoring and retention of the protein (144). Mutagenesis of the proline residue to an asparagine residue within the LPXTGX motif impaired cell wall anchoring, while mutation of the threonine to an alanine residue had no effect. The deletion analysis data suggest that all three of the domains, the LPXTGX motif, the membrane-spanning domain and the charged tail, are essential for the sorting and anchoring of protein A to the *S. aureus* cell wall. It was hypothesized, that the membrane-spanning hydrophobic domain and the charged tail may be involved only transiently during the wall-association pathway and that an undescribed pathway is responsible for anchoring proteins to the cell wall (144).

The role of the protein A-charged tail was analyzed by a serine scanning mutagenesis experiment where serine replaced each residue in the charged tail (145). The results indicated that two positively-charged arginine residues, found 31-33 residues downstream from the leucine of the LPXTGX motif, were essential for the anchoring of protein A (145). To demonstrate the universality of the protein localization mechanism for Gram-positive proteins with this characteristic C-terminal anchor, several fusion proteins were constructed in which hybrid proteins consisted of the N-terminus of protein A and the C-terminus of related proteins from different bacteria with similar C-terminal domains. These proteins included those of *S. aureus* fibronectin binding protein (FNBP) (76, 149), *S. sobrinus* surface protein antigen A (SPAA) (158), *E. faecalis* phermone

response gene B (PRGB) (77), *S. pyogenes* M proteins (EMM) (64), *S. mutans* wall-associated protein (WAP) (35), *A. viscosus* fimbrial subunit protein (FIM) (172), *L. monocytogenes* internalinzation protein (INLA) (45), *S. agalactiae* B streptococci, immunoglobulin A receptor, *c* protein (BAC) (61) and *S. aureus* collagen adhesin (CNA) (126). To demonstrate the importance of the 31-33 residue spacing between the (L)PXTGX and the first positively-charged amino acid of the charged tail, non-anchoring hybrid constructs were made functional by adjusting this spacing to 31-33 residues. Some of these hybrid proteins were localized to the cell wall and others to the cytoplasmic membrane, but in either case, the proteins were resistant to extraction by hot sodium dodecyl sulfate (SDS) (145). The ability of these hybrid proteins to localize to the cell wall or cytoplasmic membrane indicated that the mechanism for protein localization is universal and highly conserved among different Gram-positive bacteria.

The studies of protein A led to the following working hypothesis for surface protein anchoring (114); the N-terminal signal sequence of the protein designates it for transport to a subcellular location. The charged tail provides a stop transfer signal and the membrane-spanning domain prevents secretion of the protein. Retention allows for post-translational proteolytic cleavage near the C-terminus, presumably within the LPXTGX motif, which is followed by linkage to the peptidoglycan of the cell wall. This post-translational modification is presumed to occur via an unidentified enzymatic activity similar to the transpeptidation reaction operating during cell-wall synthesis (137).

To test this hypothesis further, Navarre and Schneewind (114) constructed chimeric proteins in which the LPXTGX motif, the membrane-spanning domain and the charged tail had been inserted

between the N-terminal sequence of the enterotoxin B molecule of *S. aureus* and either a maltose-binding protein or the β-lactamase enzyme, to construct the C-terminus (114). The N-terminal signal sequence of enterotoxin B molecule designates the protein for export from the cytoplasm. Over time, the chimeric protein was cleaved by a putative enzyme activity into two products, that were recognized by specific antisera. Cell fractionation and protease protection assays indicated that the N-terminal portion of the chimeric protein (enterotoxin) was translocated and located in the cell wall fraction, while the C-terminal portion (β-lactamase) was not translocated and remained in the cytoplasm. The association of the N-terminus with the cell wall was determined by using muralytic enzymes indicating that the N-terminus was linked to the cell wall. To determine the specific protein sorting-cleavage site, the C-terminal portion of the chimeric protein was subjected to sequence analysis. This demonstrated that the N-terminal amino acid was the glycine residue of the LPXTGX motif indicating that cleavage occurred between the threonine and the glycine residues. The authors proposed a model for cell wall linkage involving the recognition of the cell wall-sorting signal consisting of the LPXTGX motif, the membrane-spanning domain and the charged tail by a specific mechanism involving a putative enzyme(s) 'sortase'. This mechanism positions the membrane-spanning domain and charged tail through the membrane and stops the transfer of the protein to prevent secretion. The sortase recognizes the LPXTGX motif and cleaves the bond between the threonine and the glycine residues of the surface protein. The newly-exposed carboxyl-group of the threonine is then covalently linked to the amino-group of the N-terminal pentaglycine cross-bridge of the peptidoglycan (114).

The most recent study by Schneewind *et al.* (143) uses both molecular biology and mass spectrometry techniques to identify the amine bond between the carboxyl threonine residue of protein A and the free amino group of the pentaglycine cross-bridge. The authors propose a similar mechanism of attachment for other Gram-positive surface proteins where the free amino group of the peptidoglycan cross-bridge serves as the site of attachment. The free amino group of the uncross-linked peptide cross-bridge remains a constant feature among the wide variations observed for the structure of peptidoglycan (142a). The enzymatic activity catalysing the linkage of the protein to the peptidoglycan, referred to as 'sortase', has not been identified. Whether a similar mechanism operates in the attachment of other Gram-positive surface proteins to peptidoglycan has yet to be demonstrated but is possible on the basis of structural similarities.

P1 protein localization and anchoring signals

The carboxy terminus of P1 shows a striking similarity to the C-terminus of M protein and these similarities are now known to be present among over 60 Gram-positive bacterial surface proteins (114). A putative cell wall-spanning domain can be identified consisting of approximately 50 polar residues particularly rich in proline (80). Within the wall-spanning domain is the hexapeptide signature sequence common to Gram-positive surface proteins, which in the case of P1, consists of LPNTGV (80). The hexapeptide consensus sequence observed in *S. sobrinus* SpaA consists of LPATGD (85), and in *S. gordonii* SspA and SspB, LPKTGT (29). The *S. gordonii* proteins CshA and CshB exhibit typical C-terminal domains, including an LPRTGS motif, but are unique in possessing four internal LPXTGX motifs consisting of LPQTGT (104). The P1 membrane-spanning

domain can be identified immediately adjacent to the hexapeptide and consists of 20 hydrophobic residues. The charged tail is comprised of amino acids KAKKD (80). The spacing between the signature sequence (L)PNTGV and the first charged amino acid of the tail is, therefore, 30 amino acids, which differs from *S. aureus* protein A by only one residue (145). The subcellular location of the P1 protein anchor, either in the cytoplasm, cytoplasmic membrane or the cell wall, has not been determined. Also, the role of each of the C-terminal domains in P1 protein localization and anchoring has not been assessed. Given the similarity of the P1 C-terminus to those of other Gram-positive surface proteins, it would not be surprising for the individual domains to function by providing recognition signals similar to those of the putative cell wall attachment pathway proposed for protein A in *S. aureus*. However, whether the *S. mutans* P1 protein cell surface attachment is analogous to that for protein A has yet to be determined.

P1 protein release from the cell surface

A unique enzyme activity, designated surface-protein releasing enzyme (SPRE) that is responsible for the release of P1 has been identified in *S. mutans* (86). This enzyme activity is similar to the 'MACE' enzyme described in the release of *S. pyogenes* M protein from protoplasted cells (122). The role of SPRE in pathogenesis is unclear but it may be involved in modulation of the cell surface and the antigenicity of *S. mutans* (86).

The Implication of Surface Proteins in Vaccine Development

Apart from a need to gain a better understanding of surface proteins and their assembly to explain their role in adhesion, antigenic variation, molecular mimicry, and virulence, another significant aspect of these proteins is their potential as vehicles for the expression of protective vaccine epitopes. As we have already seen, common structural features of surface proteins allow the anchoring component of one protein to serve the same function in an unrelated species. Therefore, *S. mutans* P1 and P1-like proteins themselves could serve not only as antigens in vaccines, but also as a means of expressing immunogenic epitopes. Chimeric proteins expressed on cells of the normal flora might be convenient, permanent 'in-dwelling' sources of antigen used to stimulate the host immune system.

S. mutans P1 as a protective antigen

Based on the adherence studies with P1 negative mutants (6), P1 does play a role in adhesion and colonization, and therefore anchoring of the P1 molecule to the bacterial cell surface would be imperative to this function. Efforts to generate an anti-caries vaccine based on inhibition of P1 adhesion demonstrated that application of P1-specific monoclonal antibodies to tooth surfaces prevented colonization by *S. mutans* and the development of caries in non-human primates (90). Monoclonal antibodies that prevented colonization of *S. mutans* in humans, recognized epitopes within amino acid residues 816-1213, which overlap the central P1 proline-rich repeat region (Fig. 1.2)(95, 111). There is at least one additional site for saliva interaction within residues 186-469 (24), of the A-repeat domain since antibodies directed against this domain supressed colonization by *S.*

mutans of murine teeth (156). Kelly *et al.* (81) suggested that given these observations, protection against dental caries may be conferred by the induction of serum antibodies recognizing the receptor-binding regions of P1.

The human immune response to surface protein P1 was characterized with respect to the B-cell, T-cell and the adhesion epitopes (81, 89). Epitope scanning with synthetic peptides indicated that the immuno-dominant B-cell epitope was within the proline-rich domain and adjacent to the T-cell epitope. The adhesion epitope mapped to polypeptide 816-1213 and overlaped both the B- and T-cell epitopes. Bacterial adhesion to mucosal and tooth surfaces might be prevented through the generation of an appropriate antibody response using a P1 subunit vaccine comprised of synthetic peptides, including the B- and T-cell epitopes, coupled to adhesion epitopes. This is one approach being developed for the generation of an effective subunit vaccine against caries.

In addition to problems of poor expression and localization, recombinant antigens can also differ from the native structure, rendering them non-immunogenic. The immunogenicity of a recombinant antigen can be enhanced by coupling it to the cholera toxin A2 subunit. Cholera toxin A2/B behaves as an adjuvant by boosting humoral responses, particularly those given by mouth, which would otherwise induce oral tolerance. Protective salivary immunoglobulin responses to the *S. mutans* P1 protein have been achieved by coupling it to the cholera toxin B subunit resulting, in reduced colonization and caries in a rodent model (78). Also, a vaccine using the *S. mutans* P1 salivary binding region expressed as a recombinant genetic fusion with the cholera toxin (CT) A2/B subunit produced a long-lasting immune response in a rodent caries model (54, 62).

From a practical stand point, the ability to anchor proteins to the bacterial surface has implications for the generation of live oral recombinant vaccines for the prevention and control of disease. The expression of foreign antigens on the surface of bacteria as a means of delivering antigens for the induction of an immune response has received considerable recent attention. The important advantage in the use of live microorganisms as vaccine vectors is their ability to express target immunogens of the natural infection and to do so in a way that promotes antigen-processing and presentation that is most similar to the natural infection (105). This stimulates both systemic and, more importantly, mucosal immune responses, since most infectious diseases initiate at the mucosal tissues. Some organisms naturally target lymphoid tissues of the mucosal epithelium and multiply within these inductive sites initiating local mucosal immune responses. For example, *Salmonella typhi* gains entry to follicular lymphoid tissues of the gut and generates strong humoral responses (105). Because *Salmonella* is an intracellular parasite, it also generates cell-mediated immune responses and is, therefore, considered an ideal vaccine vector. There are a number of concerns with respect to toxic immune responses (or reactogenicity) since the immune response to the vector is usually dominant and could limit the effectiveness of subsequent immunizations of the host with the same microbial vector. For example, cross-reactive immune responses from subsequent *Mycobacterium bovis* BCG immunizations are toxic to the host, limiting its usefulness as a carrier or adjuvant (105). The possibility of generating inappropriate autoimmune reactions would also render such a recombinant vaccine unsuitable for human use.

Other potential problems also exist for antigen expression since the level of antigen can be critical to the generation of protective immunity. This was demonstrated experimentally when mice where orally immunized with two different live-attenuated *Salmonella* strains expressing the C-fragment of the tetanus toxin on recombiant plasmids (33). The presence of the *lacI* gene on one recombinant plasmid resulted in a 100-fold decrease in the level of antigen expression *in vitro*. Immunity in mice was induced by vaccination with a recombinant plasmid which did not encode the *lacI* gene. The authors suggested that the low antigen expression and the intracellular location of the antigen in *Salmonella* resulted in an antigen dose insufficient to induce immunity necessitating subsequent booster doses. However, the level of antigen expression determined *in vitro* may not correspond to its *in vivo* expression.

Vaccine stability is also an important issue since the precise antigen dose is unknown and because changes can occur in a live vaccine, such as, the aquisition of virulence genes encoded by transposable elements. It is important to recover the live vector from immunized animals and determine whether the antigen expression levels are consistent with those before immunization. However, live oral vaccines provide the advantage of surface localization of antigen for the induction of mucosal responses as opposed to direct adminstration or encapsulation of the antigen, which often results in antigen denaturation in the acid environment of the stomach. Live oral vaccines also have the potential to immunize against several diseases simultaneously through genetic engineering of antigen expression. The exploitation of the C-terminal anchor of Gram-positive surface proteins may be advantageous for the expression of heterologous antigens in commensal organisms.

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Chapter 2

Rationale and Approach

Introduction

Gram-positive bacteria express a number of proteins on their cell surface implicated in specific adhesion events (6). The consequences of adhesion may be several, but primarily adherence allows the organism to establish itself in a particular environment. Adhesion to mammalian extracellular matrix is thought to promote bacterial colonization and invasion at subepithelial tissues (2,10). In addition, specific attachment is considered an early critical phase in the establishment of infection and, therefore, is an important research focus (10). Depending on the circumstances governing colonization, infection or disease can ensue (2).

Streptococcus mutans has been implicated in the aetiology of dental caries (5). The surface protein P1 of *S. mutans* is involved in adhesion to the salivary agglutinin of the aquired pellicle covering the surface of the teeth and contributes to the tissue tropism identified for this organism (1). Deletion of the *spaP* gene encoding P1 by isogenic recombination results in a mutant impaired in the ability to adhere to saliva-coated hydroxyapatite (4), although it is not known whether this mutant is less pathogenic. Thus, because of the involvement of P1 in adhesion and tissue tropism, one can postulate a central role for this surface protein in the pathogenic potential of *S. mutans* in dental caries.

Relevance of surface-associated proteins

Currently, two approaches for the control of infectious disease involves surface proteins. The first is to develop anti-adhesion analogs to block initial adhesion events and, thereby, prevent infection. This situation is also applicable to prevention of infection occurring with implanted medical devices. With respect to the P1 adhesin of *S. mutans*, generation of

adhesion-blocking antibodies could also reduce the incidence of colonization on tooth surfaces, thereby reducing the occurence of tooth caries. Alternatively, researchers are eager to develop recombinant-live oral vaccines. Commensal organisms would be used to express foreign antigens on their surface in order to elicit the appropriate immune response. Microbial cell-surface proteins may play two important roles in this strategy. Firstly, surface proteins involved in attachment may constitute suitable antigens (or epitopes) to which antibodies are generated thus preventing colonization by the targeted organism. Secondly, surface proteins may provide the means of expressing specific vaccine antigens by the construction of protein fusions able to direct these antigens to the cell surface. The expression of heterologous antigens on the surface of commensal organisms has been recently reported in the literature (3,7,8), thus, mechanisms of surface protein localization and attachment to the cell surface are of significant interest.

The recent escalation of antibiotic resistance among Gram-positive organisms has re-focused the attention of researchers on the development of antimicrobial agents and a thorough understanding of the mechanism by which surface proteins are attached to the cell surface may suggest new targets for the development of antimicrobial agents. Since Gram-positive surface proteins share common structural elements within that portion of the protein responsible for attachment to the cell wall, and attachment of these proteins is believed to proceed by a common mechanism, it may be possible to develop antimicrobial agents which interfere with this assembly process. For example, similar to the glycopeptide antibiotics, which inhibit the transpeptidation reaction in peptidoglycan synthesis and assembly, inhibition of surface protein attachment might lead to perturbations in the

cell-wall integrity rendering the organism more susceptible to standard antimicrobial agents or promoting functional abnormalities. This strategy for the development of new antimicrobial agents was recently proposed by Schneewind *et al.* (9) after identifying the location of the *S. aureus* protein A within the glycine cross-bridge of the peptidoglycan. The authors suggest that the enzyme responsible for the cell wall linkage of surface proteins may represent a novel target for antibacterial therapy. Therapeutics directed towards protein localization would have the advantage of affecting the majority of cell surface proteins and would not require prior knowledge of protein function. In contrast, an immunolgical approach requires the systematic identification and analysis of individual virulence factors and protective antigens.

Rationale for this study

This study is concerned with the *S. mutans* P1 protein that shares the same structural attributes, including the anchoring motif, of what is now known to be a large class of Gram-positive surface proteins. With the exception of *S. aureus* protein A, there is limited information concerning the attachment of these surface proteins to the cell surface, as well as the precise subcellular location of the anchor. The differences between P1 retainer and non-retainer strains have not been studied and these may provide a basis for determining the mechanism for protein attachment. To begin to address these questions and differences, the following objectives were selected:

1) To confirm the universality of the surface-protein anchoring mechanism amongst Gram-positive cocci.

2) To determine the role of the wall-associated domain in retention of the P1 protein on the cell surface.

3) To determine the location of the P1 anchor within the cell wall of *S. mutans*.

4) To determine if differences in peptidoglycan structure or composition account for the observation that some *S. mutans* strains do not retain P1 protein on their cell surface.

Experimental approach

The universality of the surface protein attachment mechanism was demonstrated by introducing the gene encoding the P1 protein, *spaP*, into related Gram-positive organisms. In addition, the anchor structure from the *S. aureus* fibronectin-binding protein was used to localize chimeric proteins in related streptococci. Expression of recombinant proteins was detected with P1-specific antisera and/or monoclonal antibodies, and polyclonal anti-fibronectin-binding protein antisera.

Like many other Gram-positive surface proteins, P1 contains several common structural attributes, including the anchoring motif involved in attachment of the protein to the bacterial cell surface. The anchor consists of several domains, each of which is suspected to serve a function in the attachment of the protein. To assess the involvement of the wall-spanning domain, mutants were constructed by using the polymerase chain reaction (PCR) to create mutations with precise *spaP* truncations. These truncation mutants were constructed on a plasmid carrying the *spaP* gene and were

introduced into a P1-negative mutant. The cellular association of the truncated recombinant P1 protein was assessed by measuring the recombinant protein on the surface of whole cells and in spent culture supernatant with P1 specific antisera and/or monoclonal antibodies.

To determine the location of the P1 anchor, antibodies directed to the anchor were generated. This was accomplished by constructing a fusion protein expressing the anchor domain as a fusion with the maltose-binding protein of *E. coli*. This fusion protein was purified and used as the antigen to immunize rabbits. *S. mutans* NG8 and the deletion mutants were fractionated and the cell walls and membranes isolated. The presence of P1 within the cellular fractions was determined by Western blotting with the anchor-specific antisera.

To begin to assess why some *S. mutans* strains do not retain P1 on their cell surface, the cell wall of a non-retainer strain (NG5) was isolated and compared with that of the retainer strain, NG8 by Western immunoblotting. These two cell-wall preparations were also analyzed for amino acid and amino sugar content to determine whether differences in their cell wall structure or composition existed.

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Chapter 3

*Role of the C-Terminus in Antigen P1 Surface Localization in Streptococcus mutans and Two Related Cocci**

* See Journal of Bacteriology, 1996, volume 178, pages 801-807.

Abstract

The C-terminus of the major surface protein P1 from *Streptococcus mutans* is comprised of a hydrophilic domain, a LPNTGV motif, a hydrophobic domain, and a charged tail. These features are shared by surface proteins from many Gram-positive coccal bacteria. To investigate the role of the C-terminal domains in antigen P1 surface-localization, full length and truncated P1 gene constructs, expressed on the shuttle vector pDL276, were transformed into the P1-negative mutant *S. mutans* SM3352, *Streptococcus gordonii* DL-1, and *Enterococcus faecalis* UV202. Transformants were tested for the expression of P1 by ELISA and Western blotting. The results showed that full length P1 was expressed by transformants of all three bacteria and was localized on the cell surface. A fusion protein composed of the *Staphylococcus aureus* fibronectin-binding protein C-terminus and the P1 protein N-terminus was also found surface-localized in *S. mutans*. Deletion of the entire C-terminal domains resulted in P1 detection in the culture supernatant. A P1 truncated mutant, carrying only the hydrophilic domain at its C-terminus was found partially associated with the cell surface. This truncated P1 was readily removed from the isolated cell wall by hot SDS-mercaptoethanol extraction. In contrast, the full-length P1 remained associated with the isolated cell wall after similar treatment, suggesting covalent linkages between the full length P1 and the cell wall. The above results showed that antigen P1 was anchored to the cell wall by its C-terminal domains, probably via covalent linkages with the cell wall. The results also support a universal mechanism involving the C-terminal domains for protein surface-localization among this group of Gram-positive bacteria.

Introduction

More than 30 surface proteins from 12 different Gram-positive bacteria exhibit a common structural organization within the carboxy (C)-terminus (36, 37). This C-terminus includes a hydrophilic (wall-spanning) domain, a consensus LPXTGX motif, a hydrophobic (membrane-spanning) domain, and a charged tail (10, 11, 36). The contribution of the C-terminus in surface-expression of proteins has been studied by various workers. Hanski *et al.* (13) demonstrated the surface expression of the *Streptococcus pyogenes* fibronectin-binding protein in group A streptococcus and *Enterococcus faecalis*. Rathsam *et al.* (32) expressed the *Streptococcus salivarius* fructosyltransferase on the surface of *Streptococcus gordonii* cells. In addition to these studies, others have demonstrated the ability of Gram-positive bacteria to express chimeric proteins on the surface of heterologous Gram-positive organisms (14, 30). Chimeric fusion proteins utilizing the C-terminus of the *S. pyogenes* M protein (30) and the *Staphylococcus aureus* protein A (14) have been used to localize protein antigens on the surface of *S. gordonii* and *Staphylococcus xylosus*.

The roles of several of the C-terminal domains in protein surface-localization have been analyzed for the *Staphylococcus aureus* protein A (27, 35, 36, 37). These studies have demonstrated important functions for the charged tail and the LPXTGX consensus sequence in the anchoring of protein A to the cell surface of *S. aureus*. Schneewind *et al.* (35) suggested that the anchoring of *S. aureus* protein A requires the cleavage of the Thr-Gly amide bond within the LPXTGX and the subsequent linkage of the

protein to the cell wall through an amide bond formed between the C-terminal carboxyl of Thr and the amino group of the pentaglycine crossbridge.

The major surface protein P1 (ca. 185 kDa) of *Streptococcus mutans*, an etiological agent in human dental caries (23), also displays the common features at the C-terminus (4, 15). Protein P1 has been suggested to behave as an adhesin by promoting the attachment of the organism to a salivary glycoprotein, a constituent of salivary pellicle coating the tooth surface (3, 22). Protein P1 is associated with the cell wall fraction (33) and immuno-electron microscopic studies have revealed P1 as fibrillar structures on the cell surface (1, 22). The present study provides data to show that the C-terminal domain of P1 is required for the surface expression of the protein in *S. mutans, S. gordonii,* and *E. faecalis*. The results support the notion of a universal mechanism for protein surface-localization in Gram-positive coccal bacteria (11, 36, 37). The present study also addresses the possible role of the hydrophilic wall-spanning domain in anchoring the *S. mutans* P1 protein.

Materials and Methods

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 3.1. The streptococci and *E. faecalis* were grown aerobically in Todd-Hewitt broth (Becton Dickinson, Cockeysville, MD) at 37°C without agitation. *Escherichia coli* cells were cultivated in Luria Broth (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, wt/vol) at 37°C with agitation. Antibiotics were purchased from

TABLE 3.1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant markers	Bacterial host	Source or reference
BACTERIA			
E.coli XL1-Blue	Rec$^-$, Tet^r		Stratagene
S.mutans NG8	Wild type		A.S. Bleiweis (1)
S. mutans SM3352	Tet^r, *spaP*$^-$		A.S. Bleiweis (7)
S. gordonii DL-1			D. LeBlanc(18)
E. faecalis UV202	Rec$^-$		D. LeBlanc(41)
PLASMIDS [a]			
pBluescript	2.9 kb, Amp^r,	*E. coli*	Stratagene
pDL276 (pUC19, pVA380-1)	6.9 kb, Kan^r	*E.coli*, streptococci	D. LeBlanc (9)
pSMI/II (pUC18)	8.9 kb, Amp^r	*E. coli*	A.S. Bleiweis (15)
pSMI/II-3 (pDL276)	14.0 kb, Kan^r	*E.coli*, streptococci	S.F. Lee (20)
pSMΔ1490 (pDL276)	13.5 kb, Kan^r	*E.coli*, streptococci	This study
pSMΔ706 (pDL276)	11.1 kb, Kan^r	*E.coli*, streptococci	This study
pP1/FnBP (pDL276)	12.6 kb, Kan^r	*E. coli*, streptococci	This study
pSMΔ1508 (pDL276)	13.5 kb, Kan^r	*E.coli*, streptococci	This study
pSMΔ1464 (pDL276)	13.4 kb, Kan^r	*E.coli*, streptococci	This study

[a] Vectors used to construct the plasmids are shown in parenthesis.

Sigma Chemical Co. (St. Louis, Mo.) and used at the following concentrations:- ampicillin at 50 μg/ml for *E. coli*; kanamycin at 50 μg/ml for *E. coli*, 500 μg/ml for *S. mutans* and *E. faecalis*, and 250 μg/ml for *S. gordonii*; and tetracycline at 15 μg/ml and 20 μg/ml for *E. coli* XL-1 Blue and *S. mutans*.

The full length *spaP* gene cloned previously from *S. mutans* NG5 and carried on the pUC18 plasmid, was designated pSMI/II (15, 21). Plasmid pSMI/II-3 was generated by cloning the *spaP* gene from pSMI/II into the *E. coli*-streptococci shuttle vector pDL276 (Fig. 3.1A, [20]). The truncated *spaP* genes were generated as described below (Fig. 3.1B). The *S. aureus* fibronectin-binding protein (FnBP) gene, *fnb*A was kindly provided by M.J. McGavin, University of Manitoba (25).

Antisera. The rabbit polyclonal antiserum to the *S. mutans* antigen P1 were generated previously (19) while a polyclonal antibody against the *S. aureus*FnBP was produced in New Zealand white rabbits. Briefly, the FnBP (ca. 200 kDa) from the culture supernatant of an overnight culture of *S. aureus* ATCC 6538 was concentrated by ammonium sulphate precipitation and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The FnBP was isolated from the gels by excising unstained areas of the gels (19). The gel slices were minced and used to immunize two rabbits as previously described (19). These antisera were provided by Dr. S.F. Lee.

DNA isolation and Analysis. The following methods for DNA manipulations and analysis were used throughout the study except where

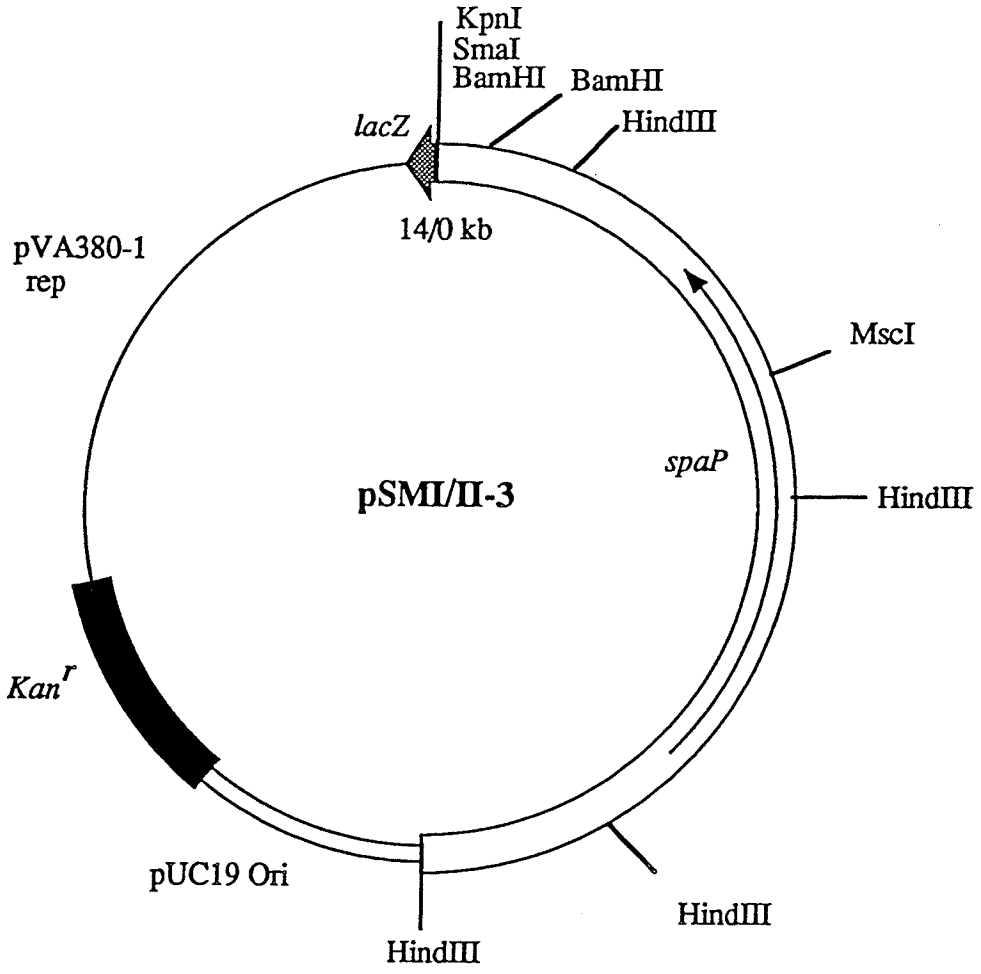

Figure 3.1.A. Map of plasmid pSMI/II-3.

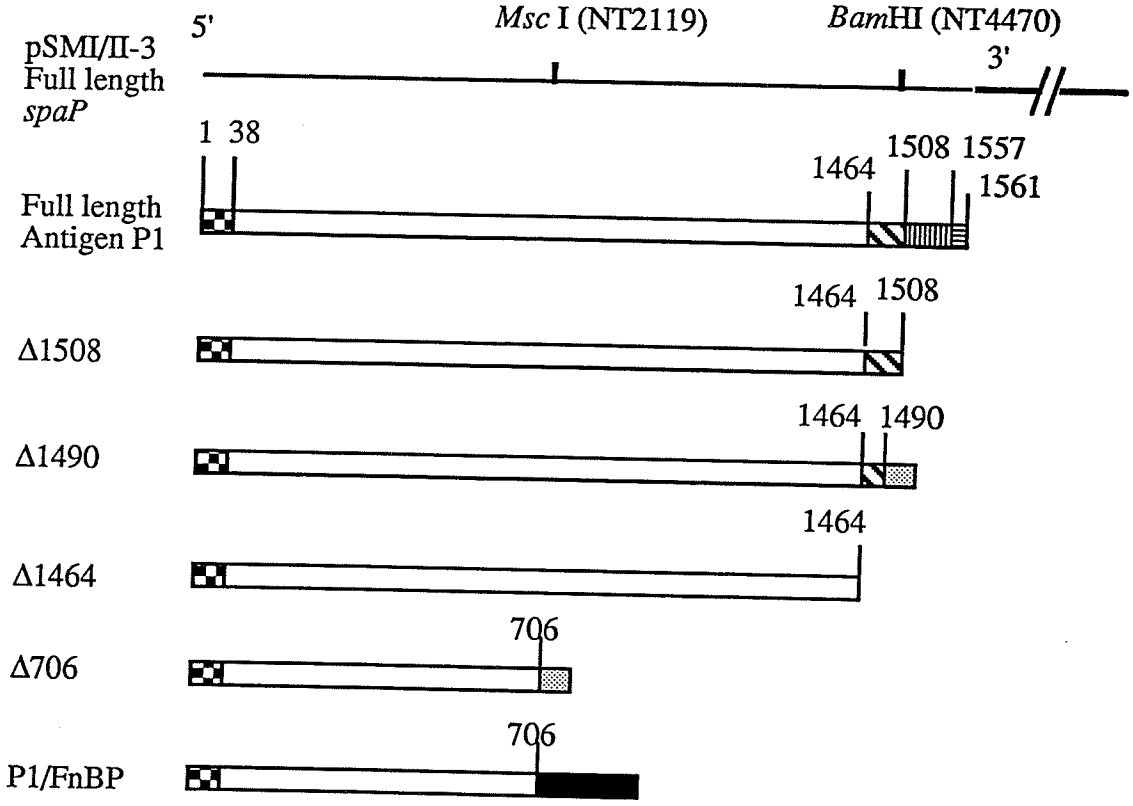

Figure 3.1B. Diagramatic representation of the full length protein P1, the various deletions at the C-terminus of P1, and the P1/FnBP fusion protein. The nucleotide number of the two relevant restriction sites are indicated in parenthesis. Other numbers above antigen P1 and truncated P1 are amino acid residue number from the start codon. Symbols : —, spaP DNA; ⫽, pDL276 DNA; ▣, leader sequence; ▨, hydrophilic wall-spanning domain; ▥, hydrophobic domain; ▤, charged tail; ■, the C-terminus of *S. aureus* FnBP; and ▩, potential LacZ fragment.

specified. Plasmid DNA was isolated from *E. coli* by the procedure of Birnboim and Doly (2) and purified by CsCl density gradient centrifugation (34). Restriction endonucleases and DNA-modifying enzymes were used according to the manufacturers instructions. All enzymes were obtained from GIBCO/BRL Life Technologies, Inc. (Burlington, ON) or New England Biolabs (Mississauga, ON), except where otherwise noted. The DNA restriction fragments were separated on horizontal 0.8% agarose (wt/vol) slab gels in Tris-acetate buffer, pH 8.6 (40 mM Tris acetate, 1 mM EDTA) at 15 V per cm of gel.

Recombinant DNA Methodology. The truncated *spaP* (pSMΔ1490 and pSMΔ706) were generated by restriction of pSMI/II-3 with *Bam*HI (for pSMΔ1490) or *Msc*I and *Sma*I (for pSMΔ706). The resulting DNA fragments were separated by agarose gel electrophoresis as described above. The DNA fragments, 13.5 kb (kilobase) and 11.1 kb representing the truncated plasmids pSMΔ1490 and pSMΔ706, respectively, were purified from the agarose gel with a Gene-Clean kit (Bio101, LaJolla, Ca.) according to the instructions by the manufacturer. The plasmids were re-circularized by ligation with T4 DNA ligase at either 4°C or 23°C for 18 h. Competent *E. coli* XL-1 Blue cells were prepared by the method of Kushner (16). Competent cells were transformed with ligated DNA as described by Sambrook *et al.* (34). *E. coli* cells harboring the plasmid were selected on LB agar plates containing appropriate antibiotics. The deletion plasmid was verified by restriction endonuclease digestion and agarose gel electrophoresis.

Transformation of Gram-positive organisms. Transformation of *S. mutans*, *S. gordonii,* and *E. faecalis* was accomplished by the method of Perry

and Kuramitsu (29), as modified by Murchison *et al.* (26). Cells were cultured in Todd-Hewitt broth, supplemented with 5% horse serum (THBS) for two consecutive days by inoculating 5 ml of THBS with 100 µl of cells. For transformation, the culture was diluted 1/40 in THBS and incubated at 37°C without aeration. The optical density at 600 nm was monitored until it reached 0.100 - 0.150. A 1 ml aliquot of cells was removed to a prewarmed, sterile glass test-tube in a 37°C water bath and ca. 25 µg of plasmid DNA in 50 µl of 15 mM sodium citrate and 150 mM NaCl was added. The cells were incubated for 30 min at 37°C and then diluted by the addition of 1 ml prewarmed THBS. The cultures were incubated for an additional 1 h and then plated directly on Todd-Hewitt agar containing the appropriate antibiotics. Plates were incubated at 37°C in an anaerobic chamber under 80% N_2, 10% CO_2, and 10% H_2. Transformants were obtained after 48 h of incubation.

Polymerase chain reaction. CsCl gradient-purified pSMI/II was used as the template for polymerase chain reaction (PCR) with oligonucleotide primers P1A (5'-*cgg ggt acc* gcc ctt cta tca gta gct tc-3'), P1B (5'-*gcc ggt acc* ***tca*** agt tga agt ccg cgg tga -3'), and P1C (5'-*cgg ggt acc* ***tca*** acc aac cgc aat acg ttt cat ttg-3'). The underlined nucleotides represent the addition of a *Kpn*I restriction site and the nucleotides in bold type represent the addition of a stop codon. The italicized nucleotides represent non-template encoded 5' additions to the PCR primers required for efficient cleavage of the oligonucleotide by *Kpn*I. The primers, P1A and P1B, amplify nucleotides (NT) 2068-4524 and primers P1A and P1C, amplify (NT) 2068-4392 of the *spaP* sequence (GenBank accession number X17390 [15]). Thus, the primers P1B and P1C were

designed to generate truncation at NT residues # 4524 (amino acid residue #1508) and # 4392 (amino acid # 1464) of the *spaP*, respectively.

PCR was carried out by using *Taq* DNA polymerase. The reaction included: 1 ng template DNA, 2 mM of each dNTPs, 1.5 mM of each primer, 1.5 mM $MgCl_2$, and 2.5 units *Taq* polymerase in 100 µl volumes overlaid with 100 µl of light mineral oil. The amplification was carried out in a DNA minicycler (MJ Research Inc., Watertown, MA) and consisted of 30 cycles of denaturation (94°C, 1 min), annealing (53°C, 2 min for primers P1A and P1B; 55°C, 2 min for primers P1A and P1C), and extension (72°C, 2 min). The mineral oil overlay was removed by chloroform extraction. The PCR product was treated with proteinase K (GIBCO/BRL) (6) and buffered-phenol (GIBCO/BRL):chloroform (1:1, vol/vol) extracted. Following digestion with restriction endonuclease *Kpn*I and purification with the Gene-Clean kit, the amplified DNA was ligated into the vector pBluescript (Stratagene, LaJolla, CA). The ligated DNA was transformed into competent *E. coli* XL-1 Blue.

The 2.4 kb and 2.3 kb DNA fragments from pPCR2.3 and pPCR2.4, respectively, carrying the truncated *spaP* sequences were isolated and subcloned, by using the *Msc*I and the *Kpn*I sites, into the shuttle plasmid pSMI/II-3 digested with the same enzymes. Restriction of pSMI/II-3 with *Msc*I and *Kpn*I cut within the *spaP* sequence and outside the *spaP* sequence, the latter within the multiple cloning site of the vector. Thus, by ligating the PCR fragments into these two sites, the 3' portion of the *spaP* gene was replaced by the truncated sequences. These recombinant plasmids were designated pSMΔ1508 and pSMΔ1464.

The DNA sequences of the entire PCR-generated fragments contained in pSMΔ1508 and pSMΔ1464 were determined by using a DNA sequencing kit (Sequenase Version 2.0, Amersham Canada Ltd., Oakville, ON) by Dr. S.F.Lee. Results confirmed the published sequence of *spaP* (15).

Construction of P1 fusion with *S. aureus*FnBp. The 3' terminal 632 nucleotides of the *fnb*A was isolated by digestion of pBTFN-1 (25) with *Hinc*II and *Sca*I. The *fnb*A fragment was then blunt-end ligated into pSMI/II-3, which had been digested with *Msc*I and *Sma*I (within the multiple cloning site). The correct orientation of the ligated DNA was verified by restriction analysis. The resulting gene fusion was 2.85 kb in length encoding a protein of approximately 102,000 Daltons. This plasmid was designated pP1/FnBP.

Enzyme-Linked Immunosorbent Assay (ELISA). The ELISA method used was that of Voller *et al.* (40). Cells were grown in Todd-Hewitt broth containing the appropriate antibiotics at 37°C for 18 h without aeration. The optical density of the culture at 600 nm was determined and adjusted to 0.2 with phosphate buffered saline (PBS). The adjusted culture was harvested by centrifugation in a microcentrifuge at 16,000 x g for 10 min, the supernatant fluid recovered and the cell pellet washed in PBS and re-centrifuged. The washed cells were resuspended in PBS, followed by two-fold serial dilution in PBS and then 100 µl /well added to polystyrene microtiter plates. The whole cells were fixed to the microtiter plates by 0.25% glutaraldehyde for 1 h at room temperature (8). The glutaraldehyde solution was decanted and the plates washed 3 times in PBS. A 3% (wt/vol) solution of bovine serum albumin (BSA) in PBS was added to each well and incubated for 2 h at 37°C. Culture supernatants were also serially two-fold diluted in PBS and

dispensed in 100 µl volumes into microtiter plates. The plates were incubated for 1 h at 37°C, washed 3 times in PBS-Tween 20 before the addition of the 3% BSA solution. The plates were washed three times with PBS-Tween 20 buffer after blocking. Antibodies were diluted in PBS-Tween 20 and added to antigen-coated plates for 2 h at 37°C or overnight at 4°C. The antisera used included the rabbit anti-P1 antisera (dilution of 1/400), the rabbit anti-FnBP antisera (1/200), and the mouse ascitic fluid containing monoclonal anti-P1 antibody 4-10A (1/5000), kindly donated by A.S. Bleiweis, University of Florida, [1]). The plates were washed again 5 times in PBS-Tween 20 before being incubated with goat anti-rabbit or goat anti-mouse immunoglobulin G (IgG) alkaline phosphate conjugates (Sigma) for 1 h at 37°C. The plates were washed 5 times with PBS-Tween 20 and developed by the addition of the substrate *p*-nitrophenyl phosphate. The absorbance at 405 nm (A_{405}) was determined by a Bio-Rad microplate reader (Model 3550, Bio-Rad Laboratories Ltd. Mississauga, ON). The results (A_{405}) represented readings from the same dilution for both fractions in side-by-side assays. Thus, the absorbance readings represented proportional distributions of antigen P1 between the two fractions. The error bars indicate the standard deviations of triplicate samples.

Preparation of samples for Western immunoblotting. One ml of overnight (18 h) culture was harvested by centrifugation at 16,000 x g for 10 min. The supernatant fluid was recovered for protein precipitation in 10 % trichloroacetic acid (TCA), washed twice with cold acetone and dissolved in 100 µl of Laemmli sample buffer (17). The cell pellet was washed in PBS and resuspended in a buffer (100 µl) containing 30 % (wt/vol) raffinose, 50 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, and 2 mM phenylmethylsulfonyl fluoride.

Cells were treated with 25 kilounits/ml mutanolysin and 270 kilounits/ml lysozyme at 37°C for 90 min (38) and the resulting protoplasts were centrifuged at 16,000 x g for 10 min. The supernatant fluid, representing mutanolysin/lysozyme released proteins from intact cells, was collected. Aliquots (20 µl) of the mutanolysin/lysozyme-released proteins and TCA-precipitated proteins from culture supernatant fluids were separated by SDS-PAGE on 7.5% PAGE gels and the buffer system of Laemmli (17). Proteins were transferred to nitrocellulose membranes with a Bio-Rad Trans-blot apparatus and the transfer buffer of Towbin *et al.* (39). After transfer, the nitrocellulose was incubated for 1 h in PBS containing 3% BSA to block additional protein-binding sites. Membranes were then incubated in appropriate antisera as described for the ELISA assay. Blots were developed in 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride dissolved in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$ (GIBCO/BRL).

Immunofluorescence. One ml of overnight (18 h) culture was harvested by centrifugation and the cell pellet washed in PBS and resuspended in 1 ml of PBS. A smear of cells was prepared by spreading a 10 µl aliquot on to the surface of a glass slide and allowing it to air dry. The cells were then heat-fixed by passing the slide through a flame. A 3% solution of BSA in PBS was applied to the smear and incubated for 30 min at room temperature to block additional protein binding sites. A 20 µl droplet of monoclonal antibody 4-10A diluted 1/500 in PBS was applied to the smear, the glass slide incubated in a humid box for 30 min at 37°C and then washed in a stream of PBS. The second antibody, an anti-mouse Fab-specific FITC-conjugated antibody (Sigma, St. Louis, MO), was diluted

1/100 in PBS and a 20 µl droplet was applied for 30 min at 37°C in a humid dark box. The smear was washed in a stream of PBS and blotted dry. A drop of emulsion containing 90% glycerol and 10% PBS, supplemented with 0.1% phenylenediamine (Sigma), was applied to prevent rapid quenching of the fluorescence and a cover slip placed on top of the emulsion. The cells were viewed with a Zeiss epifluorescence microscope (495 nm excitation, 525 nm emission) and photographed with Kodak 400 Ectachrome color slide films. Black and white prints were reproduced from color prints.

Preparation of cell walls. *S. mutans* cells were grown in 1 liter cultures of semi-defined medium (5) supplemented with 0.2% (wt/vol) tryptone. The cultures were incubated for 20 h in an anaerobic chamber (80% N_2, 10% CO_2, and 10% H_2) and the cells harvested by centrifugation (10,000 x g, 4°C, 20 min) and washed 3 times in 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mM pepstatin A. Cells (ca. 1 g wet wt/ml of phosphate buffer) were put into glass Mickle vials containing glass beads (40/60 mesh) added to a depth of 1-1.5 cm (31). A few drops of amyl alcohol were added to prevent foaming. The cells were disrupted at 4°C in a Mickle tissue disintegrator (Mickle Engineering Co., Gomshall, England) until cells stained Gram negative (1-2 h) (31). Unbroken cells and glass beads were removed by centrifugation at 8,000 x g for 15 min at 4°C and the cell walls isolated from the supernatant fluids by centrifugation at 27,000 x g for 30 min at 4°C. The pelleted walls were washed 3 times in phosphate buffer with protease inhibitors and freeze dried.

Extraction of cell wall proteins. Twenty milligrams of freeze-dried cell walls were resuspended in 300 µl of 3 times concentrated SDS-mercaptoethanol buffer (20 mM Tris, pH 6.8, 0.3% glycerol, 6% SDS, and 0.15% β-mercaptoethanol) of Laemmli (17) and boiled for 20 min. The heated suspension was centrifuged at 10,000 x g for 20 min and the supernatant fluid was recovered leaving the residual cell walls in the pellet. The residual cell walls were washed three times in distilled water and resuspended in a final volume of 150 ml. A 10 µl aliquot of residual cell walls and 5 µl aliquots of untreated cell walls and the extracted proteins were analyzed by Western immunoblotting with the monoclonal antibody 4-10A to detect antigen P1.

Results

Expression of *spaP* in Gram-positive coccal bacteria. The full length P1 gene (*spaP*) carried on the shuttle plasmid pSMI/II-3 (Fig. 3.1A) was transformed into *S. mutans* SM3352 (the P1-deficient isogenic mutant of strain NG8, [7]), *S. gordonii* DL-1, and *E. faecalis* UV202. Kanamycin-resistant transformants were tested by ELISA for the expression and distribution of the recombinant P1 protein. Initial ELISA results indicated that P1 was predominantly cell-associated in the transformants (Fig. 3.2). One transformant from each of the three bacteria were selected for further studies. The three transformants *S. mutans* SMI/II-3/SM3352, *S. gordonii* SMI/II-3/DL-1, and *E. faecalis* SMI/II-3/UV202 were treated with mutanolysin/lysozyme and the released proteins subjected to Western blot analysis. Figure 3.3 shows definite but multiple immunoreactive bands indicative of antigen P1, present in the samples.

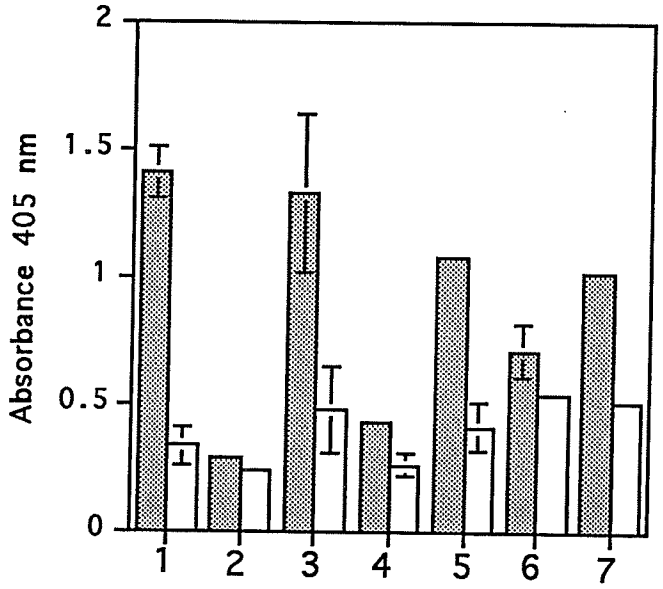

Figure 3.2. Expression of full length *spaP* in *S. mutans* SM3352, *S. gordonii* DL-1, and *E. faecalis* UV202. Antigen P1 was detected in both whole cells (▨) and culture supernatant fluids (□) by ELISA using the anti-P1 monoclonal antibody 4-10A. **Bars: 1**: *S. mutans* NG8 **2**: *S. mutans* SM3352; **3**: SMI/II-3/SM3352; **4**: *S. gordonii* DL-1; **5**: SMI/II-3/DL-1; **6**: *E. faecalis* UV202; and **7**: SMI/II-3/UV202. Absorbance readings were determined from diluted samples equivalent to culture OD600nm values of 0.05.

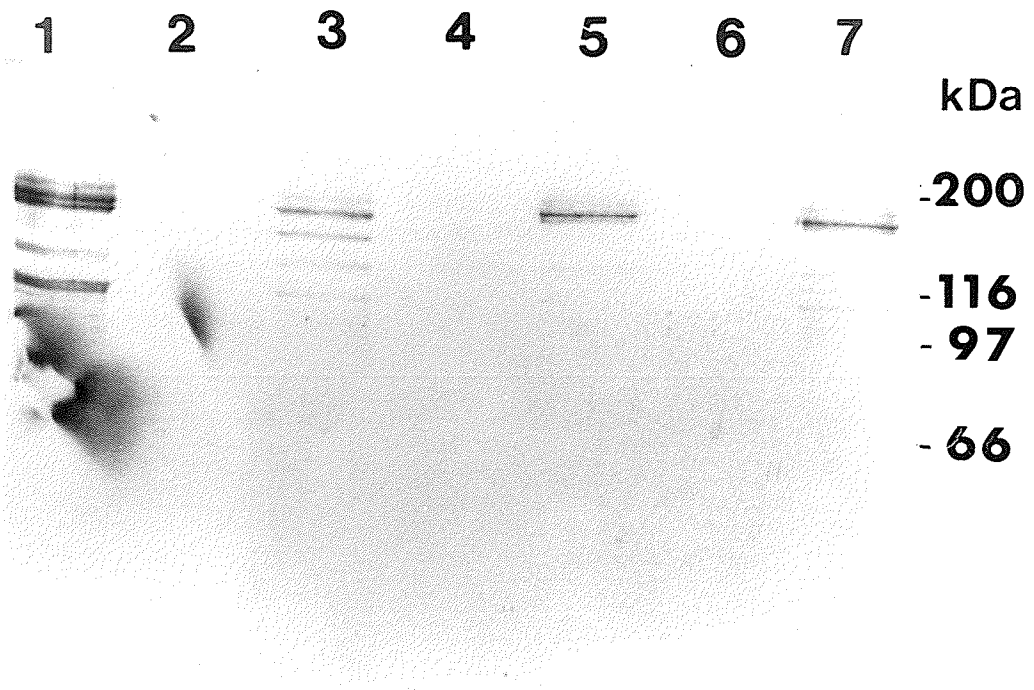

Figure 3.3. Detection of antigen P1 in recombinant *S. mutans*, *S. gordonii*, and *E. faecalis* carrying the full length *spaP* by Western blotting. Samples shown are mutanolysin/lysozyme-released material from whole cells. Antigen P1 was detected with the anti-P1 monoclonal antibody 4-10A. **Lanes: 1**: *S. mutans* NG8; **2**: *S. mutans* SM3352; **3**: SMI/II-3/SM3352; **4**: *S. gordonii* DL-1; **5**: SMI/II-3/DL-1; **6**: *E. faecalis* UV202; and **7**: SMI/II-3/UV202.

The wild-type P1 antigen from *S. mutans* NG8 also appeared as multiple bands when extracted by the same muralytic enzymes (Fig. 3.3, lane 1) with the highest molecular weight band was in the range of 185,000, the expected full size of P1 (15). These results indicate that *spaP* was expressed in the P1-deficient *S. mutans*, *S. gordonii,* and *E. faecalis.* The gene product appeared to have the expected size and be surface-localized in the transformants.

Immunofluorescence. To demonstrate further that the P1 detected in the transformants was in fact found on the cell surface, immunofluorescence studies were performed. As shown in Figure 3.4 (A, C, and E), slight to intense fluorescence was present on the cell surface of transformants *S. mutans* SMI/II-3/SM3352, *S. gordonii* SMI/II-3/DL-1, and *E. faecalis* SMI/II-3/UV202, and absent for the non-transformed bacteria (Figs. 3.4B, 3.4D, and 3.4F). These results support the surface-localization of antigen P1 in these transformants.

Expression of the P1/FnBP fusion protein. To provide further evidence in support of a common mechanism for surface localization of Gram-positive proteins, a gene fusion was created by ligating the 5' DNA from *spaP* in-frame to the 3' DNA coding for the C-terminus of the *S. aureus*FnBP (Fig. 3.1). The resulting fusion protein would therefore express the C-terminal domains from the *S. aureus*FnBP (211 amino acids) and the N-terminus of antigen P1 (706 amino acids). The fusion protein would total 917 amino acids generating a polypeptide of an estimated 102 kDa before cleavage of the signal sequence and 98 KDa after signal sequence cleavage. Using the same shuttle vector (pDL276), the construct, pP1/FnBP, was transformed into both *S. mutans* SM3352 and *S. gordonii* DL-1.

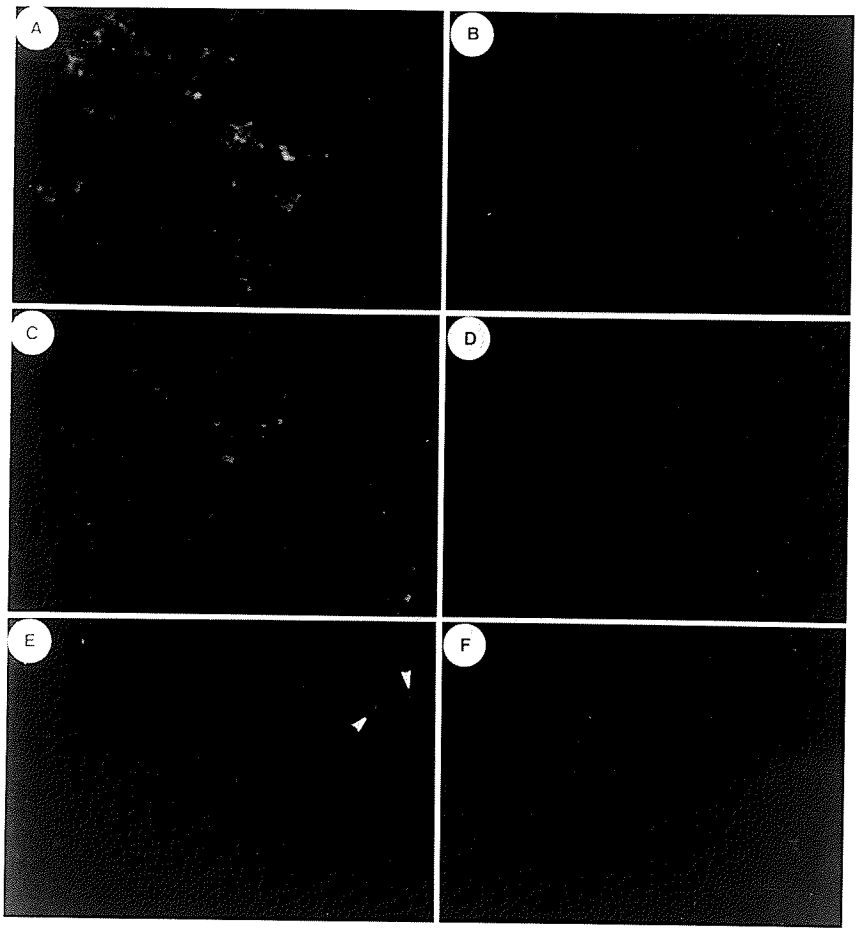

Figure 3.4. Detection of antigen P1 in *S. mutans*, *S. gordonii*, and *E. faecalis* transformants carrying the full-length *spaP* by immunofluorescence labelling. **A**: *S. mutans* SMI/II-3/SM3352; **B**: *S. mutans* SM3352; **C**: *S. gordonii* SMI/II-3/DL-1; **D**: *S. gordonii* DL-1; **E**: *E. faecalis* SMI/II-3/UV202; and **F**: *E. faecalis* UV202. Weak fluorescence in *E. faecalis* SMI/II-3/UV202 is indicated by arrowheads.

Kanamycin-resistant transformants were screened for expression of the fusion protein. ELISA analysis with anti-P1 antisera indicated that the fusion protein was primarily on the cell surface of one of the transformants, *S. mutans* P1/FnBP/SM3352 (Fig. 3.5, bar 2). In the case of transformant *S. gordonii* P1/FnBP/DL-1, the reactivity detected with the whole cells and culture supernatant fluid was marginally greater than the background *S. gordonii* DL-1. Expression of the chimeric protein P1/FnBP in the transformants was also detected by Western immunoblotting with both the rabbit anti-P1 antisera and rabbit anti-FnBP antisera (Fig. 3.6). An immunoreactive band of approximately 98 kDa, was observed with both antisera from samples prepared from the transformants. These results indicate that the chimeric protein was expressed in both *S. mutans* SM3352 and *S. gordonii* DL-1.

Construction of deletion mutants of *spaP*. Computer analysis of the *spaP* amino acid sequence with the PLOT.A/HYD MacProt program identified a 44 amino acid stretch (residues 1464 to 1508) to be the hydrophilic wall-spanning domain. This hydrophilic stretch of amino acids is found N-terminal to the LPNTGV consensus sequence at residues 1528 to 1533. Thus, the C-terminal domains of antigen P1 are designated begin at amino acid residue 1464 and terminating at the last amino acid of the protein (Fig. 3.1B).

To assist in the definition of the C-terminal domains of antigen P1 in protein surface-localization, a number of deletion mutants of P1 were generated (Fig. 3.1B). The first deletion mutant was Δ706, which had P1 truncated at amino acid residue 706. This deletion mutant contained none

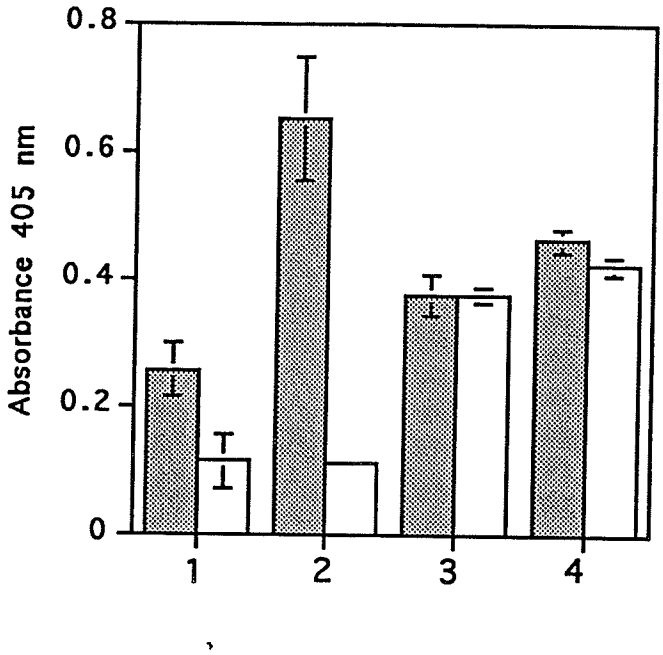

Figure 3.5. Expression of the fusion gene *spaP/fnbA* in *S. mutans* SM3352 and *S. gordonii* DL-1. The recombinant protein was analyzed by the rabbit polyclonal anti-P1 antisera in ELISA. Absorbance readings were determined from diluted samples equivalent to culture OD600nm of 0.1 and 0.025 for *S. mutans* and *S. gordonii*, respectively.
Bars: 1: *S. mutans*SM3352; **2:** P1/FnBP/SM3352; **3:** *S. gordonii* DL-1; **4:** P1/FnBp/DL-1. Whole cells (▩) and culture supernatant fluids (□).

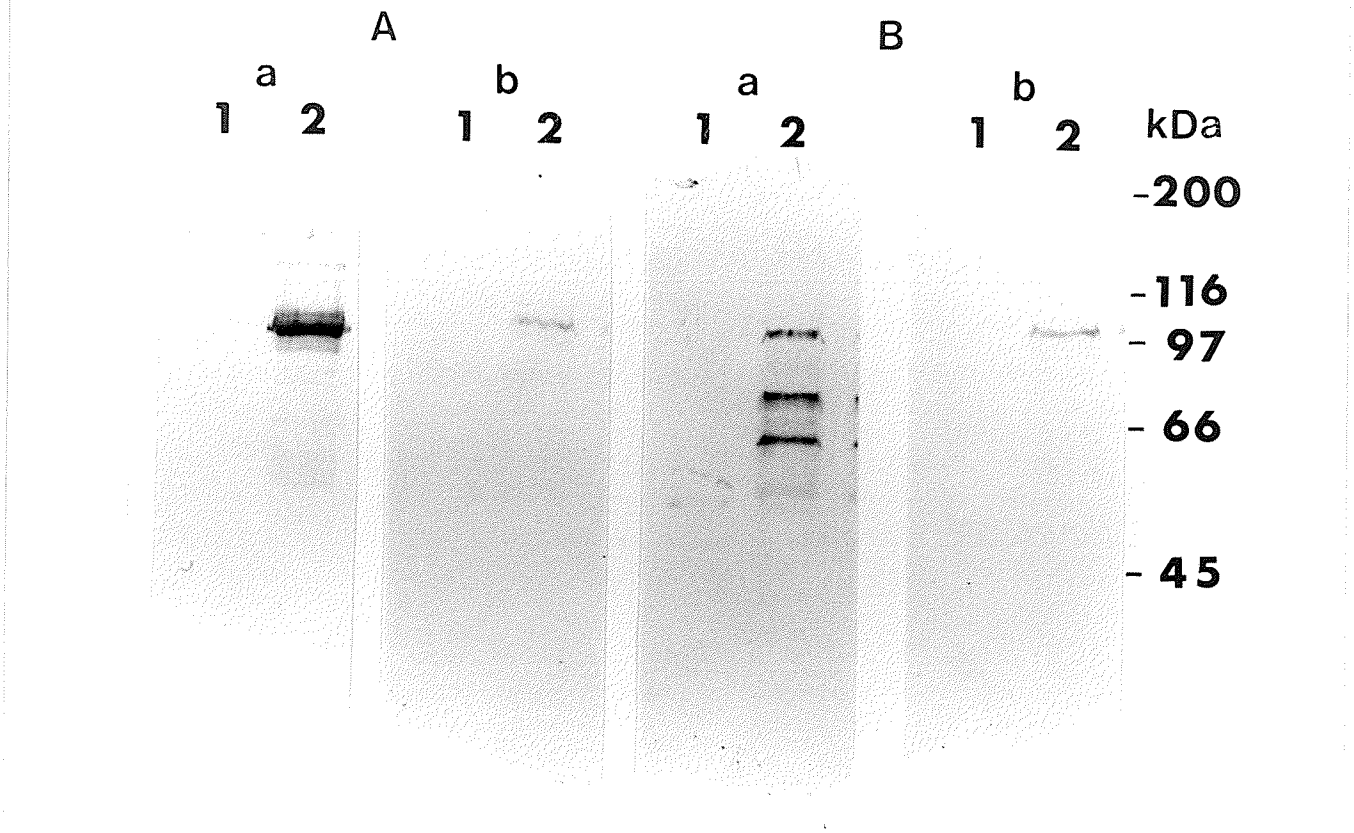

Figure 3.6. Detection of P1/FnBP fusion protein expressed by *S. mutans* (panel a) and *S. gordonii* (panel b) transformants by Western blotting. Samples are culture supernatant fluids from late exponential cultures. Antigen P1/FnBP fusion protein was detected with the rabbit polyconal anti-P1 antibody (**blot A**) and the rabbit polyclonal anti-FnBP antibody (**blot B**). **Lane 1a**: *S. mutans* SM3352; **2a** : P1/FnBP/SM3352; **1b**: *S. gordonii* DL-1; **2b**: P1/FnBP/DL-1.

of the C-terminal domains. The second deletion mutant was truncated at residue 1464 (Δ1464) N-terminal to the wall-spanning domain. Two other deletion mutants with full or partial wall-spanning domains were also created (Δ1508 and Δ1490). Deletion mutant Δ1490 still carried 26 of the 44 amino acid residues of the wall-spanning domain. Δ706 and Δ1490 were generated from the convenient restriction sites *Msc*I and *Bam*HI, respectively, and computer analysis of the DNA sequence suggested that they both carried a 3' in-framed fusion with the *lacZ* gene from the plasmid vector. Δ1508 and Δ1464 were generated by PCR as described in Materials and Methods.

Expression and localization of *spaP* deletion mutants. *S. mutans* and *S. gordonii* transformants carrying plasmid pSMΔ706 were able to express the truncated *spaP* and the bulk of the gene product was found in the culture supernatant fluids (Fig. 3.7A, bar 3 and 5). Similarly, transformants carrying pSMΔ1464 were found to express P1 in the culture supernatant fluids (Fig. 3.7B, bar 3 and 5). These results confirm a role for the C-terminus in surface-localization of antigen P1 in *S. mutans* and *S. gordonii*.

Transformants of *S. mutans* and *S. gordonii* carrying part (Δ1490) or whole (Δ1508) of the hydrophilic wall-spanning domain were analyzed by ELISA (Fig. 3.8). Results showed that the majority of the gene product was found in the culture supernatant fluids (Fig. 3.8, bars 3, 4 and 6, 7). A small amount of recombinant P1 may also be present on the cells, as indicated by the higher-than background readings exhibited by the whole cells. In contrast, in *S. mutans* and *S. gordonii* transformants carrying the

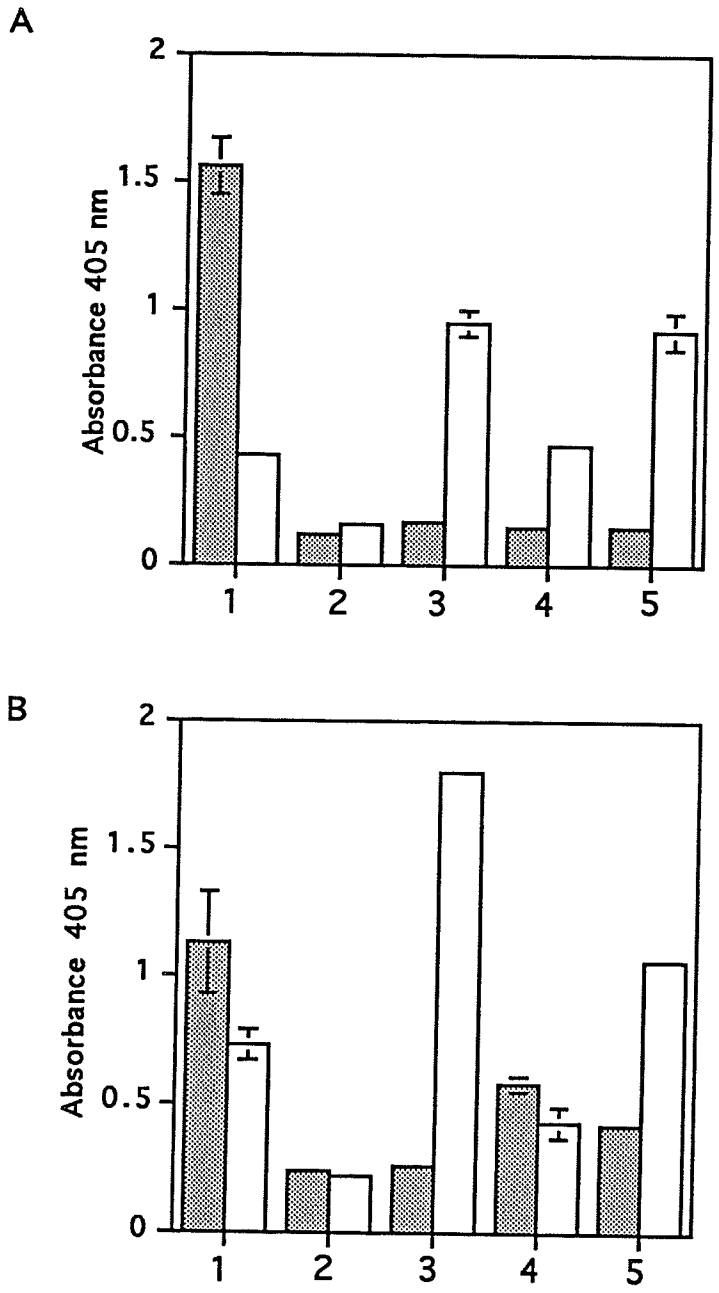

Figure 3.7. Expression of truncated *spa*P (pSMΔ706 and pSMΔ1464) in *S. mutans* SM3352 and *S. gordonii* DL-1. Panel A, antigen P1 was detected in both the whole cells (▨) and the culture supernatant fluids (□) by ELISA using the rabbit polyclonal anti-P1 antibody. **Bars: 1:** *S. mutans* NG8; **2:** *S. mutans* SM3352; **3:** Δ706/SM3352; **4:** *S. gordonii* DL-1; and **5:** Δ706/DL-1. Absorbance readings were determined from diluted samples equivalent to culture OD600nm of 0.025. **Panel B,** antigen P1 was detected with monoclonal anti-P1 antibody. **Bars: 1:** *S. mutans* NG8; **2:** *S. mutans* SM3352; **3:** Δ1464/SM3352; **4:** *S. gordonii* DL-1; **5:** Δ1464/DL-1.. Absorbance readings were determined from diluted samples equivalent to culture OD600nm of 0.05 and 0.1 for *S. mutans* and *S. gordonii* respectively.

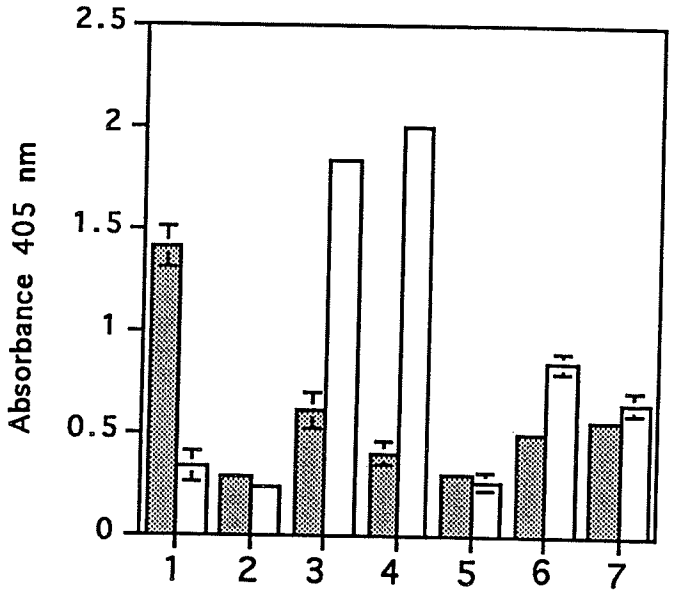

Figure 3.8. Expression of the truncated spaP (pSMΔ1490 and pSMΔ1508) in *S. mutans* SM3352, and *S. gordonii* DL-1. Antigen P1 was detected in both the whole cells (▩) and the culture supernatant fluids (□) by ELISA using the anti-P1 monoclonal antibody 4-10A. Absorbance readings were determined from diluted samples equivalent to culture OD600nm of 0.05 and 0.1 for *S. mutans* and *S. gordonii* respectively. **Bars: 1:** *S. mutans* NG8; **2:** *S. mutans* SM3352; **3:** Δ1490/SM3352; **4:** Δ1508/SM3352; **5:** *S. gordonii* DL-1; **6:** Δ1490/DL-1; and **7:** Δ1508/DL-1.

intact *spaP*, P1 was mostly cell-associated (Fig. 3.2, bars 3 and 5). Western immunoblotting with the monoclonal anti-P1 antibody, indicated the presence of multiple immunoreactive bands in the culture supernatant fluids and mutanolysin/lysozyme-released wall proteins from *S. mutans* Δ1508/SM3352 and *S. gordonii* Δ1508/DL-1 (Fig. 3.9, lane 3 and 7). The highest molecular weight band was ca. 185 kDa .

E. faecalis UV202 was also transformed with the deletion mutant pSMΔ1490. ELISA data demonstrated similar results to those found with pSMΔ1490 transformed *S. gordonii* (data not shown). The expression of the recombinant P1 antigen in *E. faecalis* UV202 was variable and decreased with continued *in vitro* subculturing.

Analysis of antigen P1 in cell walls. Cell walls from *S. mutans* NG8, *S. mutans* SM3352 harboring pSMI/II-3 and *S. mutans* SM3352 harboring pSMΔ1508 were isolated and analyzed by Western immunoblotting. Antigen P1 could be identified from wall preparations from *S. mutans* NG8 (Fig. 3.10A, lane 1), SMI/II-3/SM3352 (lane 2), and Δ1508/SM3352 (lane 3). As indicated by the intensity of the reactive protein bands, the relative amount of the truncated P1 associated with cell walls of Δ1508/SM3352 was much less than that for the wild type NG8 and SMI/II-3/SM3352. This result is consistent with the ELISA data (Fig. 3.8) showing that the reactivity of the Δ1508/SM3352 cells was marginally greater than the non-transformant SM3352. The typical multi-banding pattern of protein P1 was observed for each of the samples, although fewer bands were seen in the case of the truncated P1.

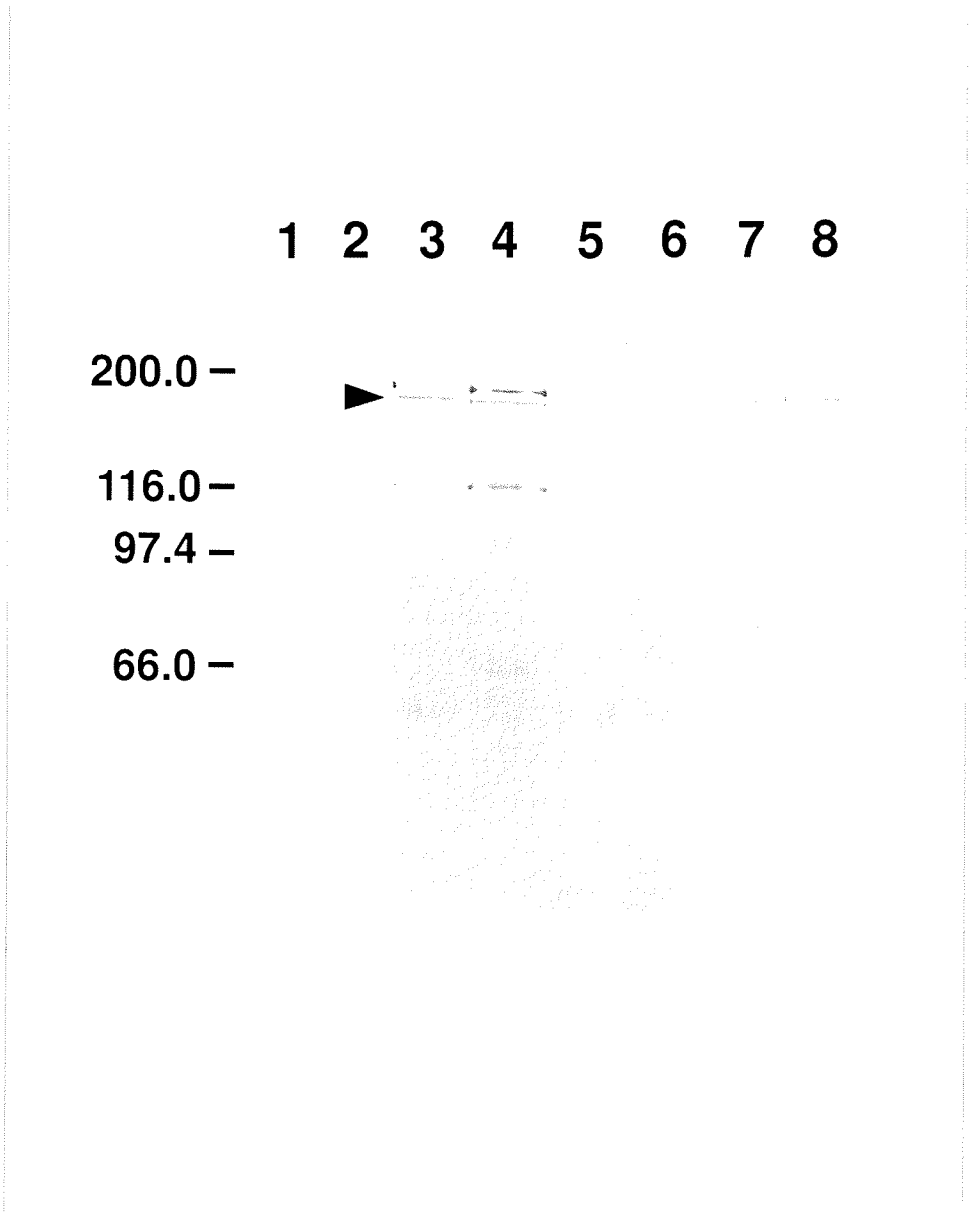

Figure 3.9. Detection of truncated P1 (pSMΔ1508) expressed by recombinant *S. mutans* and *S. gordonii* by Western blotting. Antigen P1 was detected with the anti-P1 monoclonal antibody 4-10A. **Lanes: 1**: *S. mutans* SM3352 mutanolysin-released proteins; **2**: *S. mutans* SM3352 culture supernatant fluid; **3**: Δ1508/SM3352 mutanolysin-released proteins; **4**: Δ1508/SM3352 cuture supernatant fluid; **5**: *S. gordonii* DL-1 mutanolysin- released proteins; **6**: *S. gordonii* DL-1 culture supernatant fluid; **7**: Δ1508/DL-1 mutanolysin-released proteins; and **8** : Δ1508/DL-1 culture supernatant fluid.

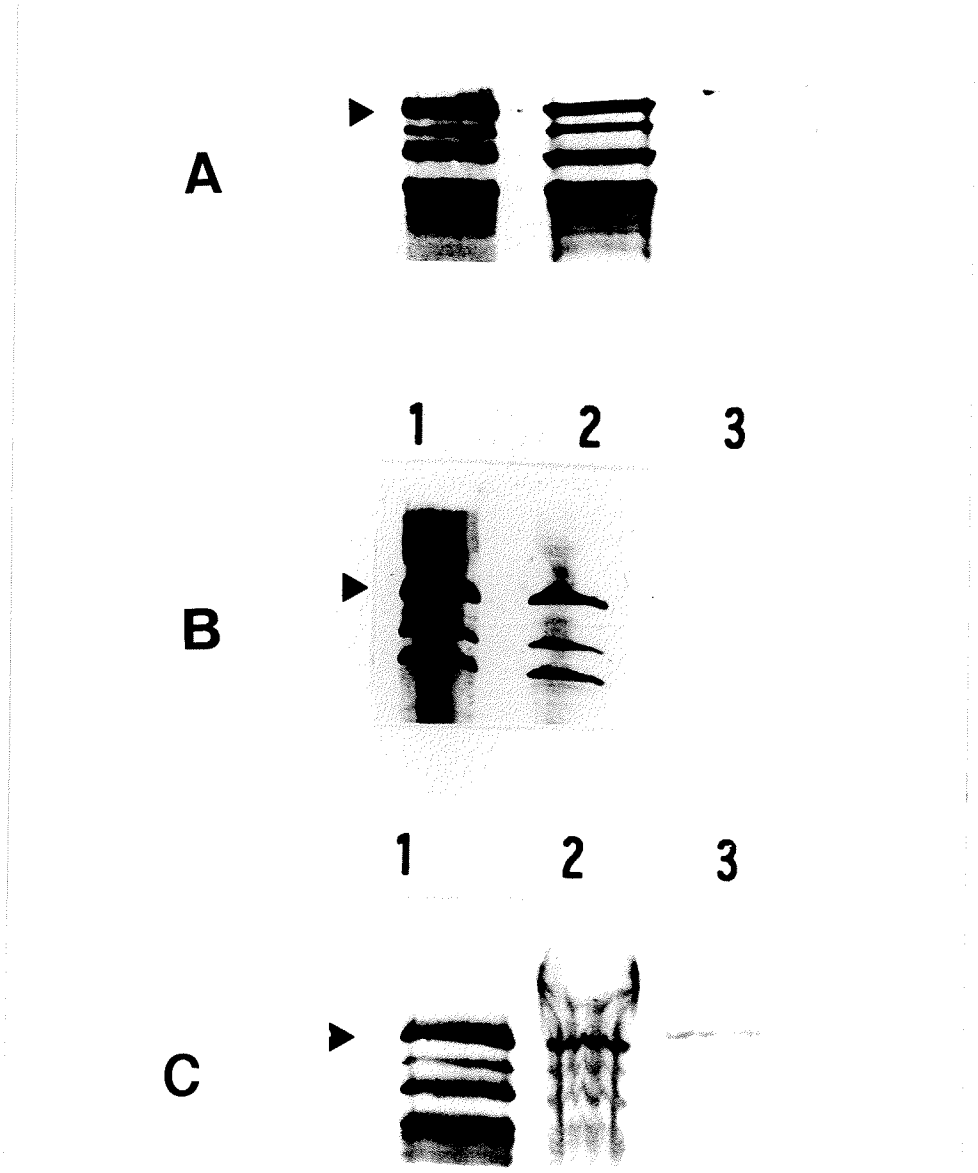

Figure 3.10. Western blotting of antigen P1 in cell-wall preparations. A: untreated cell walls; **B**: SDS-mercaptoethanol-extracted residual cell walls; and **C**: SDS-mercaptoethanol-solubilized cell wall P1. **Lanes: 1** : *S. mutans* NG8; **2**: *S. mutans* SMI/II-3/SM3352; and **3**: *S. mutans* Δ1508/SM3352. Arrowheads indicate the ca. 185 kDa full-length P1.

Cells walls from all three strains were also subjected to hot SDS-extraction to remove non-covalent proteins associated with the cell walls. The residual cell walls (Fig. 3.10B) and the SDS-extracted proteins (Fig. 3.10C) for each sample were analyzed for the presence of antigen P1. The antigen was detected in the SDS-extracted residual cell walls from *S. mutans* NG8 and SMI/II-3/SM3352 (Fig. 3.10B, lanes 1 and 2, respectively). In contrast, P1 was not detected in the residual cell walls from Δ1508/SM3352 (Fig. 3.10B, lane 3), but was detected in the SDS-extracted wall proteins (Fig. 3.10C).

Discussion

In the present study, the role of the C-terminal domains in surface-localization of P1 in *S. mutans* , as well as, in *S. gordonii* and *E. faecalis* was investigated. The results clearly indicate that the C-terminal domains of P1 are responsible for the protein surface-localization in these three bacteria. This statement is supported by the findings that the intact P1 was surface-localized in these organisms, while the C-terminus truncated P1 was not. The last (C-terminal) 97 amino acid residues of P1 appeared to be responsible for the protein surface-localization as suggested by results with deletion mutant Δ1464. The fact that P1 was able to surface-localize in *S. gordonii* and *E. faecalis* and that the *S. aureus* FnBP C-terminal domains were able to surface-localize P1 in *S. mutans* strongly indicate that the C-terminal domains from one organism were able to function in another organism. Thus, these results provide strong evidence in support of a common mechanism for surface-protein localization and anchoring among this group of Gram-positive bacteria (36, 37). The signals for

surface-protein localization and anchoring are found within the C-terminal domains and these signals are recognized by the equivalent anchoring-machinery of an alternate bacterium. Thus, a different but related organism, was able to recognize and process information encoded by the C-terminal domains from a second organism.

In the P1/FnBP-fusion protein expression studies, the immunoblotting results showed that the correct fusion was made and the chimeric protein was expressed by both *S. mutans* and *S. gordonii*. The ELISA data suggest that the chimeric protein was associated with the cell surface of *S. mutans*. However, the chimeric protein was only marginally detected in *S. gordonii*. The reason for the lack of detection of P1/FnBP on the surface of *S. gordonii* is not clear at this time. Perhaps the fusion protein, when present on the cell surface of *S. gordonii,* was folded in such a way that the antigenic epitopes were inaccessible to the antibody.

The wall-spanning domain appeared to play a role in intercalating P1 to the cell wall. Antigen P1 was observed in the cell walls isolated from the deletion mutant Δ1508 expressed in *S. mutans* SM3352, although the majority of the P1 protein was detected in the culture supernatant. Treatment of Δ1508/SM3352 cell walls with boiling SDS-mercaptoethanol resulted in the removal of the P1 from the cell walls suggesting that the protein is not covalently associated with the cell wall. The native P1 protein and the expressed recombinant full length P1 also were associated with the cell wall. In contrast, hot SDS-mercaptoethanol treatment could not completely remove the native P1 protein or the full length recombinant P1

protein from purified cell walls suggesting a possible covalent link with the cell wall.

The roles of the LPNTGV motif, hydrophobic domain, and charged tail of *S. mutans* P1 in protein surface-localization are not clear at this time. In the model proposed for protein A (35, 36), the LPXTGX motif and charged tail are required for signal recognition and post-translational modification of the protein to allow covalent link of protein A to the cell wall cross bridges. Given the similarity between the C-terminus of P1 and *S. aureus* protein A, it is likely that the two domains in P1 have similar functions.

P1 protein released from intact cells by muralytic enzymes appeared as multiple bands in Western blots (Fig. 3.3, lane 1). A ladder of protein bands has also been observed for *S. pyogenes* M protein (12) and *S. aureus* protein A (36, 37) when extracted from whole cells. In the case of protein A, the multi-banding pattern was thought to be caused by mucopeptide fragments attached to the protein. Pancholi and Fischetti (28) isolated the cell wall-associated portion of the *S. pyogenes* M protein and were unable to detect any amino sugars associated with the wall-associated fragment. Earlier studies by Russell (33) have suggested that *S. mutans* antigen P1 is a glycoprotein since cell-wall carbohydrate has been associated with the extracted protein. However, whether antigen P1 represents a glycoprotein or a protein-carbohydrate complex has remained unresolved (24). It should be pointed out that the multiple banding pattern may also be attributed to proteolytic activities.

In conclusion, the results presented in this paper showed that *S. mutans* P1 protein is anchored to the cell surface by the C-terminus, possibly via covalent linkage to the cell wall. The wall-spanning domain of the *S. mutans* P1 antigen contributes to the non-covalent retention of the P1 protein on the cell surface. The absence of the LPXTGX motif, hydrophobic domain and charged tail resulted in impaired anchoring of P1 to the cell surface thus, the findings support the model for surface-localization and anchoring of Gram-positive bacterial proteins previously described (36, 37).

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Chapter 4

Subcellular localization of the Streptococcus mutans P1 protein anchor: Comparison of P1-retainer and P1-nonretainer strains

Abstract

The association of the *Streptococcus mutans* P1 protein with the cell wall was determined by fractionation of the cell envelope into various components. Western immunoblotting of isolated cellular components revealed P1 protein in the cell wall. P1 protein was shown associated with the peptidoglycan following carbohydrate extraction with hot 10% trichloroacetic acid. To determine whether differences in the peptidoglycan were responsible for the P1-non-retainer phenotype, the cell walls of P1-retainer (NG8) and P1-non-retainer (NG5) strains were prepared for analysis. Purified cell walls of both strains demonstrated the presence of P1 protein. However, in contrast to NG8, hot SDS-extraction of NG5 cell walls removed all detectable P1 protein. This suggests that P1 protein is covalently linked to the cell wall in the P1-retainer strain NG8, but not in the P1-non-retainer strain NG5. Amino acid analysis of peptidoglycan from both P1-retainer and P1-non-retainer strains demonstrated an identical molar ratio for glu : ala : lys of 1: 3.6 :1 suggesting that the difference observed between retainer and non-retainer strains does not involve significant differences in the peptidoglycan structure. We speculate that the P1-non-retainer phenotype may result from mutations occurring within the putative enzyme responsible for anchoring P1 protein to the peptidoglycan, resulting in incomplete assembly of the P1 anchor into the peptidoglycan.

Introduction

Surface proteins of Gram-positive organisms can share a number of common features within the carboxy terminus (C-terminus) of the molecule. These common features include a hydrophilic 'wall-spanning' domain, the hexapeptide LPXTGX motif (11), a hydrophobic 'membrane-spanning' domain, and a charged tail (12). In our recent studies (16), we demonstrated the anchoring of the *Streptococcus mutans* major surface protein P1 to the cell surface via the C-terminal domains. This mechanism of surface localization and anchoring of surface proteins is common among Gram-positive cocci (13, 16, 26, 32).

The definition of the mechanism involved in the linkage of surface proteins into the peptidoglycan structure may lead to the generation of new antimicrobial agents. Agents which interfere with the anchoring of surface proteins into the peptidoglycan may compromise the integrity of the cell wall and influence the antibiotic sensitivity of the pathogen.

Staphylococcal protein A is anchored to the cell surface by similar C-terminal domains. Protein A is covalently linked, via an amide bond, to the peptidoglycan pentaglycine cross-bridge (31). Our recent studies indicate that the *S. mutans* protein P1 also appears to be covalently linked to the cell wall (16), although the cell wall component(s) which participates in the linkage has not yet been identified.

Various strains of *S. mutans* differ in their ability to retain P1 on the cell surface. *S. mutans* NG8 (serotype c) is considered a retainer strain

because the P1 protein remains associated with the cell and is removed only after treatment with muralytic enzymes (6). In contrast, *S. mutans* NG5 (serotype c) is considered a non-retainer strain, since P1 protein is found predominantly in the culture fluid rather than cell-associated. Previous studies by Brady *et al.* (9) have demonstrated that the non-retainer phenotype can not be attributed to loss of the C-terminus of the P1 protein. In addition, the sequence of the *spaP* gene from the NG5 strain did not reveal any premature termination codons (17). In fact, when *spaP* cloned from NG5 was expressed in a P1-negative mutant of NG8, the gene product was surface-localized (16). The nature of the difference in the retainer vs non-retainer phenotype is unknown. The non-retainer phenotype could be the result of a defective enzyme (sortase [34]) which fails to anchor the protein to the cell wall. Alternatively, it could be due to defects in the peptidoglycan structure, such as , the cross-bridges or within an integral structural component to which the P1 protein is linked. To examine these possibilities, the association of the P1 protein with the cell surface was investigated in a retainer (NG8) and a non-retainer (NG5) strain of *S. mutans*.

Materials and Methods

Bacterial strains and growth conditions. The source of *S. mutans* NG8 and *S. mutans* NG5 has been described previously (1). Cultures were maintained as glycerol stocks at -20°C and -70°C and checked for purity on blood agar plates. Cultures were grown in the semi-defined medium of Bowden *et al.* (7) with the amino acid mixture replaced by 0.2% (wt/vol) tryptone. The inoculated medium was incubated in an anaerobic chamber

(80% N_2, 10% H_2, and 10% CO_2) for 20 h before the culture was harvested. *E. coli* TB1 (*ara*D(*lac proAB*) *rpsL* (F80 *lacZ*DM15) *hsdR*) was grown at 37°C with agitation in either LB broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, wt/vol) or in Rich media (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose, wt /vol) with or without 100 μg/ml ampicillin.

Monoclonal antibodies and antisera. Monoclonal antibodies (MAb), 4-10A and 6-8C, were generously provided by A. S. Bleiweis (University of Florida [1]). Both MAbs were used in the form of ascitic fluid. MAb 4-10A recognizes an epitope near the middle of the P1 molecule and MAb 6-8C recognizes an epitope at the P1 C-terminus (9). Rabbit polyclonal antisera recognizing the P1 C-terminus were produced as described below.

Construction of maltose-binding fusion protein expressing the C-terminus of *S. mutans* P1 protein. The plasmid pMAL-cRI (*lacI*P_{tac}*malE*D2-26-fx-*lacZ*aAmp^r) (New England Biolabs, Beverly, MA) carrying the *E. coli* maltose-binding protein gene (*malE*) was used in the construction of the fusion protein. Plasmid DNA was isolated by the alkali lysis method (4). The *Eco*RI site and the *Xba*I site within the polylinker of pMAL-cRI were used as the cloning sites. The plasmid pMAL-cRI was digested with *Eco*RI and isolated from a 0.8 % agarose gel by cutting out the appropriate sized band and purifying it by using the Gene Clean kit (Bio101, LaJolla, CA). The *Eco*RI ragged end was subsequently blunt ended by a fill-in reaction with the Klenow fragment of DNA polymerase. To achieve this, 5-10 μg of *Eco*RI linearized plasmid was incubated with 5 units of Klenow, 10 mM Tris, pH 7.5, 50 mM NaCl, and 2 mM deoxyribonucleotides at room temperature for 40 min. The plasmid was buffered phenol-chloroform (1:1,

vol/vol) extracted and restricted with *Xba*I. Plasmid DNA was then purified with the Gene Clean kit.

The plasmid encoding the *S. mutans* antigen P1 C-terminus, pSpaP-C4, was constructed by subcloning the 3' 1.0 kb *Hin*dIII-*Kpn*I DNA fragment from pSMI/II (5, 17, 21) into pBluescript. The 600 bp *Hin*cII-*Xba*I DNA fragment within the *Hin*dIII-*Kpn*I DNA fragment from pSpaP-C4 was isolated from an agarose gel, purified by the Gene Clean kit, and ligated to pMAL-cRI (29). The *Hin*cII-*Xba*I DNA fragment encodes the last 144 amino acid residues of the P1 C-terminus. The ligated DNA was transformed into *E. coli* TB1 (19).

Plasmids from ampicillin-resistant transformants were screened by restriction endonuclease analysis. Transformants carrying the construct, designated as pMal-COOH, were then tested for expression of the fusion protein. Transformants were inoculated into 5 ml LB broth with 100 μg/ml ampicillin and allowed to grow overnight at 37°C. The following morning, 5 ml fresh medium was inoculated at a dilution of 1/100 with the overnight culture. The culture was allowed to grow at 37°C with aeration until an OD_{600} nm reached 0.5 (2-3h). One ml of the uninduced culture was removed and kept on ice, while the remainder of the culture was induced with isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 0.3 mM and the incubation continued at 37°C. After 2 h, 1 ml of the induced culture was removed to an Eppendorf tube. Both the uninduced and induced cultures were centrifuged for 2 min at 16,000 x g, and the culture supernatants removed and the cell pellets resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (20). The samples were boiled for 5 min and a 15 μl aliquot loaded onto a 7.5% SDS-PAGE gel. The size of the fusion protein was

compared with the pMAL-cRI control sample. The fusion protein, designated as MBP-P1, was clearly evident in the IPTG-induced samples as a protein band of 58.8 kDa.

Purification of fusion protein on amylose resin. The fusion protein MBP-P1 was prepared from 1 liter of *E. coli* culture harboring the plasmid pMal-COOH grown in Rich broth with 100 μg/ml ampicillin and induced for 2 h at an OD_{600} nm of 0.5 with 0.3 mM IPTG. The culture was harvested by centrifugation at 4,000 x g for 20 min at 4°C and the cell pellet resuspended in 5 ml of buffer consisting of 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol (column buffer). The cell suspension was frozen at -70°C and thawed in an ice bath. The cells were sonicated, on ice, with 6 by 30 second-bursts at maximum output for a total of 3 min (Model W-375 Sonicator, Heat Systems Ultrasoncics Inc., Plainview, N.Y.). Unbroken cells were removed by centrifugation at 4,000 x g for 5 min at 4°C and the supernatant recovered to a clean tube. The insoluble material was centrifuged at 27,000 x g for 30 min at 4°C, the pellet resuspended in 4 ml of 6 M urea dissolved in column buffer and left at 4°C overnight to solubilize the protein. The solubilized protein was dialyzed against four 3 liter changes of column buffer over 24 h at 4°C. The insoluble material was removed by centrifugation and 20 ml of soluble proteins were recovered with a protein concentration 4 mg/ml.

The MBP-P1 in the soluble protein fraction was isolated by on an amylose resin column. The amylose resin, with a capacity of 2 mg protein per ml bed volume, was packed into a 20 ml Bio-Rad econo-column (1.5 x 12 cm) and equilibrated in column buffer. After application of 2 ml of sample at a

concentration of 2.0 mg/ml protein, the column was washed with 8 bed volumes of column buffer. The MBP-P1 protein was eluted from the column with 10 mM maltose dissolved in column buffer. One-ml fractions were collected and each fraction was assayed with the Bradford protein reagent (Bio-Rad Laboratory, Mississauga, ON) for location of protein-rich fractions. A 20 µl aliquot from each of the collected fractions was analyzed by SDS-PAGE. Fractions containing fusion protein from several purifications were pooled to give a total yield of 4 mg protein (0.38 mg/ml).

Cleavage of the fusion protein with Factor Xa (New England Biolabs) was attempted but resulted in insufficient digestion. It is possible the fusion protein was folded in such a way that the Factor Xa cleavage site was inaccessible. For this reason, the MBP-P1 fusion protein was used directly as the immunogen for the production of antiserum, allowing the MBP to act as the carrier protein.

Generation of anti-P1COOH antisera in rabbits. Two New Zealand white rabbits were each injected subcutaneously with 0.2 mg of the purified MBP-P1 fusion protein emulsified in Freund's incomplete adjuvant. At 14 days, the rabbits were given a booster of the same amount of immunogen in saline, injected intramuscularly. At day 28, the animals were given an intravenous booster of 0.2 mg of antigen in 1 ml of phosphate buffered saline. The animals were test bled 7 days later to confirm reactivity with P1, by enzyme-linked immunosorbent assay (16). The blood was allowed to clot and the sera were recovered by centrifugation. The serum immunolglobulins were concentrated by ammonium sulfate precipitation (14) by the addition of 0.5 volume of saturated ammonium sulphate followed by stirring at 4°C overnight.

The precipitated proteins were removed by centrifugation at 3,000 x g for 30 min, the supernatant recovered and 0.5 volumes of saturated ammonium sulphate was added. The suspension was stirred overnight at 4°C and the antibody fraction recovered by centrifugation at 3,000 x g for 30 min. The pelleted antibodies were resuspended in a 0.3-0.5 volumes of phosphate-buffered saline (PBS) and then dialyzed against 3 changes of PBS overnight. The recovered antisera, designated rabbit anti-P1COOH, were aliquoted and stored at 4 °C or at -70°C.

SDS-PAGE and Western immunoblotting. SDS-PAGE was performed by the method of Laemmli (20). The method used for Western immunoblotting has been described previously (16, 33). The molecular weight protein markers used were from GIBCO/BRL Life Technologies, Inc. (Burlington, On).

Preparation of cell walls and membranes. Cells were grown in four 1-litre cultures until late log phase (20 h) in the semi-defined medium in an anaerobic chamber (H_2 10%, N_2 80 %, CO_2 10%). Cells were harvested by centrifugation at 10,000 x g, 20 min, at 4°C, and washed 3 times in 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mM pepstatin A to avoid proteolysis. Cells (ca. 1 g wet wt./ml of phosphate buffer) were put into glass Mickle vials containing glass beads (40/60 mesh) added to a depth of 1-1.5 cm (27). A few drops of amyl alcohol were added to prevent foaming. The cells were disrupted at 4°C in a Mickle tissue disintegrator (Mickle Engineering Co., Gomshall, England) until the cells stained Gram-negative (1-2 h). Unbroken cells and glass beads were removed by centrifugation at 8,000 x g for 15 min at 4°C. The cell walls were

isolated from the supernatant by centrifugation at 27,000 x g for 30 min at 4°C. The pelleted cell walls were washed three times in the phosphate buffer with protease inhibitors and freeze-dried. The supernatant recovered from the cell wall isolation was transferred to a clean centrifuge tube. Membranes were recovered from the supernatant by centrifugation at 50,000 x g for 1 h at 4°C. The opaque pellet, representing the membranes, was washed three times in distilled water and then suspended in 1-2 ml of phosphate buffer and stored at -70°C.

Trypsinization of cells. *S. mutans* cells were treated with trypsin to remove surface proteins prior to isolation of cell membranes. This method reduced the possibility of the contamination of membrane fraction with P1 from the cell surface. Four liters of exponential phase cells were harvested (1g/litre) and washed, without protease inhibitors as described above. The cells were resuspended in 100 ml of 50 mM potassium phosphate buffer, pH 7.4. Trypsin (Sigma Chemical Co., St. Louis, MO.) was added to a final concentration of 100 μg/ml and the cell suspension incubated 1 h at 37°C on a rotating platform at 12 rpm (Hematology mixer, model 346; Fisher Scientific, Edmonton, AB) (24). At the end of the incubation period, trypsin-chymotrypsin inhibitor (Bowman-Birk inhibitor from soybean, Sigma) was added to 150 μg/ml and the cells harvested at 8,000 x g (20 min, 4°C) (24). The cell pellet was washed in 50 mM potassium phosphate buffer (pH 6.0) and suspended in 10 mM potassium phosphate buffer (pH 7.4) as a thick suspension (1g/ml). Cell walls and membranes were prepared from the trypsinized cell suspension as described above.

SDS-mercaptoethanol extraction of NG8 and NG5 cell wall proteins. Twenty mg of freeze-dried, non-trypsinized, cell walls were resuspended in 300 µl of 3-fold concentrated SDS-mercaptoethanol buffer (20 mM Tris, pH 6.8, 0.3% glycerol, 6% SDS and 0.15% β-mercaptoethanol) and boiled for 20 min (27). The heated suspension was centrifuged at 10,000 x g for 20 min and the supernatant was recovered, leaving the residual extracted cell walls in the pellet. The residual SDS-mercaptoethanol-extracted cell wall pellet was washed and resuspended in 300 µl of distilled water. A 10 µl volume of the untreated cell walls, residual cell walls, and the SDS-mercaptoethanol extracted proteins were analyzed for P1 by Western immunoblotting with the MAb 4-10A.

Sarkosyl extraction of NG8 and NG5 membranes. Membranes prepared from trypsinized and non-trypsinized cells were suspended at a concentration of 2 mg protein in 0.5 ml distilled water. A 0.5 ml of 1 % sarkosyl (N-lauroly sarcosine, Sigma) in 10 mM potassium phosphate buffer, pH 7.5, and 1 mM EDTA was added and the samples were incubated for 1 hr at room temperature on a rotating platform (2). The extracted membranes were recovered by centrifugation at 50,000 x g for 1 hr and, suspended in 1.0 ml distilled water. The sarkosyl buffer supernatant, the sarkosyl-treated residual membranes, and the untreated membranes were analyzed for the presence of protein P1 by Western immunoblotting with MAb and polyclonal antisera.

Treatment of cell walls with mutanolysin. A suspension of trypsinized cell walls (10 mg/ml dry weight) was made in distilled water. A 50 µl aliquot of cell walls was removed to an Eppendorf tube and 50 mM Tris, pH

7.0, 10 mM $MgCl_2$, and 125 units of mutanolysin (Sigma, St. Louis, MO) were added to a final reaction volume of 100 µl. The cell walls were incubated on the rotating platform at 37°C for 1 h. The mutanolysin-digested cell walls were recovered by centrifugation at 16,000 x g for 20 min in a microcentrifuge, washed twice, and resuspended in 100 ml of distilled water. The supernatant was recovered in a clean Eppendorf tube. A 20 µl aliquot of each sample was analyzed by Western blotting.

Extraction of carbohydrate from purified cell walls. Fifteen mg of freeze-dried cell walls from trypsinized cells of NG8 and NG5 was subjected to a second trypsinization by resuspension in 5 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 100 µg/ml trypsin. After incubation for 1 h at 37°C on the rotating platform, the cell walls were harvested at 38,000 x g for 20 min at 4°C, washed in distilled water and recovered by centrifugation. The cell wall pellet was resuspended in 10 % (wt/vol) trichloroacetic acid (TCA), placed in a boiling water bath for 20 min (25, 30) and centrifuged. The extracted cell-wall pellet, representing the peptidoglycan, was washed in distilled water and resuspended in 2 ml of distilled water and dialyzed against water before lyophilization. The supernatant from the TCA-extracted cell walls was saved and the carbohydrate recovered by the addition of four volumes of cold acetone and a few crystals of sodium acetate. The carbohydrate was precipitated overnight at 4°C and harvested by centrifugation at 12,000 x g for 20 min. Residual acetone was evaporated in a stream of air and the carbohydrate dissolved in distilled water before lyophilization.

Analysis of isolated carbohydrate and the peptidoglycan. Freeze-dried carbohydrate extracted from cell walls was dissolved in distilled water at

a concentration of 2 mg/ml (dry weight). The total carbohydrate content was estimated by the phenol-sulfuric acid assay, with glucose as the standard (10). The values presented in Table 4.1 are the average of duplicate samples. A volume of 30 µl per lane was analyzed by Western immunoblotting with MAb 6-8C and the rabbit anti-P1COOH antisera.

The peptidoglycan was resuspended at a concentration of 3.25 mg/ml in 50 mM Tris, pH 7.4, and 1 mM $MgCl_2$. A 50 µl volume of peptidoglycan was incubated with 50 µl of mutanolysin (125 units) containing 4 mM phenylmethylsulfonyl fluoride to inhibit proteases. After 1 h at 37°C on the rotating platform, the digested peptidoglycan fragments were recovered by centrifugation for 20 min at 16,000 x g in a microcentrifuge. The supernatant was drawn off to a clean tube and represented mutanolysin-released proteins. The remaining peptidoglycan pellet was resuspended in 100 µl of distilled water. SDS-mercaptoethanol buffer was added and the samples were boiled for 20 min, loaded (30 µl per lane) on 12% SDS-PAGE gels and subsequently probed with anti-P1 antibodies by Western blotting.

The peptidoglycan samples of *S. mutans* NG8 and NG5 were analyzed for amino acids content at the Protein Sequencing Facility, University of Calgary. Peptidoglycan samples were hydrolyzed in a solution of 6N HCl and 0.1% β-mercaptoethanol at 150°C for 1 h *in vacuo*. Hydrolysis of peptidoglycan in 6N HCl with 4% thioglycolic acid for tryptophan (22) and performic acid oxidation for cysteine (15) were also conducted at 150°C for 1 h. Hydrolysis of peptidoglycan in 6N HCl at 100°C for 6 h *in vacuo* was completed for amino sugar analysis. The amino acids were analyzed on a Beckman 6300 amino acid analyzer with ninhydrin detection. Norleucine was added to the sample prior

to hydrolysis as an internal standard. Each amino acid was quantitated by the external standard method with a Beckman System Gold software on a Packard Bell 386SX microcomputer. The analysis data was converted to mole % and amino acid composition by using software created at the Protein Sequencing Facility.

Protein concentration determination. The protein concentrations in samples were estimated by the method of Bradford, with bovine serum albumin (Sigma, St. Louis, MO) as the standard (8).

Results

MBP-P1 fusion protein and polyclonal anti-P1COOH. The maltose-binding-P1 fusion protein (MBP-P1) eluted from the amylose resin demonstrated a single protein band on Coomassie stained gels (Fig. 4.1A, lane 2) compared to the crude P1 from culture supernatant (Fig. 4.1A, lane 1). The molecular weight of this protein was estimated at 58,800, which is within the expected size of the fusion protein (maltose-binding protein, 42 kDa, P1 C-terminus 144 amino acids, 15.8 kDa). A total of 80 mg of protein was isolated from 1 litre of culture following solubilization in 6 M urea and dialysis, with a total yield of 4 mg of purified fusion protein.

Western blotting with the rabbit anti-P1COOH antiserum generated against the MBP-P1 fusion protein, recognized the native P1 protein (ca. 185 kDa), but not other proteins present in the culture supernatant of *S. mutans* NG5 (Fig. 4.1 C, lane 1). The same antiserum also reacted strongly with the

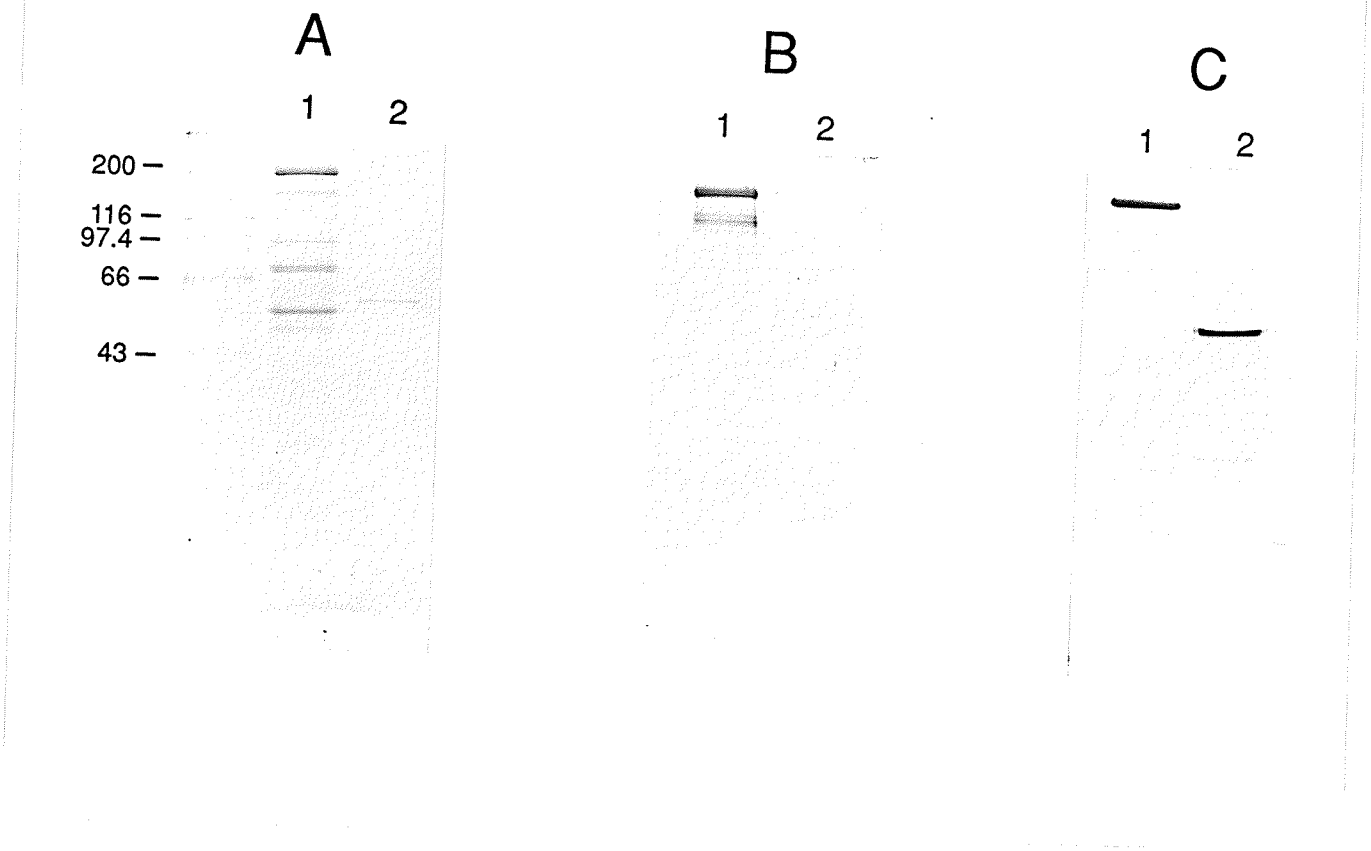

Figure 4.1. SDS-PAGE and Western blotting of native antigen P1 and fusion protein MBP-P1COOH. Panel A: A Coomassie blue R-250 stained SDS-PAGE gel; **panel B**: A Western blot reacted with the MAb 6-8C (1/3000); and **panel C**: A Western blot reacted with the rabbit anti-MBP-P1COOH (1/100). **Lane 1**: 3 µg proteins from the culture supernatant of *S. mutans* NG5, and **lane 2**: 0.1 µg of MBP-P1COOH fusion protein isolated from *E. coli*.

MBP-P1 protein (Fig. 4.1C, lane 2). MAb 6-8C also recognized the 185 kDa native P1 and two smaller proteins (Fig. 4.1B, lane 1). However, the MAb 6-8C did not recognize the MBP-P1 fusion protein (Fig. 4.1B, lane 2), presumably because the epitope recognized by 6-8C lies N-terminal to the P1 C-terminus and has consequently been deleted in the MBP-P1 fusion protein.

Localization of P1 within isolated membranes and cell walls. The isolated membranes from both NG8 and NG5, when analyzed by Western blotting, did not show any reactivity with the P1 specific MAb 4-10A or 6-8C. Subsequently, membranes from NG8 and NG5 were extracted with sarkosyl to remove integral membrane proteins. Both the residual membranes and the sarkosyl-extracted protein fractions also showed no detectable immuno-reactive bands when probed with MAb 4-10A and anti-P1COOH antibodies in either the NG8 or NG5 samples.

When the isolated cell walls from non-trypsinized *S. mutans* NG8 and NG5 cells were subject to analysis by Western immunoblotting with MAb 4-10A (Fig. 4.2A), an immunoreactive multi-banding pattern corresponding to protein P1 was observed. This multi-banding pattern was similar to previous observations (16). Based on the intensity of the bands, the amount of P1 in the cell walls of NG5 (Fig. 4.2A, lane 2) was much less than that in the NG8 cell walls (Fig. 4.2A, lane 1). This result is consistent with the P1-non-retainer phenotype of NG5, where relatively little P1 remains cell-associated.

Following treatment with hot SDS-mercaptoethanol to remove non-covalently linked proteins, extracts of the residual cell walls and the SDS-solubilized proteins from NG8 showed typical immunoreactive bands with the

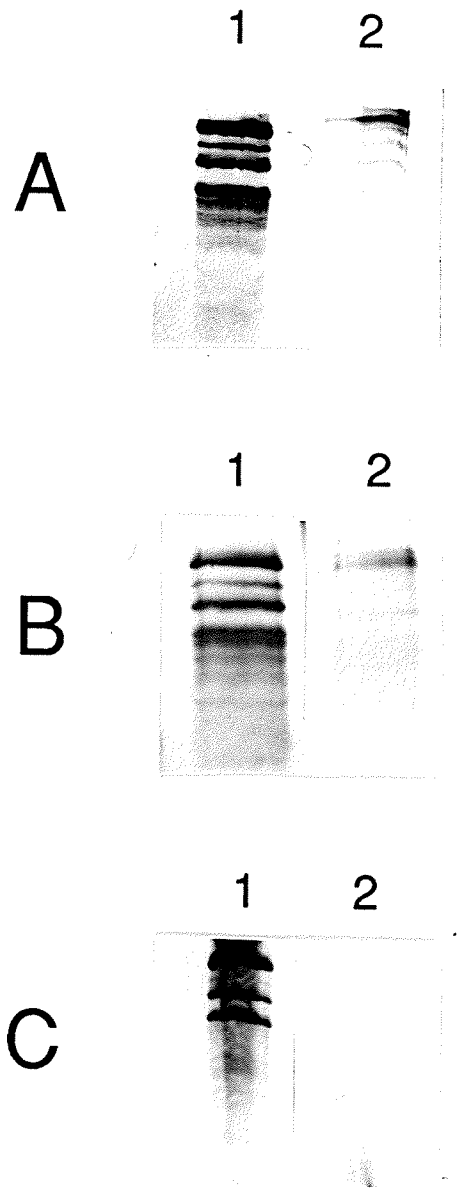

Figure 4.2. Western blotting of antigen P1 in extracts of non-trypsinized cell walls prepared from *S. mutans* with MAb 4-10A (1/5000). Panel A: untreated cell walls; **panel B**: SDS-mercaptoethanol-solubilized P1 from cell walls; and **panel C:** SDS-mercaptoethanol-extracted residual cell walls. **Lane 1**: *S. mutans* NG8, and **lane 2**: *S. mutans* NG5. Arrowheads indicate the full-size P1 (ca. 185 kDa).

MAb 4-10A (Figs. 4.2B and 4.2C, lane 1). In contrast in NG5, immunoreactive bands corresponding to protein P1 were visible only in the SDS-solubilized protein fraction (Fig. 4.2B, lane 2) and the residual cell walls were negative (Fig. 4.2C, lane 2). This demonstrates a significant difference in the nature of the association between the P1 protein and the cell wall in these two strains of *S. mutans*.

Identification of the P1 C-terminus in trypsinized cell walls. Extracts of cell walls of trypsinized NG8 cells were analyzed for the C-terminus of protein P1 by Western immunoblotting. There was one strong (ca. 110 kDa) and two (ca. 92 and 70 kDa) weakly reactive bands present in the preparation when probed with MAb 6-8C (Fig. 4.3A, lane 1). Subsequently, trypsinized NG8 cell walls were digested with mutanolysin. Extracts of the mutanolysin-treated residual cell walls contained both the 110 and 70 kDa reactive bands (Fig. 4.3A, lane 2), while the mutanolysin-released protein sample contained only the 110 kDa immunoreactive band (Fig. 4.3A, lane 3). A band of approximately 27 kDa was visible in lanes 3 and 4 and (Fig. 4.3B) and presumably represents cross-reaction with mutanolysin.

When extracts of the NG8 trypsinized cell walls were probed with the anti-P1COOH antibodies, there was negligible reactivity (Fig. 4.3B, lane 1). Also, extracts of the mutanolysin-treated cell wall did not react with anti-P1 COOH antibodies (Fig. 4.3B, lane 2). However, the supernatant recovered after mutanolysin digestion (or the mutanolysin-released proteins) demonstrated a reactive band of 110 kDa (Fig. 4.3B, lane 3). Both trypsin and trypsin inhibitor did not react with the antibodies, therefore the

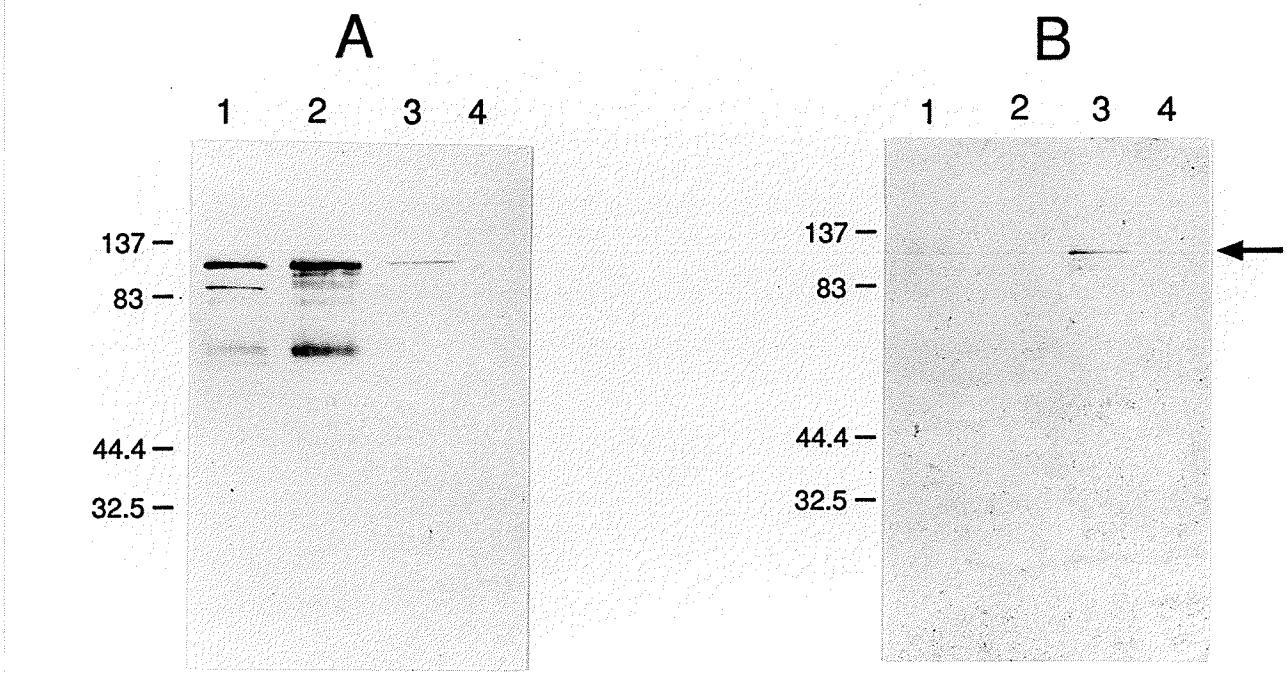

Figure 4.3. Western blotting of *S. mutans* NG8 antigen P1 C-terminus in trypsinized cell wall preparations before and after treatment with mutanolysin. Panel A: P1 C-terminus detected with MAb 6-8C (1/3000); **panel B**: P1 C-terminus detected with rabbit anti-MBP-P1COOH (1/100). **Lane 1**: untreated cell walls, **lane 2**: residual cell walls after treatment with mutanolysin, **lane 3**: mutanolysin-released P1, and **lane 4**: mutanolysin alone.

immunoreactive bands did not represent liberated trypsin previously bound to the cell walls.

Identification of P1 C-terminus in cell-wall carbohydrate and peptidoglycan. The amount of carbohydrate in 100 mg dry weight of trypsinized cell walls was determined before and after boiling in 10% TCA (Table 4.1). The cell walls from NG8 and NG5 contained a mean value of 35.9 mg and 20.9 mg total carbohydrate, respectively. TCA-extracted cell walls had the majority of the carbohydrate removed, leaving only 0.6 mg (1.7%) in the peptidoglycan of NG8 and 0.9 mg (4.4%) carbohydrate in NG5. Only 13.9 mg (39%) in NG8 and 8.6 mg (41%) in NG5 of the cell wall carbohydrate was recovered by acetone precipitation. The remainder of the extracted cell wall carbohydrate was lost, presumably as a result of degradation during boiling.

When the peptidoglycan and the TCA-extracted carbohydrate were analyzed by Western immunoblotting, there was negligible reactivity with the rabbit anti-P1COOH antibodies present in the samples (data not shown). In addition, the TCA-extracted carbohydrate did not display any detectable reactivity with the MAb 6-8C (Fig. 4.4, lane 1). However, the peptidoglycan contained a very faint immunoreactive band of approximately 70 kDa when reacted with MAb 6-8C (Fig. 4.4, lane 2,). After treatment of the peptidoglycan with mutanolysin, two immunoreactive bands, 70 and 27 kDa, were detected with MAb 6-8C in the residual peptidoglycan (Fig. 4.4 lane 3). The 70 kDa band was also observed in the mutanolysin-released protein fraction (Fig. 4.4, lane 4). As revealed earlier the 27 kDa band was likely to be a protein in the mutanolysin preparation (Fig. 4.4, lane 3 and 4). The 110 kDa

TABLE 4.1 : Quantitation of carbohydrate from trichloroacetic acid extraction of cell walls from *Streptococcus mutans*[a].

Strains	Unextracted cell walls (mg)	Carbohydrate extracted by TCA (mg)	Carbohydrate in TCA-extracted peptidoglycan (mg)
NG8	35.9 (100%)	13.9 (39%)	0.6 (1.7%)
NG5	20.9 (100%)	8.6 (41%)	0.9 (4.4%)

[a] Values for 100 mg dry wt. cell walls.

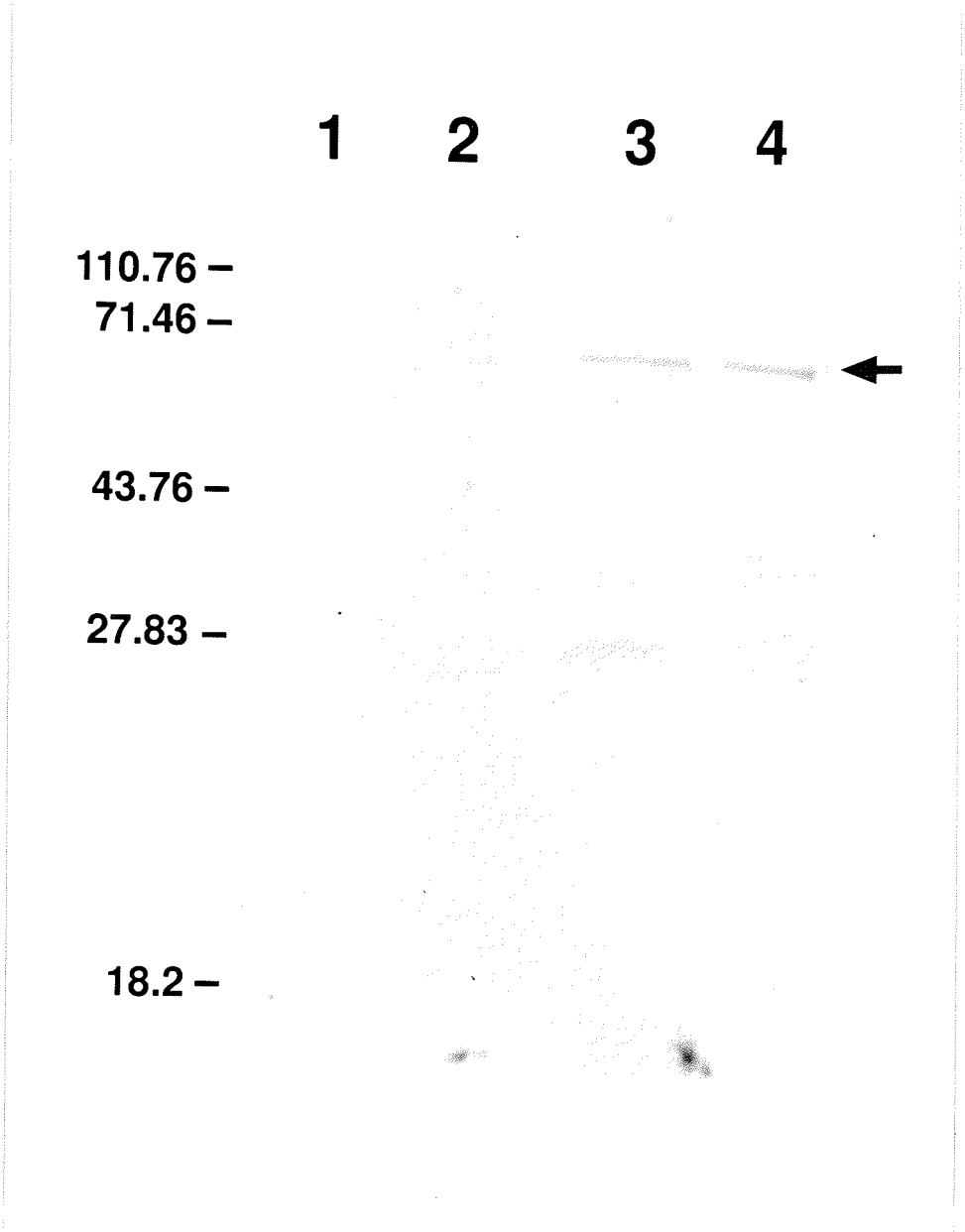

Figure 4.4. Western blotting of extracts of *S. mutans* NG8 trypsinized cell walls after carbohydrate removal with boiling trichloroacetic acid. Lane 1: carbohydrate recovered from extracted cell-walls, **lane 2**: 'carbohydrate-extracted' peptidoglycan, **lane 3**: 'carbohydrate-extracted' peptidoglycan after treatment with mutanolysin, and **lane 4**: mutanolysin-released antigen P1 from peptidoglycan. Antibodies used were 6-8C (1/3000).

immunoreactive band observed in Figure 4.3B lane 3 was not evident in the TCA extracted peptidoglycan.

The reactivity of the protein bands with MAb 6-8C was enhanced after treatment of either the cell walls (Fig. 4.3A, lane 2) or peptidoglycan (Fig. 4.4, lane 3) with mutanolysin, particularly when compared with untreated samples (Fig. 4.3A, lane 1; Fig. 4.4, lane 2).

Amino acid composition of peptidoglycan samples. The amino acid composition of the peptidoglycan from both NG8 and NG5 indicated a similar molar ratio for the predominant amino acids, glu: ala: lys were (1 : 3.6 : 1) (Table 4.2). Other amino acids were present in trace amounts after acid hydrolysis.

Table 4.2: **Amino acid composition of isolated peptidoglycan from the *Streptococcus mutans* P1-retainer strain NG8 and the P1-non-retainer strain NG5.**

	NG8			NG5		
Amino Acid	pmoles	mole%[a]	AA Ratio[b]	pmoles	mole%	AA Ratio
Asp	312	0.76		329	0.88	
Thr	184	0.45		194	0.52	
Ser	382	0.93		455	1.22	
Glu	7390	18.02	1.01	6684	17.93	1.00
Gly	236	0.58		387	1.04	
Ala	26325	64.20	3.61	23923	64.18	3.59
Val	221	0.54		295	0.79	
Met	32	0.08		73	0.19	
Ile	172	0.42		320	0.86	
Leu	216	0.53		431	1.16	
Tyr	110	0.27		137	0.37	
Phe	219	0.53		343	0.92	
His	42	0.10		60	0.16	
Lys	7284	17.77	1.00	6668	17.89	1.00
Trp	23	0.06		ND		
Arg	ND[c]			78	0.21	
Pro	92	0.23		142	0.38	
Nle[d]	236			513		

[a] Mole % was calculated as (# of pmoles of aa / total pmoles) X 100. The total pmoles for NG8 and NG5 were 41,000 and 37,276, respectively.

[b] AA ratio = Ratio of the three predominant amino acids gly, ala, and lys with the latter used as one.

[c] Not detected.

[d] Internal standard : Norleucine.

Discussion

In previous studies, we demonstrated the association of the P1 protein, via the C-terminus, with the *S. mutans* cell surface and implicated the cell wall in this association (16). In this study, the association of protein P1 with cell wall structure(s) was investigated by fractionation of the cell envelope into various components. By using the MAb 4-10A, which recognizes an epitope central to the P1 molecule (9), P1 was found associated with the cell wall and not with isolated membranes (Fig. 4.2), confirming our previous findings (16).

To assist our present study, polyclonal antibodies specific for the P1 C-terminus were generated by immunization of rabbits with a fusion protein expressing the P1 C-terminus with the MBP fusion partner. The antibodies generated against the purified MBP-P1 fusion protein reacted with the native *S. mutans* NG5 P1 protein (Fig. 4.1C). These results indicate that the correct in-frame fusion was made and the epitopes presented by the MBP-P1 protein were identical to those of the native P1 protein C-terminus. However, the yield of MBP-P1 fusion protein after purification was lower that expected. This low yield was presumably due to the difficulty in solubilizing the protein. In addition, some of the solubilized fusion protein failed to bind to the amylose column and was observed in the effluent further accounting for the low yield.

In an effort to identify precisely the site of the P1 protein anchor, the polyclonal MBP-P1COOH antisera, in conjunction with the MAb 6-8C, were used to probe cell walls isolated from *S. mutans* cells treated with trypsin to remove surface P1 (Fig. 4.3). The P1 protein anchor was also demonstrated in purified cell walls with MAb 6-8C. The P1 C-terminal anchor, however, could

not be clearly demonstrated with the polyclonal MBP-P1COOH antisera until purified cell walls were treated further with mutanolysin, thus allowing P1 fragments carrying the epitopes, previously masked by the cell wall to be exposed by the extraction with the SDS-PAGE sample buffer. These data also suggest that the P1 C-terminus is protected from proteolysis by its association with the cell wall. These results confirm our previous findings obtained from a genetic approach, indicating that the P1 protein was anchored in the cell wall via the C-terminus (16).

Whether antigen P1 was anchored to the peptidoglycan or to a carbohydrate moiety within the cell wall had not been clearly demonstrated (23). To resolve this question, the carbohydrate was removed from purified cell walls by boiling in TCA, leaving the peptidoglycan skeletal structure. These two fractions, the extracted carbohydrate and the peptidoglycan, were tested for the presence of the P1 C-terminal anchor. The P1 anchor was demonstrated from the peptidoglycan- but not the carbohydrate-fraction (Fig. 4.4). The association of the P1 C-terminus with the peptidoglycan is possibly via a covalent linkage since P1 cannot be dissociated from intact isolated cell walls by hot SDS-mercaptoethanol extraction.

The absence of the P1 110 kDa protein in the carbohydrate and peptidoglycan samples (Fig, 4.4, lanes 1 and 2) may be the result of low levels of protein associated with cell wall after TCA extraction. The amino acid composition analysis of the peptidoglycan samples indicated only pmole levels of protein, which is below the level of detection for the Western blot assay. In addition, the 110 kDa band (Figure 4.3) may have resulted from incomplete trypsinization of the P1 protein from the cells. Trypsin cleaves on the carboxy

side of lysine and arginine but cleaves more slowly when either amino acid is followed by an acid residue, and does not cleave at all if lysine and arginine are followed by proline (3). There are a large number of lysine and arginine residues that are potential trypsin cleavage sites. However, the proline-rich region of P1 would prohibit cleavage at a number of these sites. Nevertheless, two potential cleavage sites at amino acid residues 910 and 941 which would generate putative trypsin fragments of ca. 75.2 kDa and 71.8 kDa fragments (17). Thus, it is quite possible that the 110 kDa P1 fragment may have been further cleaved to the 70 kDa fragment during the trypsinization of the isolated cell walls. It is also possible that trypsin could have been present in the cell wall extracts, although it is unlikely that bound trypsin would have influenced the Western blotting results because it does not react with the antibodies used.

The treatment of the cell wall or peptidoglycan with mutanolysin generated more intense immunoreactive bands in Western blotting than untreated samples, suggesting that cleavage of the glycan chain causes the P1 C-terminus to be more readily extractable in hot SDS. This supports our hypothesis that the P1 anchor is buried in the peptidoglycan structure since epitopes for antibodies recognizing the C-terminal portions of the molecule are exposed after muralytic-enzyme treatment. These results are in agreement with previously described protein insertion sites, such as, the cell wall, for the *S. pyogenes* M6 protein (24) and the peptidoglycan, for the *S. aureus* protein A (31).

Linkage of the *S. aureus* protein A to the peptidoglycan has been identified as an amide bond between the threonine residue found in the protein

A LPETGE sequence, and the terminal glycine of the peptidoglycan pentaglycine cross-bridge (31). This linkage is believed to form following cleavage of the bond between the threonine and glycine of the LPETGE motif via a mechanism similar to the transpeptidation reaction (34). Schneewind and co-workers (31, 34) speculated that amide-bond formation required an enzyme, termed sortase, although the nature of the enzyme has not been characterized. A similar sortase enzyme may be responsible for the bonding of P1 to the *S. mutans* peptidoglycan. Linkage of P1 to the peptidoglycan may also occur via amide-bond formation involving different amino acid residues. The *S. mutans* peptidoglycan is classified as A3α, with the lysine residue of the stem-peptide participating in the peptide cross-bridge (30). The LPXTGX consensus sequence in P1 is LPNTGV (17) and the cross-bridge present in the *S. mutans* peptidoglycan is predominantly L-Lys- Ala $_{2\text{-}3}$ (30a), therefore, the amino acids participating in the P1 amide bond may involve the LPN(T)GV threonine and the terminal alanine residue of the cross-bridge. Alternatively, the P1 protein may be linked via a phosphodiester linkage to the muramyl- C6 hydroxyl group as has been described for wall teichoic acids (32a).

The difference between the retainer NG8 strain and the non-retainer NG5 strain was evident when hot SDS-mercaptoethanol cell wall extracts were examined for the presence of P1 (Fig. 4.2). P1 present in cell walls of the non-retainer *S. mutans* NG5 strain, could be readily extracted by hot SDS-mercaptoethanol, while P1 in the retainer NG8 strain cell walls remained firmly attached after similar treatment. This suggests that P1 in the NG5 strain was not covalently bonded to the cell wall. Since there are no anomalies in the gene encoding the NG5 P1 protein, structural analysis of the peptidoglycan was made. Data obtained from amino acid analysis of NG8 and

NG5 peptidoglycan indicated identical molar ratios of the predominant amino acids, glu : ala : lys, suggesting no apparent peptidoglycan structural differences. The reason why the NG5 P1 fails to anchor to the cell wall may be due to a defect in the putative assembly mechanism. Such a failure would explain the predominance of P1 in the culture supernatant of P1 non-retainer strains such as NG5. There are reports that strains of *S. mutans* lose the P1-retainer phenotype upon sub-culturing (18, 28). However, the mechanism responsible for these changes is not known. The non-retainer *S. mutans* strain may represent a mutant defective in 'sortase' activity, hence providing an ideal system for the identification and study of this putative enzyme.

Acknowledgments

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Chapter 5

General Discussion

Overview

A significant role for Gram-positive cell-surface proteins in disease and bacterial identification was first described for *Streptococcus pyogenes* by Lancefield in 1928, who recognized the antiphagocytic effect of M protein (24, 11). Later, the use of whole cell extracts, which included cell wall protein and carbohydrate antigens, proved to be of considerable value in identifying and typing *Streptococcus* species. This early demonstration of antigens in relation to disease and the identification of *Streptococcus* spp. stimulated considerable research into the nature of the antigens, and also the cell wall composition of Gram-positive bacteria. Major advances in defining cell wall structure were made in the 1960s, spurred on by interest in the activity of penicillin and its effect on cell wall synthesis (44). Concurrently, improvements in sample preparation and the resolving power of the electron microscope revolutionized the understanding of the ultrastructure of the cell wall (43), while chemical analysis of cell wall composition by bacterial taxonomists (9) has culminated in detailed structural information on wall lipid, carbohydrate and peptidoglycan (45, 46). The results of the studies by taxonomists and those involved in defining the aetiology of bacterial infections provided a firm base for further work using the evolving techniques of protein analysis and molecular biology. Today, application of these latter techniques to the determination of cell wall and surface protein composition has led to the modification of surface proteins for the expression of effective vaccine epitopes, ultimately to reduce bacterial virulence and the occurence and spread of infections.

Fundamental to further developments in these areas is an appreciation of the biology of surface proteins. This is reflected in the interest shown in the structure of cell surface proteins, especially with respect to the mechanism of cell-wall localization and anchoring (20). Conservation of structural C-terminal anchoring domains of cell-surface proteins from Gram-positive bacteria suggests a 'universal' function for the anchor. If this is so, the construction of proteins with a common anchor but with different functional domains should be possible, and the anchor of one species should be effective in another. This concept is the basis for use of human avirulent commensal microorganisms as live vaccines for the presentation of specific antigens of an infectious agent (29). The stable expression and correct localization of chimeric proteins on the surface of the cell is crucial to the success of such a vaccine strategy. The studies reported in this thesis have provided a more detailed understanding of the mechanism of protein anchoring, towards the eventual goal of live vaccine development.

Evidence for a common anchoring mechanism and implications for vaccine development

To determine whether the C-terminal protein anchor of *S. mutans* P1 protein shared a common function and structure with other Gram-positive cell surface proteins, experiments were designed to test the ability of the P1 C-terminus to anchor in other Gram-positive bacteria. The *spaP* gene was introduced into both *Streptococcus gordonii* DL-1 and *Enterococcus faecalis* UV202 and expression of P1 was evaluated by ELISA and Western blotting

assay. These experiments clearly showed that the majority of the recombinant P1 (rP1) was cell-associated. Furthermore, Western blotting of rP1 prepared from mutanolysin-treated cells produced multiple reactive bands. This was similar to the banding patterns observed for *S. aureus* protein A mutanolysin preparations (49), which were attributed to the covalent attachment of cell-wall peptidoglycan to the C-terminus of protein A. Therefore, these observations imply a common anchoring mechanism, in which the C-terminus of rP1 is able to function as a normal cell-surface protein anchor in both *S. gordonii* DL-1 and *E. faecalis* UV202.

However, unlike *S. mutans* SM3352 and *S. gordonii* DL-1 transformants, expression of recombinant P1 in *Enterococcus faecalis* diminished with subculturing, as has been noted by others (18). This phenomenon may indicate loss of the plasmid. Plasmid DNA isolations from transformed *Enterococcus* cells and southern hybridization experiments should determine whether the plasmid was present, lost or incorporated into the chromosome. The polymerase chain reaction would provide an alternate method for detecting the plasmid encoded sequences by designing primers specific for *spaP* and the plasmid encoded antibiotic resistance marker.

Reciprocal experiments were also performed, with a chimeric protein consisting of the C-terminal 211 amino acids of the *Staphylococcus aureus* fibronectin-binding protein and the N-terminal 706 amino acids of P1. As determined by ELISA and Western blotting, the chimeric protein was cell associated in both *S. mutans* SM3352 and *S. gordonii* DL-1. The results from these experiments indicated that a heterologous C-terminal anchor could function to localize P1 in streptococci and provide additional

proof of a common mechanism for the assembly of surface proteins onto the cell surface (48). This commonality of surface protein anchoring is important for the expression of heterologous antigens, and the construction of live recombinant oral vaccines based on commensal organisms. To use this strategy for the construction of vaccines, it is important to know the stability and level of expression of the heterologous antigen on the carrier cell, in order to obtain the optimal immune response in a host (10, 29). Therefore recombinant vaccine organisms should be monitored during repeated *in vitro* subculturing for stability of heterologous antigen expression prior to *in vivo* studies.

Non-pathogenic streptococcal species are part of the normal microflora that colonizes human mucosal surfaces (16), and are ideal candidates for expression of heterologous antigens for immune protection against a variety of diseases, especially where specific stimulation of mucosal immunity is desired. In one such example, the human papillomavirus E7 protein was expressed in *S. gordonii*, a common plaque organism (39). This was accomplished by construction of a gene fusion in which sequences encoding the *S. pyogenes* M protein C-terminal anchor and the E7 protein were ligated (39). The chimeric fusion protein was expressed on the *S. gordonii* cell surface and shown to be immunogenic in mice. The malaria blood-stage antigen has also been expressed as a chimeric surface protein in *Staphylococcus xylosus*, where the C-terminus of protein A anchored the fusion protein (19). It is therefore possible to exploit the ability of Gram-positive bacteria, including oral streptococci, to anchor proteins onto their cell surface for the expression of potential vaccine antigens.

Evidence in support of a role for the cell wall-spanning domain in protein retention

The carboxy terminal cell wall anchor of Gram-positive surface proteins is highly conserved with respect to the wall-spanning domain, the LPXTGX consensus sequence, the hydrophobic membrane-spanning domain and the charged tail (12, 13), suggesting a strong selective pressure for retaining this intact structure during evolution, and that the mechanism for protein anchoring is common among Gram-positive bacteria (22). Generally, the role of each of these C-terminal domains in cell anchoring is poorly understood and therefore, the role of the wall-spanning domain was investigated.

Earlier experiments by Pancholi and Fischetti (37), identified the *S. pyogenes* M protein wall-associated domain embedded within the cell wall, suggesting it was intercalated within the peptidoglycan, but not necessarily covalently linked to it. Amino acid composition and sequence analysis of the cell-wall embedded protein fragment established that the LPXTGX consensus sequence, hydrophobic domain and charged tail sequences were absent (34). Collectively, these studies implied a potential role for the cell wall-spanning domain in the retention of these proteins on the cell surface, possibly by intercalation within the rigid peptidoglycan network.

To test this hypothesis in *S. mutans*, a *spaP* deletion mutant was created, encoding a recombinant P1 protein (rP1) which included the entire cell wall-spanning domain, but not the LPXTGX motif, the hydrophobic membrane-spanning domain or the charged tail. Although, the rP1

protein was surface-expressed, in both the P1-negative mutant *S. mutans* 3352 and *S. gordonii* DL-1 as indicated by whole cell ELISA assay, the majority of rP1 was found in the culture supernatants. However, some of the rP1 expressed on *S. mutans* 3352 remained cell-associated, even during cell-wall purification, confirming that the wall-spanning domain functioned in this case, to retain some of P1 protein on the cell surface. These results are suggestive of the wall-spanning domain being intercalated within the peptidoglycan as previously suggested for M protein (37). Further data in support of a role for the wall-spanning domain in protein retention was obtained by creation of another mutant lacking the entire C-terminal anchor sequences, including the wall-spanning domain. In this situation, the recombinant protein was found entirely in the culture supernatants when expressed in *S. mutans* 3352 and *S. gordonii* DL-1.

Previous studies by Schneewind *et al.* (49), using PhoA fusion proteins had not shown a role for the *S. aureus* protein A wall-spanning domain in protein anchoring, suggesting differences between *S. mutans*, *S. pyogenes* and *S. aureus* in that respect. The high proportion of proline, glycine, threonine and serine residues found in the wall-spanning domain in *S. pyogenes* are believed to intercalate the peptidoglycan structure, presumably contributing to M protein retention (37). Further studies would be required to determine whether these specific amino acid residues are responsible for the retention-function associated with the wall-spanning domain in *S. mutans* and *S. pyogenes*.

Cell wall anchoring is not universal to all proteins sharing common C-terminal domains

Possession of the C-terminal anchor structure does not preclude other possible surface protein-cell associations. Examples exist where proteins with a typical anchor structure are found extracellularly in the culture supernatant. The extracellular location of these proteins could be the result of a protein-releasing mechanism. Generally, the reason for this extracellular location of surface proteins is unclear, although very subtle differences in the C-terminus have been identified in some cases. In one such example, the *S. mutans* enzyme fructanase, described by Burne and Penders (7), surprisingly possesses a typical C-terminal anchor but is almost exclusively found in the culture supernatant. All of the C-terminal domains could be identified in this enzyme, including an additional stretch of 4 charged amino acids, C-terminal to the LPXTGX motif. Sequence analysis comparisons with other Gram-positive surface proteins also identified a significant lack of proline and glycine residues within the wall-spanning domain of fructanase, residues that are proposed to intercalate the wall peptidoglycan. Thus, their absence may affect the anchoring of the fructanase with the cell surface. This phenomenon provides further evidence that in some cases, a wall-spanning domain with a high content of proline and glycine residues can play a role in protein retention. The function of the four charged residues preceeding the hydrophobic membrane-spanning domain is unknown, but their presence may indicate a signal for some alternative function. Since the fructanase serves a different cellular function from that of adhesins, such subtle differences in

the structure of the C-terminus and the cellular association of the enzyme might be expected.

A similar example of surface protein release is observed in *S. salivarius*. The *S. salivarius* fructosyltransferase (FTF) remains cell-associated but is released from the cell in the presence of the substrate (32). Sequence analysis of the gene encoding FTF indicated the presence of a C-terminal anchor possessing the characteristic C-terminal domains, but without the LPXTGX consensus sequence (41). The authors suggested that FTF was not anchored to the cell wall but to the membrane and that release was triggered in the presence of substrate. Anchoring and substrate-induced release of FTF was also observed when the gene was introduced into *S. gordonii*, suggesting that the processing events for anchoring and release of this enzyme were similar in these two related streptococci. Therefore, the anchoring mechanism for FTF appears different from those of other surface proteins that possess the LPXTGX within their C-terminal anchor structures. Whether ligand-binding results in a conformational change in FTF, that signals the release of the enzyme from the cell surface has yet to be investigated.

One example where changes in the C-terminal structure could not account for differences in cell asssociation is the *S. pyogenes* serum opacity activity (SOF), which is found both cell associated and also in the culture supernatant (40). The C-terminus of the serum opacity protein demonstrates a typical cell anchor with all four characterisitic domains. Rakonjac *et al.* (40) suggest that the release of the serum opacity factor protein from the cell surface is the result of a previously described specific proteolytic activity within the C-terminus (40). Alternatively, the authors

suggested multiple forms of SOF due to differential expression of SOF from more than one gene. This explanation is plausible, since tandem arrays of cell surface binding proteins have already been identified for the M and M-like genes (6,8).

In the examples given above for extracellular location of surface proteins, all proteins expressed enzymatic activities. The precise sub-cellular location of surface-protein anchoring is believed to vary, and for the majority of proteins, the subcellular anchor site is unknown. These observations suggest that retention of surface proteins on the cell surface can involve other mechanisms in addition to the C-terminal domains, although the nature of these mechanisms remains obscure. There has however, been speculation for the role of putative enzyme activities in both the anchoring and release of cell surface proteins

Putative enzyme activities involved in the attachment or release of surface proteins

The earlier experiments of Pancholi and Fischetti (38) describing the isolation of the wall-spanning fragment of M protein suggested enzymatic involvment based on the absence of the membrane-spanning hydrophobic domain and charged tail from the embedded M protein fragment. This cleavage of the C-terminal domains was thought to be due to a putative endogenous enzyme activity responsible for the release of M protein from the protoplast. This thiol- and pH-dependent enzyme was termed the membrane anchor cleaving enzyme (MACE) (38), and the LPXTGX motif

was proposed to represent a specific site for recognition and cleavage from the peptidoglycan (38, 49).

More recently, a similar enzyme activity, surface protein-releasing enzyme (SPRE), which is believed responsible for release of P1 protein from protoplasts, has been described in *S. mutans* (25). However, it has also been proposed that the release of P1 from protoplasts could be an artifact, resulting from autolytic digestion of the cell wall (22). The findings in the current study suggest that P1 is anchored to the cell wall, and not the cytoplasmic membrane. Therefore, the association of P1 with protoplasts would not be expected. As mentioned previously, it has been proposed that multiple forms of the *S. pyogenes* serum opacity factor (SOF) may exist (40). The presence of two forms of P1, a transmembrane- and a cell wall-associated form, could explain the differences in results from different workers, although this aspect of P1 has not been explored.

Experiments by Navarre and Schneewind (34) with *S. aureus* protein A have also indicated a putative enzyme activity, referred to as sortase, in attachment of protein A to peptidoglycan and the development of a model describing protein A attachment (see Fig. 5.1). Sortase is believed to catalyze the cleavage between the threonine and the glycine residues of the LPX(T)GX motif, and the subsequent formation of an amide bond with the terminal glycine of the peptidoglycan cross-bridge (34). The formation of this linkage is believed to proceed by a mechanism analogous to the transpeptidation reaction in peptidoglycan cross-linking (55). A similar mechanism may operate in *S. mutans* P1 anchoring. The regulation of these enzyme activities may also influence the association of the protein

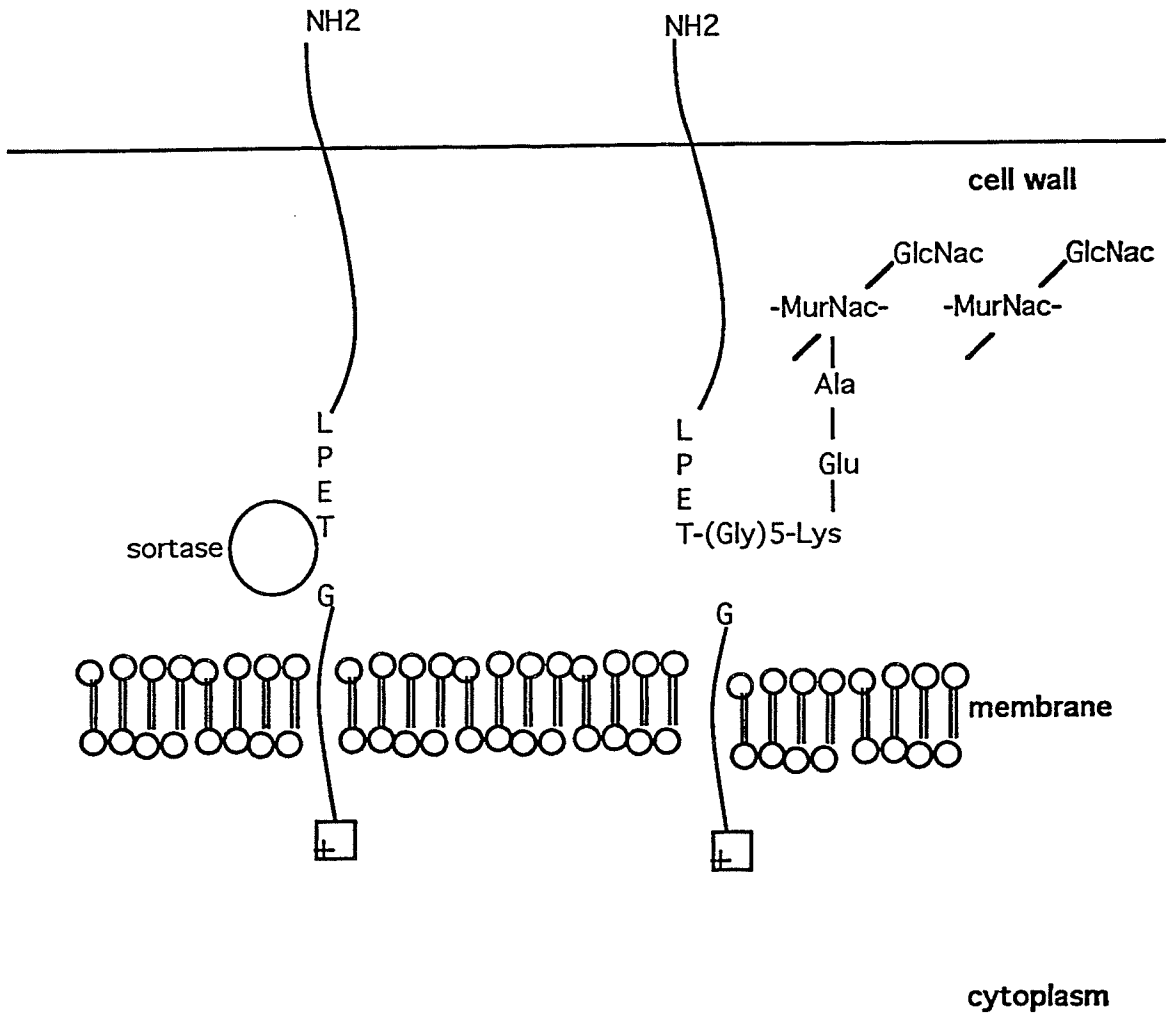

Figure 5.1. Proposed mechanism for the linking of *S. aureus* protein A to the peptidoglycan. The sortase enzyme recognizes the LPXTGX motif of protein A and cleaves the peptide bond between the threonine and glycine residues. The threonine carboxyl is amide-linked to the peptidoglycan cross-bridge, covalently linking protein A to the cell wall.

with the cell surface. To date, these hypothetical enzymatic activities have not been demonstrated.

Identification of the surface-protein anchoring site within the cell envelope

Generally, the precise cellular location of the anchor has not been identified for the majority of surface proteins. Early studies during the late 1950s, concerning the localization of M protein in streptococcal cells showed M protein bound to the cell wall (summarized in 24). Streptococcal protoplasts and L-forms devoid of cell walls, produced M protein which diffused into the medium, indicating that the protein was not bound in the absence of a cell wall (24). However, in contrast, later studies by Pancholi and Fischetti (38) indicated that newly synthesized M protein was retained by protoplasts, suggesting the presence of an anchor site in the membrane. These results conflict with more recent studies with *S. aureus* protein A and also with the data presented in this thesis, both of which are in support of a role for cell wall peptidoglycan in anchoring of Gram-positive cell surface proteins.

The advantage to having surface proteins linked to the cell wall instead of anchoring via the membrane is unknown. Kehoe (22) speculates that cell-wall linkage might prevent excessive protein bridging between the cell wall and the plasma membrane, preventing interference with cell wall growth. Wall association may also prevent membrane saturation with hydrophobic/charged tail anchors, which could otherwise interfere with other membrane functions. The cleavage of the C-terminal hydrophobic/charged tail and assembly of the surface protein into the cell wall must be coordinated with the other anchoring steps and with

peptidoglycan assembly. Kehoe (22) suggests that variations in the efficiency of this coordination might explain why some wall-associated proteins are more readily released from the surface than are others and why release can vary among strains and growth conditions. In the present study, it was hoped that characterization of P1-retainer and non-retainer strains of *S. mutans* might provide a means of explaining these discrepancies.

S. mutans strain NG8 is a retainer strain in which the P1 protein is predominately cell-associated. Cells of *S. mutans* NG8 were fractionated into cell wall, membrane and cytoplasmic fractions, and probed for the presence of P1, with both monoclonal antibodies and antisera specific for the P1 C-terminus. The anchor was demonstrated within the cell wall, but could not be detected in either the cytoplasmic- or membrane fractions. This same situation was also observed for the *S. aureus* protein A anchor. Mutanolysin, which cleaves the β(1,4)- glycosidic linkage between the N-acetyl glucosamine and the N-acetyl muramic acid residues of the peptidoglycan backbone chain (43), was used to determine whether the P1 anchor was released from treated cell walls. Reactivity in Western blotting experiments with the P1 anchor-specific antisera indicated that the epitopes recognized by the antisera were exposed only after treatment of cell walls with mutanolysin. This result suggests that the anchor is buried within the wall, causing epitope masking.

To more precisely map the location of the P1 anchor within the purified cell walls, the wall carbohydrate was extracted with trichloroacetic acid, leaving only the peptidoglycan structure. Both of these fractions were analyzed for the presence of the anchor with the monoclonal antibody and

the specific antisera. The *S. mutans* P1 anchor was identified within the peptidoglycan, also the site for *S. aureus* protein A anchoring, but was not observed in the TCA-extracted carbohydrate. Whether the assembly of P1 is analogous to that identified for protein A, into the peptidoglycan cross-bridge, is unknown. The model proposed by Navarre and Schneewind (34) could apply universally to these characteristic Gram-positive surface proteins since a certain percentage of peptide cross-bridges are not cross-linked (46) and therefore have unsubstituted amino groups (at the ε-amino group of the stem-peptide lysine residue and at the terminal amino acid of the cross-bridge) which could participate in peptide bonds with surface proteins. However, an additional protein attachment site could exist within the peptidoglycan. Since muramyl 6-hydroxyl groups can be phosphorylated (46), protein attachment could occur via a putative phosphodiester linkage at this site. The precise location of the *S. mutans* P1 protein attachment site within the peptidoglycan has not been determined.

Does S. mutans P1 assemble into the peptidoglycan by a mechanism similar to that described for S. aureus protein A?

S. mutans P1 may be assembled by a mechanism similar to that described for staphylococcal protein A, where the protein is inserted into the peptidoglycan by covalent linkage to the amino acid cross-bridge following cleavage of the LPXTGX motif (34, 47). If P1 does follow the same assembly pathway as does protein A, then the LPXTGX should be cleaved by the putative 'sortase'. It would, therefore, be important to isolate the P1 anchor from the peptidoglycan and obtain the amino acid sequence to determine whether cleavage of the LPXTGX motif has occurred. By using the antisera generated specifically to the P1 C-terminus to produce a

chromatography affinity matrix, the anchor could be purified from mutanolysin-digested peptidoglycan and subjected to amino acid sequence analysis. Alternatively, an experiment previously described by Navarre and Schneewind (34) used chimeric proteins to demonstrate the cleavage between the threonine and glycine residues of the LPXTGX motif. The chimeric proteins consisted of the N-terminus of enterotoxin B, followed by the LPXTGX, membrane-spanning domain, and charged tail of protein A, with the C-terminus consisting of either maltose-binding protein or β–lactamase. Placement of the wall-anchoring signal in the middle of the polypeptide resulted in cleavage of the precursor polypeptide at the LPXTGX motif, and cell-wall anchoring of the N-terminal fragment, while the C-terminal fragment remained in the cytoplasm. The C-terminal fragment was isolated from the cytoplasm and the N-terminus subjected to sequence analysis to identify the cleavage site within the LPXTGX motif (34). The cleavage site, between the threonine and the glycine of the LPXTGX motif was later confirmed by electrospray mass spectrometry (47).

Subsequent studies established that muramidase-solubilized *S. aureus* protein A is observed as multiple bands on coomassie-stained SDS-polyacrylamide gels (PAGE) (48). However, when muramidase-solubilized *S. aureus* protein A is further treated with lysostaphin, to cleave the peptidoglycan pentaglycine cross-bridge (43), protein A runs as a uniform band on stained SDS-PAGE gels (48). These observations suggest that protein A is cross-linked to the peptidoglycan via the pentaglycine cross-bridge and the multiple bands seen in muramidase-solubilized protein A are due to retained peptidoglycan fragments. Muramidase-solubilized *S. mutans* P1 protein is also observed as multiple bands on

stained SDS-PAGE gels suggesting a similar peptidoglycan linkage, although there is no enzyme activity equivalent to lysostaphin that could specifically cleave the *S. mutans* peptidoglycan cross-bridge. Thus, it is not possible to determine whether P1 would run as a uniform band on SDS-PAGE gels after subsequent enzyme treatment. Amino sugar composition analysis of the purified P1 wall-embedded fragment might identify peptidoglycan fragments that remain associated with muramindase-purified P1 protein.

To confirm that *S. aureus* protein A was linked to the peptidoglycan cross-bridge, Schneewind *et al.* (47) constructed a hybrid protein from the enterotoxin B and maltose-binding protein sequences utilizing the protein A LPXTGX motif, membrane-spanning domain and charged tail sequences at the C-terminus. The hybrid protein, designated MalE-Cws, had two engineered trypsin-cleavage sites and was isolated from *S. aureus* cell walls with muramidase and lysostaphin and purified on amylose resin. Some of the purified MalE-Cws was treated with trypsin, generating peptide fragments. By using electrospray mass spectrometry, the mass of the full length MalE-Cws and the trypsin fragments were calculated. The calculated mass for the C-terminal trypsin fragment, which included the LPXTGX, was larger than expected, indicating the addition of 3 linked glycine residues, which was confirmed by amino acid sequence analysis. These experiments demonstrate that the *S. aureus* protein A is amide-linked via the C-terminal carboxyl of threonine to the free amino group of the pentaglycine cross-bridge within the *S. aureus* cell wall. Similar experiments with the *S. mutans P1* may help to confirm the covalent linkage of P1 to the peptidoglycan.

Comparison of P1-retainer and non-retainer strains as a means of identifying defects in cell wall chemistry and in the protein localization pathway

The proposed model (34) for the mechanism of surface protein anchoring is similar to the penicillin-sensitive transpeptidation reaction of cell wall synthesis (55). Transpeptidases preferably cleave peptide bonds composed of D-amino acids (15). The transpeptidase cleaves the stem peptide at the D-alanyl-D-alanine residues, and catalyses the formation of a peptide bond between the newly liberated carboxyl of D-alanine, and the free amino-group of another wall peptide (56). Further evidence would be required to confirm this model for surface protein anchoring in *S. aureus*. Whether this model applies to the *S. mutans* P1 protein is unknown. Isolation and characterization of the enzymatic activity involved in the LPXTGX cleavage and amide bond formation to the peptide cross-bridge in *S. mutans* would provide evidence in support of this model. This may be studied by use of naturally occuring strains of *S. mutans* which liberate P1 into the culture medium.

S. mutans non-retainer strains which naturally do not retain P1 protein on the cell surface may be useful in determining the mechanism for P1 protein anchoring. In previous studies, sequence analysis and hybridization experiments detected no obvious differences in the P1 gene or P1 anchor of the *S. mutans* non-retainer NG5 strain, when compared to the wild-type NG8 strain (3). The underlying cause(s) responsible for these two phenotypes is unknown. However, studying the differences between retainer and non-retainer strains may give some clues on the mechanism

of P1 anchoring, especially if the non-retainer strain represents a natural mutant defective in a specific enzyme involved in the assembly mechanism. The non-retainer phenotype may represent structural differences in the cell wall, and not necessarily a defective or absent assembly enzyme. To address this possiblity, the retainer and non-retainer strains were compared in order to identify any differences in P1 association and cell wall chemistry. Initial experiments involved subcellular-fractionation of both strains to determine whether the site for P1 anchoring differed between the two strains. The high level of P1 protein detected in culture supernatants of the non-retainer strain may have resulted from increased gene expression and high cell wall turnover. The fractionation of the non-retainer strain did not indicate the association of P1 in either the cytoplasm or membrane fractions. Some of the P1 was identified with the walls but unlike the retainer strain, the P1 was removed by boiling with SDS. These results suggest a difference in the cell association of P1 between the retainer and the non-retainer strains.

The cell walls from both strains were purified to begin comparison of the wall chemistry. Wall carbohydrate was removed by boiling trichloroacetic acid, and the carbohydrate recovered by acetone precipitation. Quantitation of the carbohydrate, before and after extraction did not reveal any dramatic differences in carbohydrate content. The purified peptidoglycan from both the retainer and non-retainer strains was also analyzed for amino acid composition. The amino acid composition and the molar ratios (1 : 3.6 : 1) of the predominant amino acids (glu : ala : lys) were similar for both peptidoglycan samples. These values are very similar to those reported by Bleiweis *et al.* (4) for three serotype c strains and the

results of Schleifer *et al.* (46a). Therefore, these results suggest that structural differences in peptidolgycan cannot account for the non-retainer phenotype.

In spite of these similarites, other subtle structural differences may exist in the peptidoglycan that could influence P1 retention, but would not have been detected by composition analysis. For example, some stem peptides contain D-alanyl-D-alanine at their C-terminal ends, while others lack the terminal D-alanine. These differences are usually due to the activities of a transpeptidase or a carboxypeptidase (51). Structural differences can also be associated with the extent of N-acetylation and O-acetylation of the muramic acid residues. Loss of acetyl groups can occur via specific peptidoglycan hydrolases, which can render the peptidoglycan structure more (or less) susceptible to hydrolysis by other intrinisic or extrinisic enzymes (51). The O-acetylation of muramic acid residues in some peptidoglycans makes the peptidoglycan resistant to the muramidase, egg-white lysozyme (43, 51). The O-acetyl and N-acetyl content of peptidoglycan can be determined by measuring the amount of acetate liberated from solubilized peptidoglycan after mild alkali or acid treatment (52). How O- or N-acetylation might affect P1 assembly is unknown, possibly excessive acetylation of peptidoglycan could sterically hinder the enzyme responsible for cross-linking P1 into the cross-bridge resulting in P1 secretion. If however, phophodiester linkages were involved in P1 assembly, then the level of peptidoglycan phosphorylation may be relevant.

Another factor that could influence cell-surface protein assembly is the extent of peptidoglycan cross-linking. Cross-linking of peptidoglycan

occurs via a transpeptidation reaction involving the stem peptide and the amino acid cross-bridge. It is postulated that the loss of the terminal D-alanine residue is directly coupled to cross-linking, and that the bond energy is utilized in the transpeptidation reaction (51, 55). The free amino group of the terminal bridge amino acid is cross-linked with the penultimate D-alanine of the stem peptide, displacing the terminal D-alanine. The degree of cross-linking can be determined by HPLC gel filtration fractionation of muramidase-treated peptidoglycan into monomers and peptide cross-linked dimers, trimers and other oligomers (53). The relationship between the degree of polymerization of peptidoglycan oligomers and the elution time is a linear one which can be used to give a cross-linking index (53) Cross-linking is calculated as the ratio of monomeric to dimeric plus trimeric oligomers. Changes in the degree of cross-linking have been shown to occur in laboratory-generated penicillin-resistant *Streptococcus pneumoniae* mutants (50). A decrease in cross-linking, presumably due to reduction in transpeptidation, could also affect the incorporation of P1 into the wall, since a similar transpeptidation mechanism has been postulated for the assembly of proteins into the peptidoglycan. Alternatively, the degree of cross-linking would be reduced if the terminal D-alanine-D-alanine stem peptides were absent. Given these circumstances, the energy required for transpeptidation and presumably P1 assembly, would then be unavailable. The degree of cross-linking could also affect the retention of P1 on the cell surface by not allowing for sufficient intercalation of the wall-spanning domain within the peptidoglycan. Presumably the three dimensional peptidoglycan structure provides the necessary framework for protein attachment.

There are other means for determining peptidoglycan structure which include X-ray diffraction, nuclear magnetic resonance and infrared spectroscopy (51, 33). Both X-ray and NMR studies suggest that the glycan strands run parallel to the cell surface and that the peptide bonds extend from the glycan chains in different directions (51). Data obtained from NMR studies suggest that the glycan chains are relatively rigid while the peptide crossbridges are mobile allowing for the flexibility required for expansion and contraction of the wall (51). These methods would be useful for comparison of the retainer (NG8) and non-retainer (NG5) cell wall structures and in providing additional evidence that no structural differences exist between the peptidoglycan of the P1 retainer (NG8) and non-retainer (NG5) strains.

Variation in peptidoglycan structure has also been associated with acquisition of antibiotic resistance (1, 14, 17, 50). Significant structural alterations in the peptidoglycan stem and cross-bridge amino acids have been documented to occur in both vancomycin- and penicillin resistance (14, 17). Penicillin resistance in *S. pneumoniae* has been associated with a change from a linear to a branched hydrophobic stem/cross-bridge structure caused by the addition of amino acids at the epsilon amino group of the lysine at stem position 3. These changes in structure can result from the production of enzymes with altered substrate specificity, as has been demonstrated in vancomycin resistant enterococci. The mode of action of glycopeptide antibiotics is based on their ability to bind to the carboxy terminal L-amino acid D-alanyl-D-alanine of the stem peptide in the disaccharide-peptide repeating unit (DSP), the precursor of the peptidoglycan structure. Glycopeptide binding inhibits transglycosylation

and consequently, formation of the backbone glycan chain (42). Subsequently, the transpeptidation reaction which imparts rigidity to the cell wall is also inhibited, and results in the termination of cell wall growth. Vancomycin-resistant enterococci avoid inhibition through synthesis of a modified carboxy terminal stem peptide consisting of D-alanine-D-lactate which results from the activity of novel D-ala:D-ala ligase-related enzymes (32). The new peptidoglycan precursor, D-alanine-D-lactate has reduced vancomycin affinity (31) and is accepted in the subsequent steps leading to peptidoglycan synthesis and assembly (17). It would be interesting to compare the antibiotic sensitivties of the *S. mutans* retainer and non-retainer strains to determine whether the inability to retain P1 on the cell surface rendered the non-retainer strain more susceptible to antibiotics.

Enzyme activities as explanation for the P1 non-retainer phenotype

The non-retainer *S. mutans* strain may represent a mutant defective in the putative enzyme activity (sortase) which incorporates P1 into peptidoglycan, providing an ideal system for the identification and study of this putative enzyme. If the defect were due to the lack of sortase activity, then it may be possible to rescue the mutation by complementation experiments. This would involve the introduction of DNA sequences encoding the enzyme activity into the non-retainer strain. Transformation of *S. mutans* with exogeneous DNA can be readily achieved, since *S. mutans* is naturally transformable. A genomic DNA library constructed from DNA isolated from the retainer strain could be readily introduced into the non-retainer strain via a shuttle vector. Screening for P1-retaining and

P1-non-retaining phenotypes could be accomplished by using appropriate antisera and/or saliva-coated magnetic beads (36).

The release of proteins from the cell surface may be due to an endogenous protease activity. Non-retention of P1 by *S. mutans* could be due to the over production of SPRE. If P1 assembly into the cell wall proceeds by a mechanism similar to that described for the *S. aureus* protein A, then cleavage of P1 at the LPXTGX motif would be expected, and P1 isolated from the non-retainer strain would be truncated at the LPXTGX. However, this is not the case, since P1 released from the surface of the non-retainer strain is the full length protein (3). The site of SPRE cleavage within the P1 molecule is unknown but cleavage may occur at a site other than the LPXTGX. If release of P1 from the non-retainer involves SPRE activity, then it should be possible to isolate peptidoglycan-anchored P1 before it is released by the SPRE.

There are other enzymatic activites which have been identified in cell wall turn-over which modify the cell wall structure. Peptidoglycan autolysins are endogenous enzymes that hydrolyze the bonds responsible for maintaining the peptidoglycan integrity. Peptidoglycan hydrolases, transglycosylases, transpeptidases and carboxypeptidases are involved in the reproduction of cells and therefore, require precise regulation. The non-retainer phenotype could therefore, result from abnormal regulation of these enzyme activities.

The universality of protein anchoring represents a target for development of therapeutic agents

A potential benefit to understanding the mechanism of surface protein anchoring is in providing new targets for antimicrobial therapy. This is of particular relevance to the escalation of antibiotic resistant Gram-positive organisms (35). Enzymes responsible for the insertion of proteins into the peptidoglycan are potential targets, since they may influence the cell wall structure. For example, inability to anchor cell surface proteins could influence the integrity of the cell wall, rendering the organism more susceptible to other antimicrobial agents. Inhibiting P1 anchoring could render the organism more susceptible to chlorhexidine. Chlorhexidine has been shown to reduce adhesion of *S. mutans* to saliva-coated HA and to rupture membranes leading to cell lysis (30), although the specific mode of action is unknown. However, there is speculation that chlorhexidine reacts with negatively-charged membrane components (23). Therefore perturbation of cell wall assembly, exposing the membrane, could facilitate the action of chlorhexidine and enhance its effect on Gram-positive bacteria.

Another potential benefit would be to interfere with the ability of the pathogen to colonize host tissues. This critical step in the initiation of infection typically depends on expression of a number of different cell-surface adhesins. Therefore, the 'universality' of the protein anchoring mechanism among Gram-positive organisms represents an ideal target for the development of antimicrobial agents, since a large number of surface proteins with characteristic C-terminal anchors have been identified and

implicated in adhesion events. As organisms may possess the ability to express multiple adhesins, an antimicrobial agent directed at the mechanism for protein anchoring would target all surface proteins with a comparable C-terminus. Moreover, the anchoring structure investigated in this study also provides some specificity for Gram-positive bacteria, since it has not been identified in Gram-negative organisms.

Since colonization of the oral cavity by *S. mutans* (21, 27, 28, 54) can be diminished or abrogated by P1-specific antibodies, then disruption of the P1 anchoring mechanism could also abrogate adhesion to salivary pellicle-coated tooth surfaces (2, 5, 26). Eliminating *S. mutans* from the oral community by inhibiting P1 adhesion could influence the ecology of the mouth and reduce the cariogenic potential of the plaque community. However, there are a number of oral bacteria which possess similar anchor structures for assembly of surface proteins. If the assembly of surface proteins involves a common mechanism, inhibition of this mechanism could also interfere with colonization of the oral cavity by commensal organisms which help to exclude potentially pathogenic organisms from the plaque population. Therefore, specific prevention of *S. mutans* colonization would require the identification and targeting of the assembly step(s) unique to P1 anchoring. This strategy of modulation of surface-protein anchoring may be of greater benefit in the prevention or elimination of mono infections with Gram-positive organisms, such as invasive *S. pyogenes* tissue infections. In infections associated with the resident flora, modulation of surface protein-anchoring may, as mentioned previously, have the potential to upset the normal balance of the resident microbial community.

Summary

In summary, evidence in support of a common mechanism for Gram-positive surface protein anchoring is presented. The role of the individual anchor domains, including the wall-spanning domain, the LPXTGX motif, the hydrophobic membrane-spanning domain and the charged tail remains speculative. However, evidence is presented to suggest that the wall-spanning domain of *S. mutans* P1 protein facilitates retention of the protein on the cell surface, possibly by intercalation with the peptidoglycan. This observation is supported by data for *S. pyogenes* M protein where the wall-spanning domain was purified from trypsin-treated cell walls. The site of *S. mutans* P1 anchoring was determined as the peptidoglycan, as has also been shown for *S. aureus* protein A. Collectively, the C-terminal protein domains, recognized in a large number of Gram-positive surface proteins, function to anchor the protein to the cell surface. A model describing the mechanism for anchoring of the *S. aureus* protein A has been proposed and involves recognition of the C-terminal domains, enzymatic cleavage of the LPXTGX motif and subsequent amide linkage to the peptidoglycan cross-bridge (Fig. 5.1). This is an attractive model and is based on the transpeptidation reaction of cell wall assembly. However, the enzyme activity required for assembly has not been identified.

The natural occurrance of *S. mutans* P1 non-retainer strains suggests a defect in protein assembly. There are no obvious differences between the retainers and non-retainers in the *spaP* gene or in the protein isolated from culture supernatants. The defect in P1 anchoring may involve differences in the cell wall structure or in the assembly

mechanism. However, examination of the wall chemistry did not identify any apparent differences in the non-retainer strain when compared to the retainer strain. These results suggest that the non-retainer represents a mutant in surface protein assembly which could be used to facilitate future studies on the mechanism of P1 protein anchoring. Further analysis of the cell wall structure will be required to ascertain whether the non-retainer phenotype represents a mutation in protein assembly.

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