Streptococcal Adhesion and Colonization

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STREPTOCOCCAL ADHESION AND COLONIZATION

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ABSTRACT: Streptococci express arrays of adhesins on their cell surfaces that facilitate adherence to substrates present in their natural environment within the mammalian host. A consequence of such promiscuous binding ability is that streptococcal cells may adhere simultaneously to a spectrum of substrates, including salivary glycoproteins, extracellular matrix and serum components, host cells, and other microbial cells. The multiplicity of streptococcal adherence interactions accounts, at least in part, for their success in colonizing the oral and epithelial surfaces of humans. Adhesion facilitates colonization and may be a precursor to tissue invasion and immune modulation, events that presage the development of disease. Many of the streptococcal adhesins and virulence-related factors are cell-wall-associated proteins containing repeated sequence blocks of amino acids. Linear sequences, both within the blocks and within non-repetitive regions of the proteins, have been implicated in substrate binding. Sequences and functions of these proteins among the streptococci have become assorted through gene duplication and horizontal transfer between bacterial populations. Several adhesins identified and characterized through *in vitro* binding assays have been analyzed for *in vivo* expression and function by means of animal models used for colonization and virulence. Information on the molecular structure of adhesins as related to their *in vivo* function will allow for the rational design of novel acellular vaccines, recombinant antibodies, and adhesion agonists for the future control or prevention of streptococcal colonization and streptococcal diseases.

Key words. Streptococcus, adhesion, bacterial cell wall proteins, colonization, salivary glycoproteins, oral infections.

A *Streptococcal Success Story*

Over 20 species of streptococci are currently designated, all of which are uniquely associated with the colonization of warm-blooded mammals. Most species of streptococci may be considered commensals—organisms that are, for the most part, tolerated by, and live in relative harmony with, the host. As commensals, streptococci are found usually at mucosal surfaces such as those within the oral cavity, upper respiratory tract, and gastro-intestinal tract. Accordingly, it is at these sites that streptococci can, given the appropriate conditions, initiate localized or systemic diseases. In recent years, a greater understanding has been acquired of the factors contributing to both the commensal success and pathogenic potential of streptococci. This information has come about partly as a result of technological advances in genetic and molecular analyses of Gram-positive bacteria, and partly because the emergence of highly virulent or drug-resistant strains of streptococci has caused concern and focused attention in this arena. In essence, the streptococcal success story can be attributed to three factors: (1) the ability of these organisms to adhere to almost any surface present in their natural environment; (2) their ability to rapidly utilize available nutrients under fluctuating environmental conditions; and (3) their ability to tolerate, resist, or even destroy host immune defenses. However, it is an indictment of our current lack of knowledge that many streptococcal diseases still cannot be controlled adequately. For example, rheumatic heart disease, a sequela to *Streptococcus pyogenes* (a group A streptococcus) infection, is once again on the increase worldwide. Recently, what has perhaps caused the greatest alarm and attracted much media attention are outbreaks of group A-streptococcal-associated necrotizing fasciitis and toxic shock syndrome, life-threatening complications of streptococcal disease. Since the late 1980's, descriptions of group A streptococ-

cal bacteremia, shock, and organ failure have increased. Another example is that, second to common cold viral infections, middle ear infections caused by *Streptococcus pneumoniae* (known as pneumococcus) account for the most frequent childhood visits to physicians in the USA. Perhaps the most far-reaching example is the failure to prevent, or in many instances even control, dental caries and inflammatory periodontal disease. These are the most prevalent diseases throughout the world and are the direct result of the activities of bacterial cells that have colonized and accumulated to form plaque on teeth. Among the earliest and most abundant colonizers of such surfaces are the streptococci.

Streptococci mostly cause disease from an initially commensal state, so the crucial factors that induce the disease state could be considered to be, at least in part, those that act to perturb the balance between microbe and host. These factors in many instances are well-recognized. They include environmental influences such as diet (*e.g.*, sugar promoting dental caries), breakdown of host defenses (*e.g.*, immunodeficiencies), or microbial determinants such as virulence components (*e.g.*, cytotoxins). For the purposes of this article, we will distinguish between virulence factors of microbes that operate in establishing the disease state, and microbial colonization factors that may be considered as prelude players to disease. The activities of the colonization determinants are highly significant: Without them, the streptococci would neither colonize in the first instance, nor, in some cases, be able to progress from a commensal to a pathogenic state. Hence, without the appropriate colonization factors, many of the streptococcal-associated diseases would not eventuate.

It is generally accepted that to become established within the human body, *i.e.*, colonize, a micro-organism must undergo a series of interactive events. The first of these is a loose physical association with the surface of a tissue. This permits stronger and more permanent bonds to be established through the binding activities of adhesins (on the microbial cell surface) to complementary receptors on the host surface. Once the microbial cells are anchored, ensuing growth and cell division result in accumulation. As these processes are repeated at distant sites, the host becomes colonized. Colonization implies a long-term association and may precede invasion of the tissue or of host cells. Tissue or cell invasion can contribute to localized disease or facilitate the spread of the organisms to distant body sites. It is important to note that the train of events *viz.* association, adhesion, colonization, invasion, and spread is not rigidly demarcated temporally, but is overlapping. This is why it is necessary to appreciate the mechanisms involved at all these stages if the diseases caused by organisms such as streptococci are to be ultimately combated successfully.

Colonization factors may be defined as those determinants that allow bacteria to: (1) associate with and adhere to host tissue surfaces; (2) utilize available nutrients and undergo cell division *in vivo*; (3) compete or cooperate with other species of micro-organisms in the immediate environment; and (4) contend with host defense mechanisms. With respect to the process of adhesion, which much of the following article will address, the streptococci in general have a quite extraordinary repertoire of adherence properties (see Jenkinson, 1995b). Most of the substrates to which streptococcal cells have been shown to bind are depicted in Fig. 1. This diagram has been constructed to help summarize the spectrum of substrate recognition by streptococci. A given host environment, such as the human oral cavity, may contain most, if not all, of the receptors shown in Fig. 1. A consequence of such promiscuous binding ability is that streptococcal cells may adhere simultaneously to many different receptors. This is accomplished through the streptococci expressing on their cell surfaces multiple adhesins (Hasty *et al.*, 1992) and receptors for adhesins on other cells. Streptococcal adhesion *in vivo* may thus involve an intricate network of interactions, the greater the number of which, the tighter the adhesion. Presumably, organisms with multiple adhesion-mediating molecules have an evolutionary advantage within an environment containing multiple cognate receptors. The activity of a series of distinct adhesins may have implications for the outcome of the adhesive interaction in regard to both the formation of structural units such as aggregates and the nature of molecular "cross-talk" with other cells.

Streptococcal Adhesion to Salivary Components

The main portal of entry by streptococci into the human body is through the oral cavity. Some species preferentially colonize not the oral cavity but the nasopharynx and enter the upper respiratory tract. A few species colonize the skin and can penetrate at this site, *e.g.*, group A streptococci. Streptococci adhere avidly to all the various surfaces present at these sites. Salivary components coat the tooth surface, buccal and lingual epithelial cells, and all other biological or non-biological (*e.g.*, prosthetic) surfaces within the mouth. Streptococcal adhesion to oral surfaces results primarily from initial binding of cells to deposited salivary components. These include not only the secretory products of the salivary glands but also: bacterial products, such as glucan polysaccharides, to which streptococci can bind *via* glucan-binding proteins (GBPs) (Banas *et al.*, 1990) (Table 1); dietary components, which may include lectins and other molecules, that interact with bacterial cell surfaces; serum products that originate as an exudate in gingival crevicular fluid; and other compounds entering whole saliva from gastric

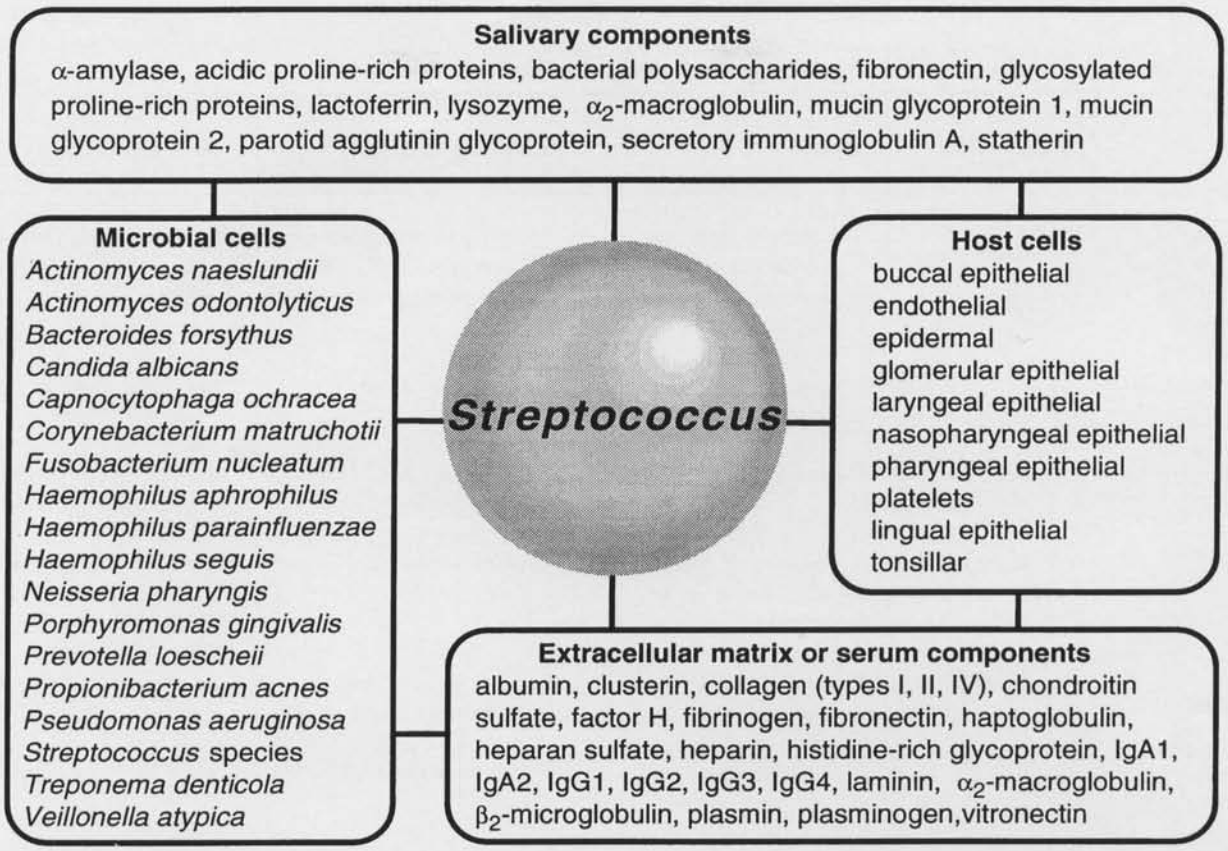

Figure 1. Multiple adhesins expressed by streptococci enable them to adhere to a wide range of substrates. Not all streptococcal species adhere to all the substrates shown, and streptococci may utilize different mechanisms in binding to a specific substrate. The adherence interactions depicted are discussed in the text or in other recent reviews cited. The Fig. is reproduced from Jenkinson (1995b) in updated form with permission from the publisher (Stockton Press).

or respiratory reflux (Gibbons, 1984; Malamud, 1985; Terpenning *et al.*, 1993). Streptococci also bind glycoproteins in the fluid phase, and this may enhance aggregation, which is part of the clearance process. It is also important that the deposition of salivary proteins or glycoproteins onto an adherent organism provides a substrate for additional microbial adherence. Salivary protein and glycoprotein binding is relevant both to the initiation of colonization and to ensuing microbial proliferations and accumulations resulting in the complex biofilm known as dental plaque. The pivotal nature of streptococcal interactions with salivary proteins and glycoproteins in oral colonization has been well-recognized, and the identification of the streptococcal adhesins and receptors involved has been studied intensely. In addition, there are components of saliva, such as lysozyme, that may participate in streptococcal adhesion to salivary pellicle without the need for a specific cognate receptor (Tellefson and Germaine, 1986). In these cases, overall electrostatic charge interactions may be more important than specific adhesin-receptor binding. An extensive review of oral bacterial interactions with salivary molecules was published relatively recently (Scannapieco, 1994), and much of that information will not be repeated here. Instead, we will discuss briefly new developments in the understanding of the multi-faceted nature of streptococcal-saliva interactions, and evidence that streptococci may distinguish between fluid-phase or immobilized forms of salivary glycoprotein receptors.

Sanguis group streptococci (S. *sanguis*, S. *gordonii*, S. *oralis*, S. *parasanguis*, and related species) are the predominant colonizers of salivary pellicle, and diverse adhesins for salivary molecules have been identified in these organisms (see Table 1). In many instances, the salivary components of the binding interactions are also known. Although a large number of distinct adhesins have been described, many of the studies have utilized different streptococcal strains, and hence the range of adhesins possessed by individual organisms remains to be determined. Nonetheless, it is apparent that sanguis group streptococci are endowed with multiple adhesins for salivary molecules. As with adherence to host cells described below, this configuration can be theorized to confer numerous advantages to the streptococci. Multiple adhesins will result in more avid binding and may improve the probability of an individual cell engag-

TABLE 1

Streptococcal Protein Adhesins Recognizing Salivary Components

Adhesin	Approx. Mol. Mass (kDa)	Species	Substrate	Reference
Antigen I/II family [Ag I/II, AgB, P1 (SpaP), PAc, Sr, SpaA, PAg, SspA, SspB, SoaA]	160-175	*S. mutans, S. sobrinus, S. gordonii, S. oralis, S. intermedius*	Parotid salivary agglutinin glycoprotein (SAG); salivary glycoproteins; proline-rich proteins	Jenkinson and Demuth (1997)
LraI family [FimA, SsaB]	35	*S. parasanguis, S. sanguis*	Salivary component(s) in pellicle	Jenkinson (1994)
Antigen complex	80, 62, 52	*S. gordonii* G9B	73-kDa submandibular salivary protein	Lamont *et al.* (1988a,b); Rosan *et al.* (1989)
Amylase-binding proteins	65, 20, 15, 12.5	*S. gordonii, S. mitis, S. salivarius, S. crista, S. anginosus*	salivary α-amylase	Douglas (1990); Scannapieco (1994); Haase and Scannapieco (1996)
Surface lectins	96, 70, 65	*S. oralis, S. mitis*	Salivary glycoprotein (N-acetylneuraminic acid)	Murray *et al.* (1986)
Fimbrial adhesin	*ca.* 200	*S. parasanguis* FW213	Salivary component(s) in pellicle	Elder and Fives-Taylor (1986)
EP-GP binding protein	27	*S. salivarius* HB	Extra parotid glycoprotein	Schenkels *et al.* (1993)
Glucan-binding protein (GBP_{74})	59	*S. mutans* Ingbritt	Dextran, glucan (1,6-α-linked)	Banas *et al.* (1990)
GBP_{59}	*ca.* 59	*S. mutans* SJ	Glucan (1,6-α-linked)	Smith *et al.* (1994)
Dextranase inhibitor (Dei)	31	*S. sobrinus* UAB108	Dextran	Sun *et al.* (1994)

ing a salivary receptor. Furthermore, adhesins of differing specificities may allow the bacteria to adhere to bind different saliva-coated surfaces, since there is evidence to suggest that salivary molecules are not uniformly distributed in the oral cavity (Dawes and MacPherson, 1993). Adhesion to saliva-coated surfaces may exhibit positive co-operativity (Nesbitt *et al.*, 1982), inasmuch as initially bound streptococci can alter the salivary substrate either by creating new receptor sites for other organisms or by excreting bacterial molecules that will attach to the substrate and also promote streptococcal attachment (Van der Mei *et al.*, 1993). The availability of more than one adhesin would tend to favor this arrangement. Finally, the sequence in which the streptococcal adhesins engage the salivary molecules may be important. Kinetic analysis of S. *sanguis* attachment to saliva-coated hydroxylapatite suggests that there is a time-dependent shift from low-affinity binding to an interaction mediated by higher-affinity adhesins (Cowan *et al.*, 1986).

The streptococcal salivary adhesins that have been described in most detail are the LraI family of polypeptides and the antigen I/II family of polypeptides. Extensive information on both of these families has been provided in recent reviews (Whittaker *et al.*, 1996; Jenkinson and Demuth, 1997). LraI polypeptides contain the consensus signal peptidase II cleavage site Leu-x-x-Cys for lipid modification (Jenkinson, 1992) and subsequent anchoring in the cytoplasmic membrane. It is worth noting, however, that FimA (Table 1), a member of the LraI family and implicated in the binding of S. *parasanguis* to salivary pellicle and to fibrin (Burnette-Curley *et al.*, 1995), might be situated membrane-distal at the tips of fimbriae (Fenno *et al.*, 1995). In brief, antigen I/II family polypeptides are of similar sizes [1500-1566 amino acid (aa) residues] and contain an amino (N)-ter-

minal signal sequence and a conserved carboxyl (C)-terminal region, including the motif Leu-Pro-x-Thr-Gly considered important for cell-wall anchorage (Schneewind *et al.*, 1995). Members of this family have been variously named and include Ssp (S. *gordonii*), AgB/P1/SpaP/PAc (S. *mutans*), and SpaA/PAg (S. *sobrinus*) (Table 1). Antigen I/II polypeptides bind a mucin-like salivary component named salivary agglutinin glycoprotein (SAG) in a lectin-like interaction (Demuth *et al.*, 1990). As mentioned above, binding to salivary glycoproteins deposited on oral surfaces is likely to promote colonization, whereas binding to salivary glycoproteins in solution will lead to the formation of bacterial aggregates that are more readily expelled from the mouth by expectoration or by swallowing. Several physically distinct salivary glycoprotein-binding regions are present within antigen I/II polypeptides (reviewed in Jenkinson and Demuth, 1997). Differing affinities of these regions for the immobilized or soluble forms of salivary glycoproteins may allow cells to adhere to immobilized glycoproteins despite the presence of excess fluid-phase receptors in saliva. This may represent a means by which the streptococci have subverted a host defense mechanism to enhance their own colonization, and partially explain the predominance of streptococci in early plaque.

Another means by which bacteria may be able to bind to more than one receptor simultaneously is by spatially separating adhesins on the cell surface. For example, α-amylase bound from solution localizes to the polar and septal regions of S. *gordonii* cells, and hence may not impede the activities of other cell-surface adhesins (Scannapieco, 1994). This arrangement may also prevent amylase-mediated cross-linking of cells and subsequent aggregation. Amylase deposited in the enamel salivary pellicle can thus promote the adhesion of streptococci possessing amylase-specific adhesins (Scannapieco *et al.*, 1995).

Secretory immunoglobulin A (sIgA) in saliva inhibits bacterial cell adhesion and promotes bacterial agglutination. Many of the early colonizers of the human oral cavity produce IgA1 proteases which cleave specifically at the hinge region of human IgA1. This may indicate that IgA1 protease-producing streptococci have an ecological advantage, particularly in early life when they become established in the oral cavity under exposure to sIgA antibodies in mother's milk. Cleavage of IgA1 by IgA1 protease separates the immunoglobulin Fc portion from the monomeric antigen-binding domain. In principle, the proteases are therefore capable of destroying the bacterial-agglutinating activity of sIgA which is important for the antibody-mediated inhibition of colonization of mucosal and tooth surfaces. In addition, although streptococci may become coated with Fab fragments as a result of IgA1 protease activity, this does not appear to interfere with adhesion (Kilian *et al.*, 1996).

Streptococcal Adhesion to Other Cells

As befits their status as successful colonizers of the human host, streptococci possess multiple adhesins for human cells (see Fig. 1 and Table 2). Although *in vitro* experiments have demonstrated adhesion to a variety of epithelial cells, such cells *in vivo* will be coated usually with mucus or salivary secretions and matrix proteins. The extent to which there is direct contact between the bacteria and the host cells is therefore uncertain. Nonetheless, the dynamic interaction of streptococci with host cells and their extracellular molecules is one factor that will determine the outcome of the encounter between bacteria and host. Direct bacterial cell binding to host cells may also, in addition to facilitating colonization, be a precursor to intracellular invasion and immune modulation (see below), events that may presage the development of disease. Adherence may therefore be one mechanism by which the streptococci can manipulate the biological activity of the host cell. Thus, streptococci (and other adherent bacteria) possess a variety of adhesins with differing receptor specificities and affinities that can potentially impinge to various degrees upon a diversity of receptor-dependent host cell biochemical pathways. The ability to orchestrate host cell responses will have numerous advantages for streptococci in the complex ecosystems in which they must survive.

In the case of pneumococcal adherence, host respiratory epithelial cells undergo inflammatory activation that enhances adhesion and invasion of virulent pneumococcal strains (Cundell *et al.*, 1995a). Pneumococci can adhere to two classes of host glycoconjugate receptors present on buccal epithelial cells, resting pneumocytes and vascular epithelial cells of the alveolar capillaries (Cundell *et al.*, 1995c). Oligopeptide permease components encoded by the *plp*A and *ami* genes may be involved in these interactions, which appear to predominate during asymptomatic carriage of the organism. Stimulation of pneumocytes or vascular cells with the pro-inflammatory cytokines tumor-necrosis factor (TNF)-α and interleukin-1 (IL-1), or the chemokine thrombin, induces expression of a G-protein-coupled receptor, platelet activating factor (PAF). Virulent strains of pneumococci can interact with PAF *via* bacterial cell wall phosphorylcholine, an event that effects elevated cell adherence and also subsequently induces intracellular invasion by the pneumococci (Cundell *et al.*, 1995a). Inflammatory activation of the host cells to express the PAF receptor is thus an important step in the transition from asymptomatic carriage to pathogenicity.

Group A streptococci are responsible for a number of clinical conditions, including pharyngitis, impetigo, puerperal sepsis, and the sequelae acute rheumatic fever and acute glomerulonephritis. Group B streptococci are the leading cause of neonatal pneumonia, sepsis, and

TABLE 2

Streptococcal Protein Adhesins Recognizing Eucaryotic or Procaryotic Cells

Adhesin	Approx. Mol. Mass (kDa)	Species	Substrate	Reference
Emm6 (M6)	48	*S. pyogenes* M-type 6	human laryngeal epithelial cells (HEp-2 cell line); human keratinocytes (HaCat cell line)	Wang and Stinson (1994); Perez-Casal *et al.* (1995); Okada *et al.* (1995)
Emm24 (M24)	54	*S. pyogenes* M-type 24	HEp-2 cells; mouse oral epithelial cells	Courtney *et al.* (1994)
PrtF1	70	*S. pyogenes* M-type 6	Hamster tracheal epithelial cells	Hanski *et al.* (1992)
Platelet aggregation-associated factor (PAAP)	150	*S. sanguis*	Platelet surface glycoproteins	Erickson and Herzberg (1987, 1995)
Glucosyltransferase	*ca.* 153	*S. gordonii* G9B	Human umbilical vein epithelial cells (HUVEC)	Vacca-Smith *et al.* (1994)
Antigen C fibrillar glycoprotein (HAF)	220-280	*S. salivarius* HB	Human buccal epithelial cells	Handley (1990)
Fibrillar antigen B (VBP)	320	*S. salivarius* HB	*Veillonella parvula*	Weerkamp and Jacobs (1982)
CshA, CshB	259, ca. 245	*S. gordonii* DL1 (Challis)	*C. albicans, A. naeslundii, S. oralis*	Holmes *et al.* (1996); McNab *et al.* (1996)
Antigen I/II family [SspA, SspB]	171, 160	*S. gordonii* DL1, M5	*S. mutans, P. gingivalis, C. albicans, A. naeslundii*	Lamont and Rosan (1990); Lamont *et al.* (1994); Holmes *et al.* (1996); Jenkinson and Demuth (1997)
Lral family [ScaA]	35	*S. gordonii* PK488	*A. naeslundii* PK606	Whittaker *et al.* (1996)
Coaggregation-mediating adhesin	100	*S. gordonii* DL1, PK488	*S. oralis, Streptococcus* spp.	Clemans and Kolenbrander (1995); Whittaker *et al.* (1996)

meningitis. The pathogenesis of these streptococcal infections is multi-step, but adhesion of group A and B streptococci to epithelial cells may be a crucial determinant. In addition, both of these streptococcal groups have been shown to be able to invade epithelial cells (Rubens *et al.*, 1992; LaPenta *et al.*, 1994). The presence of lipoteichoic acid (LTA), composed of a repeating poly(glycerol phosphate) and covalently attached fatty acids, on the streptococcal cell surface has long been implicated in adhesion of streptococci to epithelial cells (see Ofek and Doyle, 1994). While the evidence is convincing that LTA is involved in adhesion of group A streptococci to buccal and pharyngeal cells, and to HEp-2 (laryngeal) tissue culture cells (Table 2), it seems that LTA plays little role in group B streptococcal adhesion (Tamura *et al.*, 1994). Fibronectin, deposited on the surfaces of human buccal cells, is a major receptor for group A streptococci (Courtney *et al.*, 1996). Adhesion of bacteria to buccal cells is thought to occur by the sequential binding of LTA and one (or more) of the fibronectin-binding adhesins (see Table 3) present on the streptococcal cell surface (discussed in the next section). M protein, a fibrous cell-surface protein on S. *pyogenes* with antiphagocytic properties, is not essential for binding of streptococci to human buccal, pharyngeal, or tonsillar cells (Caparon *et al.*, 1991; Courtney *et al.*, 1994). However, binding of S. *pyogenes* to HEp-2 cells may be mediated in part by M protein which recognizes two fucose-containing glycoprotein receptors (Wang and Stinson, 1994). This M-protein-mediated adhesion may be augmented by LTA and the fibronectin-binding adhesins. M6 protein also mediates adhesion of S. *pyogenes* to keratinocytes

(Table 2) which may be significant in skin infections (Okada *et al.*, 1995).

Upon gaining access to the bloodstream, many streptococci (particularly oral strains) infect the heart valves and endocardium, causing endocarditis (Weinberger *et al.*, 1990). In addition to adherence to extracellular matrix proteins, as described below, streptococcal pathogenicity in endocarditis and related diseases involves interaction with platelets. The molecular nature of S. *sanguis*-induced platelet aggregation has been studied extensively by Herzberg and colleagues. S. *sanguis* is capable of inducing platelet aggregation through a variety of distinct surface components (Herzberg, 1996). A trypsin-sensitive class I adhesin mediates initial attachment of the bacteria to the platelets. Aggregation is then induced by an antigenically distinct class II adhesin, platelet aggregation-associated protein (PAAP). PAAP is a 150-kDa glycoprotein, readily broken down into smaller fragments that retain activity, and contains a collagen-like immunodeterminant (Erickson and Herzberg, 1987; Gong *et al.*, 1995). The relationship of PAAP to the S. *gordonii* laminin-binding protein (Table 3), which also carries a collagen-like immunodeterminant (Sommer *et al.*, 1992), has not been established. Binding of PAAP to its cellular receptor activates signal transduction pathways that result in secretion of granule contents, including ATP and Ca^{2+}, actin-myosin-dependent shape changes, and expression of receptors for soluble plasma proteins such as fibrinogen (Herzberg *et al.*, 1993). The functional domain of PAAP that mediates binding to platelets may reside in the PGE(P/Q)GPK sequence consensus motif shared by the platelet-binding domain of collagens (Erickson and Herzberg, 1993). The outcome of these sequential binding and aggregation steps is the irreversible cross-linking of platelets into a thrombus, a major pathogenic determinant in severe streptococcal endocarditis and occlusive vascular disease.

An event important in determining the consequences of bacterial colonization, that occurs early in the interactive process, is the induction of pro-inflammatory or anti-inflammatory cytokines and chemokines in response to the bacteria. Although bacterial lipopolysaccharide (LPS) is the best-studied cytokine-stimulating cell component [for which the generic term "modulin" has recently been proposed (Henderson *et al.*, 1996)], Gram-positive molecules including streptococcal adhesins may also act as modulins. Binding of the S. *mutans* Sr (antigen I/II) protein to the carbohydrate moieties of monocyte membrane glycoproteins elicits the production of TNF-α, IL-1β, and IL-6 (Soell *et al.*, 1994). Furthermore, antigen I/II polypeptides and cell-surface rhamnose-glucose polymers may mediate the ability of oral streptococci (including mutans group streptococci, S. *anginosus*, S. *constellatus*, S. *gordonii*, S. *intermedius*, S. *milleri*, S. *mitis*, and S. *salivarius*) to stimulate the production of IL-8 in epithelial (KB) cells and of IL-8 and IL-6 in endothelial cells (Vernier *et al.*, 1996). These adhesive events thus serve to activate the immune response, which may be a double-edged sword. In the periodontal tissues, this level of stimulation may be required to maintain a balance with the mass of streptococci in plaque. Alternatively, cytokine stimulation may contribute to tissue pathology once the bacteria have gained access to the bloodstream, and the effects of cytokines on adherence and invasion of virulent pneumococci are described above.

Streptococci that inhabit the mucosal and related areas of humans will encounter not only host cells but also other microbial cells. This is particularly relevant in the oral cavity, where over 300 species of bacteria can be found densely packed in dental plaque. Although oral streptococci can adhere avidly to the salivary pellicle on the tooth surface, and comprise a major proportion of early plaque, they possess the ability to adhere to numerous other plaque organisms (see Fig. 1). These co-adherence partners include other early plaque colonizers such as *Actinomyces* species and later colonizers such as *Porphyromonas gingivalis* and *Bacteroides forsythus* (Lamont *et al.*, 1992; Whittaker *et al.*, 1996; Yao *et al.*, 1996). The later colonizers often require an anaerobic environment and hence rarely appear in plaque until after a layer of initial colonizers has developed. It is unlikely, therefore, that the late arrivals make contact with enamel salivary pellicle, and so adherence to streptococci is considered an important colonization mechanism (Slots and Gibbons, 1978). Since many of the co-adhering organisms are potential periodontal pathogens (such as P. *gingivalis*, B. *forsythus*, and *Treponema denticola*), inter-bacterial binding to streptococci has significant implications for the development of periodontal diseases. In addition to assisting colonization, adherence among different species of bacteria may also facilitate nutritional relationships between organisms. For example, *Veillonella* species utilize as a carbon source the organic acid metabolic end-products of the streptococci (Distler and Kröncke, 1981).

In some cases, the binding between bacterial cells can result in the formation of co-aggregates (*e.g.*, *Streptococcus-Actinomyces* interactions) (Whittaker *et al.*, 1996), but in others aggregates do not appear to be formed consistently (*e.g.*, S. *gordonii*-P. *gingivalis* interaction) (Lamont *et al.*, 1992). Factors that influence the formation of aggregates subsequent to initial binding can be hypothesized to include the number of adhesins participating in the interaction and the avidity of their binding, the ratio of the cells of each species available for binding, and steric constraints. Co-aggregation may, like saliva-mediated aggregation of streptococci, facilitate clearance of organisms from the oral cavity. It seems plausible, then, to propose that co-adherence mecha-

nisms may differ, depending upon whether the partner cells are in suspension or bound to a surface. This may explain why some *Actinomyces* species that co-aggregated with streptococci *in vitro* did not colonize streptococcal-coated surfaces *in vivo* (Skopek *et al.*, 1993). By contrast, P. *gingivalis* has been shown to localize on streptococcal-rich plaque when introduced into the mouths of human volunteers (Slots and Gibbons, 1978).

The molecular basis of streptococcal adhesion to other microbial species is under investigation in a number of laboratories. Cell-surface structures such as fibrils on S. *crista* and S. *salivarius* are important for binding to Gram-negative partners and to *Actinomyces* species (see Handley, 1990; Whittaker *et al.*, 1996), although the adhesins themselves may be distinct from the structural components of the fibrils (Correia *et al.*, 1996; Jameson *et al.*, 1995). Streptococcal adhesins for *Actinomyces* that have been identified are documented in Table 2. The best-characterized adhesins are the multi-functional proteins SspA, SspB, and CshA. These polypeptides act in concert to mediate adhesion of S. *gordonii* to A. *naeslundii*, S. *oralis*, and *Candida albicans* (Holmes *et al.*, 1996; McNab *et al.*, 1996). Binding of streptococci to C. *albicans* further involves recognition, by the yeast cells, of a linear streptococcal cell-wall phospho-polysaccharide (Holmes *et al.*, 1995). Similar polysaccharides mediate co-aggregation of streptococci and *Actinomyces* cells and are generally referred to as receptors for the *Actinomyces* adhesins. The structures of these receptors, which contain N-acetyl-galactosamine (GalNAc) linked to galactose (Gal), either GalNAcβ-(1→3)Gal or Galβ-(1→3)GalNAc, have been described in detail elsewhere (Whittaker *et al.*, 1996). The Ssp (antigen I/II family) proteins also mediate binding of S. *gordonii* to P. *gingivalis*. In this reaction, antigen I/II protein cleavage products, that could be generated by P. *gingivalis* proteases, have functional activity (Lamont *et al.*, 1994). Therefore, it is possible that P. *gingivalis* may induce the presentation by streptococci of adhesins that promote co-adherence. Deletion analysis has localized the P. *gingivalis* binding domain of SspB to a region spanning amino acid (aa) residues 1168-1250 at the C-terminal end of the molecule (Lamont *et al.*, 1995).

Co-adherence among strains of oral bacteria *in vivo* occurs in the presence of oral fluids such as salivary secretions and gingival crevicular fluid. Molecules present in these fluids that are bound by bacteria have the potential to modulate co-adherence. Salivary molecules including lysozyme and histatins, together with serum molecules such as fibrinogen, can inhibit adhesion of S. *mitis*, S. *oralis*, or S. *crista* to P. *gingivalis* (Murakami *et al.*, 1991; Nagata *et al.*, 1994). Hence, these molecules may comprise part of a host defense mechanism against colonization by P. *gingivalis*. Adherence of S. *crista* to B. *forsythus* and T. *denticola* is also inhibited by saliva, but not by serum (Yao *et al.*, 1996). These interactions may be favored, therefore, in the gingival crevice or periodontal pocket, the sites of pathogenic action of B. *forsythus* and T. *denticola*. By contrast, S. *gordonii* adhesion to P. *gingivalis*, and most *Streptococcus-Actinomyces* co-aggregations can proceed in the presence of saliva (Kolenbrander and Phucas, 1984; Lamont *et al.*, 1992).

In addition to a potential protective role in the inhibition of co-adherence, salivary molecules can also enhance such binding by acting in a bridging capacity. Bacterial products such as glucans present in saliva are important in this respect, and GBPs present on mutans group streptococci promote inter-bacterial binding (Drake *et al.*, 1988) as well as adhesion of these bacteria to glucans within pellicle. Saliva promotes binding between P. *gingivalis* and some strains of S. *gordonii* (Lamont *et al.*, 1992), while SAG mediates binding between S. *mutans* and S. *gordonii* (Lamont and Rosan, 1990; Lamont *et al.*, 1991). These diametrically opposed activities of saliva serve to illustrate the complexity of the oral ecosystem and also provide important insights into possible mechanisms to control the colonization of pathogenic organisms.

Streptococcal Adhesion to Extracellular Matrix Molecules and Serum Components

The eucaryotic extracellular matrix is composed of a complex mixture of macromolecules involved in cell-cell adhesion, proliferation, migration, and differentiation. Matrix components such as fibronectin, fibrinogen, collagen, and proteoglycans (*e.g.*, heparin) serve not only as receptors for host cell functions but also for the attachment of micro-organisms. Damage to host tissues exposes matrix components, and this then allows microbial colonization to occur. Microbial cell-surface proteins that interact with extracellular matrix components have been designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), and a comprehensive review of the structure and function of MSCRAMMs was published quite recently (Patti *et al.*, 1994). Accordingly, we will consider only new information on streptococcal MSCRAMMs and describe their structural and functional relationships to other prominent surface proteins of streptococci that bind human serum components such as albumin, plasmin, and immunoglobulins.

Species representative of all streptococcal Lancefield groups have the ability to adhere to extracellular matrix components, whereas the ability to bind immunoglobulins and other serous proteins is really a feature of the more virulent group A, B, and C organisms. Interactions between streptococcal cells and host tissue proteins are highly complex. Matrix or serum proteins can present more than one binding site for bacterial cells. Availability of these binding sites may be influenced by whether the proteins are in soluble phase or

bound to a surface, or complexed with other host proteins or proteoglycans. Furthermore, it is now apparent that the various species of streptococci, and even different strains within a species, may express different arrays of adhesins. Thus, streptococci do not all use the same mechanisms to adhere to host proteins.

Fibronectin (Fn) is found in soluble form in most body fluids or as a component of the extracellular matrix. Fn is localized to the fibrin matrix during wound healing, and binding to Fn is used by streptococci in skin wound colonization. Streptococcal adherence to Fn exposed or deposited at sites of endothelial damage may be important in the pathogenesis of infective endocarditis (Baddour, 1994). Salivary Fn coats oral epithelial cells and is present in salivary pellicles, where it may be bound by oral streptococci (Babu and Dabbous, 1986). Group A streptococci can bind both soluble and bound forms of Fn, whereas strains of S. *gordonii*, S. *pneumoniae*, and group B streptococci appear to bind Fn only when this is immobilized onto a surface (Lowrance *et al.*, 1988; Tamura and Rubens, 1995; van der Flier *et al.*, 1995; McNab *et al.*, 1996). This could be explained by the bacteria recognizing determinants that are revealed only when Fn is bound to a surface. Alternatively, the affinity of Fn-binding may be low, and so binding cannot be demonstrated with Fn in solution. In some cases, the presence of soluble Fn increases the extent of cell binding to immobilized Fn, suggesting that cells have different affinities for soluble and insoluble forms.

There are conflicting data on the proteins involved and on the mechanisms of binding of group A streptococci to Fn. For many years, a cell-surface complex of LTA and fibrillar M protein was thought to be the major Fn-binding component. While M (Emm) protein is now known not to be essential for Fn binding by S. *pyogenes* (Caparon *et al.*, 1991), a role for LTA in conjunction with other Fn-binding proteins such as FNB54 (Table 3) is still recognized (Courtney *et al.*, 1996). The identification, to date, of six different genes encoding Fn-binding proteins in S. *pyogenes* (see Table 3) has cast confusion over the relative roles of LTA and these various proteins in the overall process. An inherent problem with attempts to decipher this puzzle [and a similar problem arises later in discussions of the immunoglobulin-binding proteins (IGPs)] lies in the genetic diversity that has evolved across the streptococcal species. Strains of streptococci have acquired different complements of Fn-binding protein genes, thus making it difficult to define genetic or functional groupings clearly.

Protein F1 (PrtF1), which is a homolog of SfbI, was first identified as a major Fn-binding determinant, and sequences are present in approximately 70% of S. *pyogenes* isolates (Kreikemeyer *et al.*, 1995). A second adhesin, PrtF2, accounts for a high level of Fn-binding activity in S. *pyogenes* strains that do not express PrtF1 (Jaffe *et al.*, 1996). Some strains lack PrtF1 and PrtF2, and in these strains low-level Fn-binding may be due to SOF (SfbII) or FBP54 (see Table 3). All of these proteins, with the exception of FBP54, are cell-wall-anchored at the C-terminus and conform to the overall structure of MSCRAMMs (Patti *et al.*, 1994). The distribution and levels of expression in group A streptococci of other Fn-binding proteins SDH and the 28-kDa antigen (Table 3) are not known. M3 protein is unusual in that it is the only Emm-like protein that has been shown to bind Fn. Expression of PrtF1 and PrtF2 in S. *pyogenes*, and hence binding of cells to Fn, is induced by aerobic conditions. This might be significant in promoting initial adherence of cells to the pharynx or skin (VanHeyningen *et al.*, 1993). Superoxide stress, under O_2-limited conditions, also stimulates PrtF1 expression (Gibson and Caparon, 1996). Another Fn-binding activity has been recently identified which requires oxygen (or oxidants) for pH-sensitive activation of Zn^{2+}-dependent Fn-binding. This Fn-binding activity, called ZOP, might be activated at later infection stages and aid invasion of deeper tissues (Lee and Caparon, 1996). Interestingly, ZOP is inhibited by LTA, so it may be that ZOP binding is the activity previously attributed to LTA.

Much less is known about the mechanisms and proteins involved in Fn-binding by other streptococci. Fn-binding proteins have been identified in S. *anginosus* and S. *gordonii* (Table 3), species that can cause endocarditis. The multi-functional adhesin CshA mediates, in part, the heparin-insensitive binding of S. *gordonii* to Fn (McNab *et al.*, 1996). Binding of S. *gordonii* and group B streptococcal cells to immobilized Fn is inhibitable by collagen, so these organisms may share common Fn-binding mechanisms that could be different from those established for the group A streptococci. Adhesion to collagen has been demonstrated for S. *pyogenes* (Visai *et al.*, 1995) and S. *mutans* (Switalski *et al.*, 1993). The significance of this remains speculative, but it could be that S. *pyogenes* binding to collagen type IV is a factor in glomerulonephritis, along with the production of a small cationic protein that binds glycosaminoglycans (GAG-BP) and localizes to glomerular laminae (Glurich *et al.*, 1991). The binding of S. *mutans* to type I collagen fibers mediates streptococcal adhesion to dentin in exposed root surfaces and so may influence the development of subgingival plaque.

Group A streptococci express cell-surface fibrillar proteins that bind a range of host plasma components such as fibrinogen, plasmin(ogen), factor H, and the Fc-domains of IgG and/or IgA (see Kehoe, 1994). Their sequence similarities and coiled-coil dimeric fibrillar structure (Nilson *et al.*, 1995) distinguish this family of proteins (referred to as the M protein family) from the MSCRAMMs and IGPs such as protein G (Table 3). M proteins have been considered as major virulence factors in S. *pyogenes* because of their anti-phagocytic properties.

TABLE 3

Streptococcal Protein Adhesins Recognizing Extracellular Matrix or Serum Components

Adhesin	Approx. Mol. Mass (kDa)	Species	Substrate	Reference
SfbI family [PrtF1, SfbI, GfbA]	70	*S. pyogenes* (group A), group G streptococcus	Fibronectin	Talay *et al.* (1994); Kline *et al.* (1996); Ozeri *et al.* (1996)
SfbII, SOF	113	*S. pyogenes*	Fibronectin	Kreikemeyer *et al.* (1995); Rakonjac *et al.* (1995)
PrtF2	115	*S. pyogenes* M-type 49	Fibronectin	Jaffe *et al.* (1996)
FnbA	117	*S. dysgalactiae* (group C)	Fibronectin	Lindgren *et al.* (1993)
FnbB	122	*S. dysgalactiae*	Fibronectin	Lindgren *et al.* (1993)
Fnb	120	*S. equisimilis* (group C)	Fibronectin	Lindgren *et al.* (1994)
FBP54	54	*S. pyogenes* M-type 5	Fibronectin, fibrinogen	Courtney *et al.* (1996)
SDH	39	*S. pyogenes*	Fibronectin, lysozyme, myosin, actin	Pancholi and Fischetti (1992)
28-kDa antigen	28	*S. pyogenes*	Fibronectin	Courtney *et al.* (1992)
14-kDa protein	14	*S. anginosus*	Fibronectin	Willcox *et al.* (1995)
CshA	259	*S. gordonii*	Fibronectin (immobilized)	McNab *et al.* (1996)
Laminin-binding protein	145	*S. gordonii* 2316	Laminin	Sommer *et al.* (1992)
Collagen adhesin	90	*S. mutans* UA140	Collagen	Lawry and Switalski (1996)
Collagen adhesin	57	*S. pyogenes* 6414, *S. zooepidemicus* (group C)	Collagen (type II)	Visai *et al.* (1995)
GAG-BP	9	*S. pyogenes* M-type 12	Heparin, heparan sulfate	Glurich *et al.* (1991)
SIC	31	*S. pyogenes* M-type 1	Clusterin, histidine-rich glycoprotein (HRG)	Åkesson *et al.* (1996)
M3	60	*S. pyogenes* M-type 3	Fibronectin, fibrinogen, human serum albumin (HSA)	Schmidt *et al.* (1993)
Plr	36	*S. pyogenes*	Plasmin	Lottenberg *et al.* (1992)
Enolase	45	*S. pyogenes*	Plasminogen	Pancholi and Fischetti (1996a)
PAM	40	*S. pyogenes* M-type 53	Plasminogen, plasmin	Wistedt *et al.* (1995)
Emm family [M1, M6, M12, etc.]	48-55	*S. pyogenes*	Factor H, fibrinogen, IgG3, HSA	Kehoe (1994); Retnoningrum and Cleary (1994); Fischetti *et al.* (1995)
FcRA (Mrp) family [FcRA76, FcRA49, Mrp4, Mrp60]	35-50	*S. pyogenes*	IgG1, IgG2, IgG4, fibrinogen	Boyle *et al.* (1994); Cleary and Retnoningrum (1994); Kehoe (1994)
Protein H (Sph)	38	*S. pyogenes*	IgG1, IgG2, IgG3, IgG4	Gomi *et al.* (1990)
Enn family [Enn4, Enn49, ML2.2]	43	*S. pyogenes*	IgA	Bessen (1994)
Arp family [Arp4, Arp60, ML2.1, Sir]	40	*S. pyogenes*	IgA1, IgA2, IgG3, C4BP (IgG1, IgG2, IgG4)	Bessen and Fischetti (1992); Stenberg *et al.* (1992, 1994); Thern *et al.* (1995)
β antigen	123	*S. algalactiae* (group B)	IgA	Jerlström *et al.* (1996)
Grp family [Protein G, MIG, MAG, ZAG]	45-70	*S. dysgalactiae*, *S. zooepidemicus*	IgG, α_2-macroglobulin, serum albumin	Jonsson and Müller (1994); Jonsson *et al.* (1995)

These have been attributed to the ability of M proteins to bind fibrinogen (Horstmann *et al.*, 1992), complement factor H (Fischetti *et al.*, 1995), and complement regulatory factor C4BP (Thern *et al.*, 1995). Mutants deficient in expression of M proteins are sensitive to phagocytosis (Courtney *et al.*, 1994; Podbielski *et al.*, 1996a), whereas in the absence of type-specific antibodies, M protein, probably in conjunction with the hyaluronate capsule, confers phagocytosis resistance. The hyaluronate capsule might act as a physical barrier that prevents C3b bound to the bacterial cell surface from interacting with the phagocyte receptor (Dale *et al.*, 1996).

Genes expressing anti-phagocytic M proteins (*emm* genes) are members of a larger *emm*-like gene family comprised of two additional sub-families, *fcr* (alternative designation *mrp*) and *enn*. The genes are the result of duplication and reside at a single chromosomal locus called the *mga* or *vir* regulon (see Whatmore *et al.*, 1994). The genetic structure of the *mga* (*vir*) regulon correlates, more or less, with production of an opacity factor (OF) which turns serum opalescent. OF^+ strains contain a triplet of *emm*-like genes *mrp-emm-enn*, whereas in OF^- strains, the *mga* regulon may contain only a single *emm* gene, or two (*emm-prt* or *emm-enn*), or more than two genes. A detailed review of M protein gene structural and evolutionary relationships was provided by Kehoe (1994). In Table 3, we have summarized sub-family groupings of Emm and Emm-like-proteins, but the groupings are not entirely satisfactory. Groupings may be attempted on the basis of primary sequence homologies and/or substrate-binding phenotypes. However, because the genes encoding these proteins have arisen by duplication and horizontal transfer between S. *pyogenes* populations, both genetic structure and protein functions have become assorted. Thus, proteins that exhibit closest overall sequence identities do not necessarily turn out to be those that have similar binding functions (Bessen and Fischetti, 1992).

The presence or absence of OF correlates with two classes of anti-phagocytic M protein, that are distinguished by variance of a short epitope within a highly conserved C-terminal region of Emm. The N-terminal regions contain type-specific epitopes, and only antibodies to these are able to overcome the anti-phagocytic function. The *emm* gene products may bind factor H, HSA, fibrinogen, and IgG3 (Table 3), but not all M proteins bind all of these substrates. Fibrinogen competes with factor H for binding M protein while retaining its own binding affinity for factor H; thus a three-molecule anti-phagocytic complex is believed to form on the streptococcal cell surface (Horstmann *et al.*, 1992). The products of both *emm* and *mrp* genes contribute to anti-phagocytic potential (Podbielski *et al.*, 1996a). It seems then that not all group A streptococci utilize the same anti-phagocytic mechanism, just as not all strains utilize the same mechanisms for adhesion. Mrp or FcRA proteins bind IgG1, IgG2, and IgG4, but not IgA, which is bound by Arp and Enn proteins (Table 3). Despite extensive sequence homology of Arp4 to M proteins, no anti-phagocytic activity could be attributed to Arp4 (Husmann *et al.*, 1995). Of all the proteins characterized, Sir has the broadest Fc-receptor activity and binds IgA and IgG of all sub-classes (Stenberg *et al.*, 1994). Protein H (Sph) binds all four sub-classes of human IgG (Gomi *et al.*, 1990). Group B and C streptococci also express a variety of IGPs that have no homology with Protein H and that bind various combinations of IgG sub-classes from different animals (Table 3).

The ability to bind plasmin or its zymogen plasminogen has long been recognized as a virulence factor in group A, C, and G streptococci. Plasmin and/or plasminogen is bound by PAM (an Emm-like protein), Plr, and a 45-kDa cell-surface protein recently identified as enolase (Table 3). Streptococcal cell-bound plasmin, derived from streptokinase-cleavage of plasminogen, influences bacterial infections by degrading fibrin and breaking down soft-tissue glycoproteins. In support of this concept, strains causing skin infections show high-level binding of plasmin (Wistedt *et al.*, 1995). The significance of the cell-surface-located forms of the glycolytic enzymes enolase and Plr (a glyceraldehyde-3-phosphate dehydrogenase similar to SDH) is not yet known. The SDH enzyme has been reported to function as an ADP-ribosylating enzyme, and the interaction of group A streptococcal SDH with pharyngeal cells causes activation of cytosolic tyrosine-phosphokinase and protein kinase C, and phosphorylation of specific pharyngeal cell membrane proteins (Pancholi and Fischetti, 1996b). This host cell signaling activity of SDH could play a critical role in determining the streptococcal infection process.

Clearly, these virulent micro-organisms have an extraordinary capacity to bind serum proteins, but generally it is not yet understood how all these binding properties contribute to virulence. It is particularly important to determine if there are relationships between binding protein activity and disease state. For example, plasmin-binding correlates with skin infections as mentioned above, while binding of clusterin by protein SIC (Table 3) may be relevant to glomerulonephritis, since possession of the *sic* gene is associated with glomerulonephritic isolates (Åkesson *et al.*, 1996). Although the significance of the numerous IGPs elaborated by streptococci is not known, passage of group A streptococci through blood increases IGP activity (Raeder and Boyle, 1993). It could be postulated, therefore, that IgG binding contributes to the anti-phagocytic activity of M protein (Cleary and Retnoningrum, 1994). Expression of streptococcal protein binding function may also be related to mode of colonization. Within class I M proteins (from OF^- strains), the ability to bind IgG

distinguishes between nasopharyngeal and impetigo isolates (Bessen and Fischetti, 1990). It is possible, then, that the ability to bind IgA or IgG immobilized on a surface may assist streptococcal adhesion to epithelia in the nasopharynx. A role for the multitude of IGPs as sensor molecules has been advanced (Cleary and Retnoningrum, 1994). The ability to distinguish among specific tissues or fluids would be advantageous to streptococci that are within the host, allowing physiological responses to the local environment to occur. Binding of a streptococcal surface protein to its cognate receptor could (by analogy to integrin-mediated intracellular signaling in eucaryotic cells) relay a signal to an intracellular signaling cascade. Evidence for this conceptually appealing process has yet to be obtained. However, it is potentially significant that a G protein (SGP) has been found in S. *mutans* (Wu *et al.*, 1995). In a variety of other cells, G proteins can function in trans-membrane signaling pathways. As more information on the role of specific proteins in disease accumulates, the development of anti-streptococcal strategies that will target specific functions becomes more feasible.

Regulation of Adhesin Expression

Bacteria that colonize the human body are subjected to continually changing environmental conditions. In the oral cavity, bacteria experience dramatic environmental changes as a consequence of host eating patterns and salivary flow rate variability. In response to these dynamic processes, bacteria often regulate gene expression in order to maintain optimal phenotypic properties. The ideal numbers and types of adhesins expressed at, for example, the mucous membranes may not be the most advantageous configuration if the bacteria gain access to deeper tissues where such adherence might promote uptake by the host's professional phagocytic cells.

Streptococci have been shown to regulate expression of adhesion- and virulence-associated genes at the transcriptional level. Transcription of *prt*F1 encoding a Fn-binding protein in group A streptococci is positively regulated by a trans-acting factor RofA (Fogg *et al.*, 1994). Expression of PrtF1 is up-regulated by atmospheric O_2, but this is not effected directly through RofA. While expression of Fn-binding protein PrtF2 is also up-regulated by atmospheric O_2, levels of M protein decrease in elevated O_2. Expression of M protein is highly regulated by the environmental concentration of CO_2 (Caparon *et al.*, 1992). C5a endopeptidase (*scp*A gene product) and, in some strains, IGPs are co-regulated with M protein at the level of transcription (Podbielski *et al.*, 1992). The control pathway requires Mga, a trans-acting positive regulatory protein product of the *mga* gene situated immediately upstream of the adjacent *emm* and *scp*A genes. The Mga protein binds to a 45-bp region upstream of a site that overlaps the -35 box of a promoter for both genes (McIver *et al.*, 1995). The Mga protein possesses features common to bacterial transcriptional regulators (helix-turn-helix domains) and contains motifs shared by the response regulators of two-component signal transduction systems, although the significance of this is unconfirmed (McIver *et al.*, 1996). Unique features of Mga include its large size (62 kDa) and a DNA binding site containing two converging repeat sequences with a 6- or 7-nucleotide separation from two central adenines. Mga expression itself is autoregulated in response to CO_2 levels (Okada *et al.*, 1993), although the involvement of the consensus sequence for Mga binding in this pathway is unknown.

Adherence-associated gene expression is also regulated in the oral streptococci. S. *mutans* produces several glucosyltransferase enzymes that catalyze the formation of glucan polymers from sucrose. Water-insoluble glucans may be involved in promoting the accumulation of S. *mutans* on solid surfaces and in mediating S. *mutans* cell-to-cell attachment (reviewed by Kuramitsu, 1993). Several studies have revealed that transcription of the S. *mutans gtf* genes is regulated in response to environmental conditions (Hudson and Curtiss, 1990; Wexler *et al.*, 1993; Kiska and Macrina, 1994). Expression of the *gtf*BC operon increases in the presence of sucrose, a substrate for glucosyltransferase, and when S. *mutans* cells are attached to experimental salivary pellicle. In S. *gordonii*, the *rgg* gene located 66 bp upstream of *gtf*G positively regulates expression of glucosyltransferase (GtfG) (Sulavik and Clewell, 1996). The existence of at least two other regulatory sites for S. *gordonii* glucosyltransferase production are predicted from the work of Haisman and Jenkinson (1991). The *pag* gene, encoding an antigen I/II polypeptide in S. *sobrinus*, appears to be under negative control from a 609-bp gene (*par*) lying 1.7 kb upstream of *pag*. The *par* gene product exhibits sequence similarities to a sensor component of an *Escherichia coli* two-component system (Takahashi *et al.*, 1993); however, the extent to which *pag* expression might be influenced by a sensor-regulator system remains to be determined.

Oligopeptide permease (Opp) transport systems in streptococci are responsible for uptake of peptides and probably are essential for growth of these organisms in the animal host. The ATP-binding protein-dependent transport systems responsible for uptake of oligopeptides by S. *gordonii* (Jenkinson *et al.*, 1996) and S. *pneumoniae* (Alloing *et al.*, 1994) have also been associated with determining adhesion of these bacteria to other oral micro-organisms (Jenkinson, 1992) and to epithelial cells (Cundell *et al.*, 1995c), respectively. Adhesion deficiency of Opp$^-$ mutants might be because the substrate (oligopeptide)-binding proteins normally function dually as adhesins. Alternatively, or in addition, *opp* mutations, which have pleiotropic effects, may affect expression of other cell-surface adhesins. Thus, Opp function may be involved in the regulation of adhesin and virulence gene

expression in streptococci. This conclusion is further supported by the demonstration that Opp⁻ mutants of S. *pyogenes* are considerably reduced in cysteine protease (SpeB) production (Podbielski *et al.*, 1996b).

There is good evidence for environmental or genetic regulation of adhesin expression in several other instances, but these have not yet been investigated at the molecular level. For example, the presence of collagen in the growth medium up-regulates expression of cell surface PAAP in S. *sanguis* (Erickson and Herzberg, 1995), and a 145-kDa laminin-binding protein in S. *gordonii* (Sommer *et al.*, 1992), while phase variation of adhesion phenotype has been demonstrated in S. *gordonii* (Jones *et al.*, 1996) and S. *pneumoniae* (Cundell *et al.*, 1995b). It is anticipated that a number of additional regulatory pathways will be elucidated in the future.

Adhesin Structure and Function

(A) Common Themes in Adhesin Design

Many of the adhesins listed in Tables 1 to 3 belong to a superfamily of Gram-positive bacterial proteins that are linked to the cell surface *via* a C-terminal cell-wall-anchor domain (Schneewind *et al.*, 1995). A separate family of putative adhesins are lipoproteins that are associated with the outer face of the cytoplasmic membrane through N-terminal lipid modification (Jenkinson, 1994). Other proteins listed that do not have recognized surface-anchor sequences (*e.g.*, FBP54, SDH) may be found associated either with other surface components such as LTA or polysaccharides, or be anchored through some as-yet-unidentified mechanism (see, for example, Rathsam *et al.*, 1993).

The C-terminally anchored adhesins come in a variety of sizes and designs, but they seem to be assembled in part from a relatively defined range of modules that are related in primary sequence and substrate-binding function. The overriding feature of these proteins is that they contain amino acid repeat blocks. These can be quite small—for example, the five blocks of 14 aa residue A repeats within M6 protein (Fischetti, 1989)—or extensive, such as the 13 blocks of 101 aa residue repeats within CshA (McNab *et al.*, 1994). Different strains of streptococci sometimes show variations in repeat block number within a given surface protein. Different lengths of repeat blocks elicit different antibody repertoires (Gravekamp *et al.*, 1996), so lower repeat number variants (with loss of epitopes) might be able to escape host antibodies generated to higher repeat number variants. The aa repeat blocks often contain substrate-binding domains, but, as described in the next section, the sequences necessary for efficient substrate binding are in many instances not localized just to the repeat blocks.

In Fig. 2, the structures of six streptococcal surface proteins are depicted and have been selected to cover many of the possible design features. Each of the precursor proteins contains a typical leader peptide (approximately 30-40 aa residues) that is necessary for secretion of the respective protein through the cytoplasmic membrane. Each of the six proteins, with the exception of glucosyltransferase GtfB, has a C-terminal wall-anchor domain containing the motif LPxTG, believed to be necessary for cell-wall covalent linkage (Schneewind *et al.*, 1995). In a number of proteins, a proline-rich segment of about 40 aa residues is found just N-terminal to the wall-anchor domain (Fig. 2). Of the six polypeptides depicted, M6 is the only one predicted to form an entirely α-helical coiled-coil structure and therefore to be in extended conformation. The N-terminal one-third of SspA is predicted to adopt the α-helical coil, but none of the other proteins contains extensive regions of α-helix. Representative sequence features within these proteins are as follows: M6 (Fig. 2A) contains three aa residue repeat blocks, designated A, B, and C. The C repeats are associated with factor H binding, while the B repeats bind HSA and IgG and possibly also fibrinogen. Strong sequence similarities to the Emm C repeats are found in Arp and Sph. However, the so-called B repeats present in Arp and Sph are not similar to those in M6 polypeptide. Protein MIG (Fig. 2B) contains five repeat blocks of 70 aa residues that bind IgG but have no sequence homology to Sph (Table 3). These Ig-binding repeat block sequences are present also in MAG, ZAG, and protein G. The N-terminal region of MIG contains non-repetitive aa sequence and the binding site for α_2-macroglobulin (Fig. 2B).

The third protein depicted in Fig. 2 is a typical Fn-binding protein (SfbI). Binding of soluble Fn by SfbI, and its homolog PrtF1, is associated with the presence of aa residue repeat blocks at the C-terminal end of the polypeptide. The numbers and sizes of these blocks vary within the different Fn-binding proteins. SfbI contains four blocks of 37 aa residues, whereas SfbII contains two blocks of 39 aa residues (plus one incomplete block). Sequences within these regions are well-conserved across a number of Fn-binding proteins and are discussed in the next section. On the other hand, regions of these proteins N-terminal to the Fn-binding repeat domain are diverse in sequence. SfbI carries four proline-rich repeat blocks of 24-26 aa residues in the central region (Fig. 2C), while SfbII contains a non-repetitive serine-rich sequence of 104 aa residues at the N-terminus and a short proline-rich region just N-terminal to the Fn-binding repeats (Kreikemeyer *et al.*, 1995). There is much still to be learned about the functional significance of these various sequences. Protein SfbI also contains a domain within the N-terminal region that has homology to an aromatic aa sequence found in the C-terminal repeating unit present in glucosyltransferases (Fig. 2D) (Giffard and Jacques, 1994). These "YG" blocks of approx-

Figure 2. Structural features of six streptococcal cell-surface proteins that function in adhesion and colonization. (A) *S. pyogenes* M6 (Emm6) (483 aa residues); (B) *S. dysgalactiae* MIG (664 aa residues); (C) *S. pyogenes* SfbI (638 aa residues); (D) *S. mutans* GtfB (1,475 aa residues); (E) *S. gordonii* SspA (1,577 aa residues); and (F) *S. gordonii* CshA (2,508 aa residues). Precursor polypeptides are drawn N-terminal end (left) to C-terminal (right). With the exception of D, all of the mature proteins are postulated to be held at the cell surface *via* a specialized C-terminal wall anchor (see text). Specific structural features and aa residue repeat block regions are indicated (see key to shading). Note that like-shaded regions across the different proteins indicate only similarities in aa composition and/or predicted secondary structure, and not sequence homologies. Conversely, aa sequences of repeat blocks within an individual polypeptide are usually highly conserved. Non-repetitive sequences are unshaded. Substrates bound by the polypeptides, and the approximate locations of the binding sites, are indicated above each structure. Abbreviations are: Ig, immunoglobulins; HSA, human serum albumin; fH, complement factor H; α_2MG, α_2-macroglobulin; IgG, immunoglobulin G; Fn, fibronectin; $(CHO)_n$, polysaccharide; SAG, salivary agglutinin glycoprotein; Ca^{2+}, calcium; Pg, *Porphyromonas gingivalis*; An, *Actinomyces naeslundii*; Ca, *Candida albicans*; So, *Streptococcus oralis*.

imately 20 aa residues are found also within some GBPs (Banas *et al.*, 1990; Sun *et al.*, 1994), *Clostridium difficile* toxins, and pneumococcal autolysin (LytA), and are thought to be attachment sites for carbohydrates (von Eichel-Streiber *et al.*, 1992). The catalytic domain of the streptococcal glucosyltransferases is located within the N-terminal region (see Russell, 1994).

Two protein structures further included in Fig. 2 are produced by S. *gordonii*, and both are associated with adhesion of this organism to oral substrates. SspA (Fig. 2E) has the general characteristics of the antigen I/II protein family polypeptides (Jenkinson and Demuth, 1997). It contains four repeat blocks of alanine-rich sequence, within the N-terminal domain, that bind fluid-phase salivary glycoproteins, and three repeat blocks of proline-rich sequence in the central region that are highly immunogenic. Just C-terminal to the proline-rich repeats are sequences that may mediate species-specific binding of cells to salivary glycoproteins (Kelly *et al.*, 1995). Further C-terminal to these sequences is a domain implicated in the binding of Ca^{2+} and P. *gingivalis* (Jenkinson and Demuth, 1997). The other S. *gordonii* adhesin, CshA polypeptide (Fig. 2F), is one of the largest streptococcal cell-wall-anchored proteins known (2508 aa residues). The aa repeat block region, which makes up 60% of the total polypeptide sequence, is rich in proline, glycine, and threonine, and evidence suggests that this region is involved in conferring cell-surface hydrophobicity (McNab *et al.*, 1995). Demonstration of a specific binding function associated with the PGT repeats has so far eluded detection. Instead, the binding functions attributed to this polypeptide, which include adherence to other microbial cells (Table 2) and to Fn (Table 3), appear to reside chiefly within the non-repetitive N-terminal domain (Holmes *et al.*, 1996; McNab *et al.*, 1996).

From the aforementioned, it must be apparent that the streptococci cell-surface proteins have acquired tailored functions through appropriate genetic assortment of ancestral binding sequences. However, it might seem rather puzzling as to why so much genetic information has been retained for the production of adhesins. This will be clarified when we understand more about the

alternate physiological functions of these proteins. For example, Fn-binding is localized to short sequences relative to the overall sequence lengths of the Fn-binding proteins. Why then are the Fn-binding proteins so large and their N-terminal sequences so variable? Perhaps the Fn-binding repeats, like the carbohydrate-binding domains present in Gtfs and lysins, provide a binding function linked to a catalytic activity within the N-terminal region. It is known that streptococcal cell-wall-anchored proteins may be actively released from the cell surface (Lee, 1995), a process that could help bacterial infection through shedding of surface-bound antibodies, or by deflecting immune defenses (Jenkinson, 1995a). Fn-binding sequences might be significant in assisting retention of released proteins within the bacterial matrix of infection, and maintaining other protein functions (possibly catalytic) related to growth and survival of the colonizing organisms. This raises one further issue relating to bacterial colonization, that of the role of adhesins in intercellular communication. Sequence analysis and circular dichroism spectroscopy of M1 protein and protein H suggest that they are almost entirely in α-helical conformation. These proteins are thermally unstable, with the coiled-coil dimers unfolded at 37°C, but are stabilized in the presence of ligands such as albumin and immunoglobulins. Temperature fluctuations within a narrow range around 37°C might then change dramatically the binding properties of the bacterial cell surface as the M proteins unfold and refold. Interestingly, refolding of chains in an antiparallel arrangement, such as might occur between M proteins on adjacent cells, is predicted to be more favorable. This would provide a mechanism for direct cell-cell interactions among colonizing S. *pyogenes* (Nilson *et al.*, 1995).

(B) Binding domains

Recent advances in gene expression and peptide synthesis technologies have facilitated quite precise delineation of sequences within streptococcal proteins that are necessary for binding functions. By expression of segments of cloned streptococcal protein-coding sequences as fusions with affinity-purifiable tags such as glutathione-S-transferase or hexa-histidine, substrate-binding domains have been isolated. Then, overlapping synthetic peptides covering these domains, and sometimes antibodies generated to these peptides, have been used to pinpoint, by inhibition assay, the linear sequences necessary for substrate binding. A general feature of the results to date is that the functional binding units can be narrowed down to relatively short linear amino acid sequences of around 50 aa residues or less.

The C repeats of M6 polypeptide comprise two repeat blocks of 23 aa residues separated by a spacer of 19 aa residues. A sequence of 40 aa residues, not identical among M serotypes, and comprising part of the spacer and the second C repeat, was identified as being necessary for the binding of factor H (Fischetti *et al.*, 1995). This defined the site that was believed to be responsible, at least in part, for M protein anti-phagocytic function. However, isogenic mutants of S. *pyogenes* expressing modified M6 protein with the C repeats deleted were found to be still phagocytosis-resistant (Perez-Casal *et al.*, 1995). A current view, then, is that the C repeats are neither necessary nor sufficient for anti-phagocytic function.

Other substrate-binding sites that have been assigned to linear sequences include those for plasmin, IgA, and Fn. Plasmin-binding by PAM was localized to a 133 aa residue N-terminal segment, more specifically to two 13-16 aa residue repeats followed by a highly conserved 45 aa residue containing essential lysines (Wistedt *et al.*, 1995). IgA-binding by the β antigen was associated with a 73 aa residue region within which the sequence MLKKIE was essential (Jerlström *et al.*, 1996). IgA-binding by Arp4 was assigned to a region comprised of aa residues 14-71 within the N-terminal portion and to an essential sequence ALxGENxDLR (where x is any aa residue) (Bessen, 1994). These IgA-binding regions have no significant sequence homologies. On the other hand, sequences within Fn-binding repeats are quite well-conserved across the proteins that bind soluble Fn (see Patti *et al.*, 1994) and contain the core consensus sequence E-D-(T/S)-(x,7-10)-G-G-(x,4)-(I/V)-(D/E)-(F/I/T). It is now known that for Fn-binding by PrtF1 and PrtF2, not just the C-terminal repeats (the FBRD domain) but also a non-repetitive sequence domain (UFBD) found N-terminal to the repeats are required. The minimal functional unit of FBRD in PrtF1 is 44 aa residues spanning two repeats and flanked by the sequence MGGQSES, the diglycine being that within the core consensus. The UFBD domain in PrtF1 contains 49 aa residues and is immediately N-terminal to FBRD (Ozeri *et al.*, 1996). The UFBD domain in PrtF2 contains approximately 100 aa residues and is located much further (100 aa residues) N-terminal to FBRD (Jaffe *et al.*, 1996). Each of the domains FBRD and UFBD is capable of binding Fn when expressed as a separate protein. This suggests that adherence of cells to Fn may involve not only multiple adhesins but also the co-operative effects of at least two linearly-distinct binding sites on the adhesins.

The identification of aa residue motifs involved in mediating adhesion and virulence-factor functions is important for the development of effective vaccines, passive immunization protocols, and new anti-microbial compounds. Obviously, given the molecular complexity of streptococcal adhesion to even a single tissue protein, developing effective means to combat streptococcal adhesion and colonization looks to be a tall order. So far, though, we have been mainly concerned with researching the adherence and virulence properties of streptococcal cells *in vitro*, outside their natural environment. It is

important to ascertain which of the multitude of adherence mechanisms defined *in vitro* is in fact important for colonization *in vivo*, and which may not be. Very little is known about this aspect of streptococcal biology, primarily because of difficulties associated with developing animal models suitable for the study of bacterial colonizers of humans. However, this is arguably a critical issue that must be addressed in future streptococcal research if we are to develop usefully our understanding of the molecular basis for streptococcal success.

Streptococcal Colonization

Streptococcal species vary widely in their abilities to attach to different surfaces. The relative adherence of a number of oral streptococcal species has been found to correlate positively with their natural intra-oral distribution. Consequently, there is often an implicit assumption that adhesins defined by utilizing *in vitro* binding assays are functional *in vivo* and necessary for the organism to colonize the host. While that may indeed be true in a number of instances, such assumptions have generally not been rigorously tested. In fact, when animal models have been utilized, many interesting and initially surprising observations have been made.

Animal studies on oral streptococcal colonization have concentrated mainly on the cariogenic potential of mutans group streptococci. Almost all studies have utilized germ-free or specific-pathogen-free rodents fed a sucrose-containing diet which promotes massive oral accumulations of streptococci. Under these conditions, the development of caries seems to depend on the ability of streptococcal species both to form plaque and to acidify the plaque *milieu* (van Houte and Russo, 1986). Some organisms, *e.g.*, strains of S. *sanguis* and S. *mitis*, do not accumulate or form plaque well under this regimen, and therefore a meaningful evaluation of their cariogenic potential, *i.e.*, virulence, cannot be obtained. Accumulation seems to depend on the ability of the organisms to utilize sucrose for the synthesis of extracellular polysaccharides. Some of these water-insoluble polymers promote bacterial adherence and accumulation, while other, more soluble, polymers may be important for provision of a ready source of metabolizable carbohydrate if conditions become nutrient-limited. The genetics and significance of extracellular polysaccharide production by mutans-group streptococci have been reviewed recently (Kuramitsu, 1993).

The importance of polysaccharide production in colonization and cariogenesis by mutans group streptococci has been demonstrated by animal-model experiments utilizing isogenic mutants. Mutants of S. *mutans* disrupted in the *gtf*B and *gtf*C genes, normally encoding enzymes GTF-I and GTF-IS synthesizing water-insoluble and water-semi-insoluble glucans, respectively, are much less cariogenic (Table 4). On the other hand, knockout of the *gtf*D gene encoding an enzyme synthesizing water-soluble glucan had little or no effect on cariogenicity (Table 4). However, conflicting data have been obtained on the relative roles of these glucan-synthesizing, and the fructan-synthesizing (Ftf), enzymes in colonization and cariogenesis, partly because different researchers have used different bacterial strains and different animal models (Table 4). It does appear now that the various enzymes may be important at different stages of colonization and influence site-specific caries. Knockout *gtf*BC mutants are able to make only soluble glucans or fructans and are clearly depressed in their ability to cause buccal lesions, but still form sulcal lesions. Such strains actually make more of the extracellular polymers that can be readily degraded to provide nutrients. Recent evidence supports the idea that colonization and cariogenesis result from an interplay of Gtf enzyme activities, and Ftf (when present). Thus, animals colonized by mutants defective only in GtfB have much increased numbers of sulcal lesions (Table 4). The absence of GtfB may proportionally increase the amount of degradable storage polymer available for metabolism and thus promote increased acid production (Munro *et al.*, 1995).

As we seek to identify colonization factors of streptococci, new animal models need to be developed. In this respect, we have recently established a murine model for the study of oral streptococcal colonization in the absence of dietary sucrose. Our system depends on utilizing mice with a reconstituted commensal microbiota that lacks, specifically, lactobacilli, enterococci, and streptococci which otherwise out-compete the exogenously inoculated streptococcal species (Loach *et al.*, 1994). In this way, colonization studies are carried out with animals that still harbor a complex microbiota, rather than with animals that are germ-free or fed antibiotics to suppress the microbiota. We have focused our studies on S. *gordonii*, which is found at most sites within the human oral cavity (Frandsen *et al.*, 1991). This species has well-characterized genes for surface protein adhesins (Jenkinson, 1994; Whittaker *et al.*, 1996) and glucan synthesis (Sulavik and Clewell, 1996), and is of current interest as a bacterial vector for the expression of heterologous antigens (Medaglini *et al.*, 1995). Utilizing isogenic mutants of S. *gordonii* deficient in the production of specific surface proteins, we have demonstrated that several genes identified as being involved in the expression of *in vitro* binding properties are also necessary for *in vivo* colonization of the murine oral cavity (Table 4). Interestingly, the CshB polypeptide, which appears to have little or no adhesin activity *in vitro*, was nevertheless essential for colonization *in vivo* (McNab *et al.*, 1994). Likewise, inactivation of the *ssp*A gene encoding an antigen I/II polypeptide did not affect adherence levels of S. *gordonii* to experimental salivary pellicle (Jenkinson *et al.*, 1993), but mutants were ablated in their ability to colo-

TABLE 4

Gene Knockouts Identifying Colonization or Virulence Factors in Streptococci

Gene(s) Inactivated	Wild-type Gene Product and Molecular Mass	Organism	Animal Model (diet)[a]	Colonization or Virulence of Isogenic Mutant Compared with Parent Strain	Reference
spaP	antigen I/II salivary adhesin, 166 kDa	*S. mutans* NG8	SPF[b] rat (hs)	No effect on oral colonization or on level of smooth-surface caries	Bowen *et al.* (1991)
sspA	antigen I/II salivary adhesin, 171 kDa	*S. gordonii* DL1	LF[c] mouse (c)	> 90% reduction in numbers of animals colonized orally	Tannock, Loach, and Jenkinson (unpublished)
cshA	multi-functional adhesin, 259 kDa	*S. gordonii* DL1	LF mouse (c)	> 90% reduction in numbers of animals colonized orally	McNab *et al.* (1994)
cshB	surface protein, *ca.* 245 kDa	*S. gordonii* DL1	LF mouse (c)	No animals colonized orally	McNab *et al.* (1994)
hppA	oligopeptide-binding lipoprotein, 76 kDa	*S. gordonii* DL1	LF mouse (c)	No animals colonized orally	Jenkinson *et al.* (1995, 1996)
hppG	oligopeptide-binding lipoprotein, *ca.* 78 kDa	*S. gordonii* DL1	LF mouse (c)	No effect on oral colonization	Jenkinson *et al.* (1995)
gtfB	glucosyltransferase (GTF-I), *ca.* 159 kDa	*S. mutans* UA130	SPF rat (hs)	No effect on oral colonization; > 80% reduced level of smooth-surface caries	Yamashita *et al.* (1993)
gtfC	glucosyltransferase (GTF-SI) *ca.* 140 kDa	*S. mutans* UA130	SPF rat (hs)	No effect on oral colonization; > 80% reduced level of smooth-surface caries	Yamashita *et al.* (1993)
gtfD	glucosyltransferase (GTF-S) *ca.* 155 kDa	*S. mutans* UA130	SPF rat (hs)	No effect on oral colonization or on level of smooth-surface caries	Yamashita *et al.* (1993)
gtfBC	GTF-I and GTF-SI	*S. mutans* V403	GF[d] rat (ls)	> 95% reduced oral colonization; reduced caries on smooth and sulcal surfaces	Munro *et al.* (1991)
gtfB	GTF-I	*S. mutans* V403	GF rat (ls)	Increased smooth (buccal)-surface caries; similar caries on sulcal surfaces	Munro *et al.* (1995)
ftf	fructosyltransferase *ca.* 87 kDa	*S. mutans* V403	GF rat (ls)	No effect on oral colonization; reduced caries on smooth and sulcal surfaces	Schroeder *et al.* (1989)
gtfBC, gtfD, ftf	glucosyl- and fructosyltransferases	*S. mutans* V403	Catheterized rat (c)	> 80% reduction in numbers of animals developing endocardial disease	Munro and Macrina (1993)
gtfG	glucosyltransferase *ca.* 170 kDa	*S. gordonii* CH1	Catheterized rat (c)	No effect on numbers of animals developing endocarditis	Wells *et al.* (1993)
fimA	LraI family, salivary pellicle adhesin, 35 kDa	*S. parasanguis* FW213	Catheterized rat (c)	> 90% decreased incidence of vegetation (endocarditis)	Burnette-Curley *et al.* (1995)
scaA	LraI family, lipoprotein 35 kDa	*S. gordonii* DL1	LF mouse (c)	No effect on oral colonization	Tannock, Loach, and Jenkinson (unpublished)
emm24	cell-surface fibrillar M protein, 54 kDa	*S. pyogenes* M-type 24	Mouse (c)	> 95% reduction in numbers of animals colonized orally	Courtney *et al.* (1994)
emm6.1	cell-surface fibrillar M protein, 48 kDa	*S. pyogenes* D471 M-type 6	SPF rat (c)	No effect on oral colonization short-term; > 90% decreased persistence after 2 wks	Hollingshead *et al.* (1993)
ply	intracellular pneumolysin, 53 kDa	*S. pneumoniae* D39 (capsular-type 2)	Mouse (c)	Increased median survival time of animals; 100-fold increase in i.p. LD_{50}	Berry *et al.* (1989a)
lytA	cell-surface-associated autolysin, 36 kDa	*S. pneumoniae* D39	Mouse (c)	Increased median survival time of animals; 10^5-fold increase in i.p. LD_{50}	Berry *et al.* (1989b)
pspA	cell-surface protein *ca.* 65 kDa	*S. pneumoniae* D39	Mouse (c)	20-fold reduced rate of blood clearance following i.v. challenge	McDaniel *et al.* (1987)

a hs, high-sucrose (56%) diet; ls, low-sucrose (5%) diet; c, conventional (no sucrose) rodent diet.

b Specific pathogen-free.

c Lactobacillus-, enterococcus-, and streptococcus-free.

d Germ-free (gnotobiotic).

nize the murine oral cavity (Table 4). These data emphasize that *in vitro* assays are not necessarily adequate for identifying the streptococcal colonization-associated factors. It is also important to appreciate that the nature of the animal model can bias interpretations of experimental data. For example, *spa*P mutants of S. *mutans* are defective in adherence to salivary pellicle *in vitro*, yet their colonization ability and cariogenicity in rodents were unaltered (Table 4). This somewhat enigmatic result may be explained by proposing that in rats fed a high-sucrose diet, the ability to express antigen I/II polypeptide SpaP is of lesser importance than that of the ability to synthesize extracellular polysaccharides.

Rodents have also been utilized for assessing pneumococcal and group A streptococcal virulence. Several studies have defined pneumococcal virulence-related factors (Table 4) with a view to improving the efficacy of the capsular polysaccharide vaccine (see Paton *et al.*, 1993). In addition, animal colonization experiments have confirmed the requirement for M protein expression for group A streptococcal virulence (Table 4). It has also been possible, by means of catheterized animals, to investigate the streptococcal factors necessary for the development of infective endocarditis. Effects on streptococcal virulence (dramatic or subtle) can be demonstrated for almost every gene that has been inactivated. Unfortunately, if an inactivated gene is found to have no effect on virulence, it may simply suggest that the model is inadequate. Similarly, a positive result should be regarded with caution until it has been demonstrated that the mutation does not affect expression of neighboring or downstream genes. Ideally, mutant strains should be complemented with the homologous wild-type gene and tested for restoration of phenotype. Nevertheless, some interesting and useful comparative data have been obtained, particularly from analyses of putative endocarditis-related factors. For example, expression of FimA by S. *parasanguis* is essential for production of endocardial vegetations, but the homologous protein ScaA in S. *gordonii* is not necessary for oral cavity colonization (Table 4) by these bacteria. In another comparison, Gtf$^-$ mutants of S. *mutans* were severely reduced in their ability to cause endocardial disease in catheterized rats, whereas *gtf*G mutants of S. *gordonii* were unaffected (Table 4). Taken collectively, these results support the general view that a colonization or virulence factor identified for a specific streptococcal strain may not necessarily be significant for colonization or virulence of a different strain, even though the latter expresses the homologous factor. Furthermore, different factors may vary in importance according to the animal model being used. It is already known, from extensive *in vitro* data, that different adhesins are brought into play, depending upon the host tissue type and host environment being colonized. Despite some of the shortcomings evident with animal model studies of streptococcal colonization or virulence, it is imperative that steps be taken to determine which adhesins identified *in vitro* are truly expressed, and are colonization factors, *in vivo*.

Control and Prevention of Streptococcal Infections

Reliance on the current classes of antibiotics alone to control streptococcal infections is becoming increasingly imprudent. Thus, novel methods to prevent or treat these diseases are desirable and indeed may soon become imperative. One obvious such approach is the development of vaccines. There are several obstacles to the development of successful vaccines against streptococcal infections. Many pathogenic species are closely related antigenically to streptococci that exist in commensal harmony with, and may even benefit, the host. Antibodies induced by vaccination that cross-react with the resident streptococci may, therefore, upset the delicate balance between the commensals and the host, with adverse consequences. Another repercussion of undesirable antibodies that can be induced by streptococcal antigens is cross-reactivity with host proteins, particularly in heart tissue, and the generation of immune-complex-mediated diseases such as acute glomerulonephritis. Additionally, many streptococcal diseases are localized to the mucus membranes or surface layers of the skin, sites where the production of a potent and long-lasting immune response is difficult to accomplish. Indeed, few infectious diseases of such sites can be currently controlled by vaccination. These constraints do not, of course, preclude the development of vaccines against streptococcal infections, but rather require that parameters such as the choice of antigen(s), delivery method, use of adjuvant, and timing of immunizations be thoroughly investigated. A notable success has been the production of a vaccine against pneumococcal disease, although this polysaccharide-based vaccine lacks efficacy in infants and young children.

S. *mutans*, a primary etiological agent of dental caries, is one organism for which much effort has been expended to develop a vaccine. Although a safe and effective vaccine is not yet available, significant progress has been made in this area. In addition to whole cells of S. *mutans*, components important in the initial adherence or subsequent accumulation of the bacteria have been assessed for vaccine suitability, including glucosyltransferase (Smith and Taubman, 1987) and antigen I/II polypeptide (Lehner *et al.*, 1981). As more becomes known about the functional and immunogenic domains of these molecules, immunization with an appropriate synthetic peptide is more feasible (Kelly *et al.*, 1995; Taubman *et al.*, 1995). A refinement of this approach is to create a hybrid molecule comprising the streptococcal component fused to the B-subunit of cholera toxin that

will more efficiently target cells of the gut-associated lymphoreticular tissue by binding to GM1 ganglioside (Michalek *et al.*, 1995). As an alternative to vaccination, passive immunization has the advantage that possible undesirable antibodies are not evoked. Promising results have been obtained by passive immunization of human subjects with monoclonal antibodies to S. *mutans* antigen I/II (Ma *et al.*, 1990). Particularly encouraging was the finding that the inhibitory effect of the antibodies on S. *mutans* colonization appeared to be more than transient and persisted after the antibodies had been eliminated from the oral cavity (Ma *et al.*, 1990). This mode of passive immunization, therefore, may induce long-term changes in the ecological relationships within the oral cavity that persist without the need for the continual presence of antibodies. Other creative approaches to passive immunization have included the use of immune bovine milk (Michalek *et al.*, 1987) and egg yolk antibody (Hamada *et al.*, 1991).

Since many oral commensal streptococci are efficient colonizers, it may be possible to engineer these organisms to express protective antigens derived from pathogenic strains. For example, recombinant S. *gordonii* have been generated that express portions of S. *pyogenes* M protein (Pozzi *et al.*, 1992). These engineered strains of S. *gordonii* can induce both systemic and mucosal immune responses to the heterologous antigen, therefore potentially providing more effective protective immunity. If this kind of approach proves successful, it could be adapted to control a number of infections. Related systems entail the expression of protective antigens from streptococci in other vectors that are inherently efficient at stimulating the immune system, such as attenuated strains of *Salmonella typhimurium* (Michalek *et al.*, 1995) or vaccinia virus (Fischetti *et al.*, 1989).

Replacement therapy is another pathway to controlling S. *mutans* infection that is being actively pursued. Now that genetic manipulation of S. *mutans* is possible, one goal has been to engineer a strain that is colonization-competent, produces high levels of bacteriocin active against other mutans group streptococci, and is deficient in lactic acid production. When introduced into the oral cavity, such a strain might grow and survive at the expense of other mutans group streptococci, but would be non-cariogenic because of the defect in lactic acid production. Strains that can colonize teeth and produce appropriate bacteriocin have been created, but abolishing lactic acid production has been more difficult to achieve, because mutations in the *ldh* gene (encoding lactate dehydrogenase) appear to be lethal in S. *mutans*. Nonetheless, it may be possible to circumvent this problem by (genetic) manipulation of the physiology of the organism (Hillman *et al.*, 1996), and replacement therapy could become a very powerful means of controlling colonization by cariogenic mutans group streptococci.

Interference with adherence mechanisms of streptococci is a worthwhile direction that could be extended beyond the use of antibodies to target streptococcal adhesins or their functional domains. As more information concerning the molecular interactions of the adhesins with their cognate receptors becomes available, it may be possible to synthesize agonists capable of irreversibly blocking adhesin-receptor interactions. The multiplicity of streptococcal adhesins would tend to limit the utility of a "shotgun" approach. However, in instances where activity of certain adhesins correlates with pathogenicity (*e.g.*, pneumococcus), it may be possible to block the pathogenic adherence mechanisms and/or stimulate adherence systems that maintain a commensal relationship. The need for novel agents to control and prevent streptococcal infections is unlikely to become less compelling.

Potential exciting avenues for future research abound. Determination of the complete nucleotide sequence of a group A streptococcal genome is well under way (Ferretti *et al.*, 1996), and upon completion of genomic sequencing of other streptococcal species in the future, it should be possible to gain broad insight into the genetic complements of these organisms, especially as related to adhesion, colonization, and virulence properties. Streptococcal genetics is now sufficiently advanced that the identification of all the bacterial genes and gene products that elicit specific host responses will be readily attainable. These responses include the intercellular signaling mechanisms that may be prerequisite for, or accompany, epithelial cell invasion by bacteria, the induction of cytokines, and the secretion of mucosal antibodies. The host-cell response mechanisms are poorly characterized, yet all of the above (and more) are significant in determining the final outcomes of streptococcal infections. More research is necessary to completely unravel the mechanisms by which streptococci are able to evade host defenses, such as by physical or enzymic protection, or through antigenic variance. In addition, investigation into mechanisms of antibiotic resistance and the search for new therapeutic agents is a sisyphean task that is imperative to pursue. Finally, it is pertinent to emphasize that adherent streptococci present in dental plaque, or colonizing an epithelial surface, differ phenotypically from their planktonic, non-adherent, counterparts. Adherent organisms in biofilms have been shown to express new genes and down-regulate expression of other genes, so future research on streptococcal cells that are growing in "biofilm mode", rather than planktonically, may be more relevant to the *in vivo* situation. The ongoing research into the adhesion and colonization mechanisms of these organisms bodes interesting times ahead with the potential for significant advances in therapeutics for streptococcal infections.

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