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The Collagen Binding Proteins of *Streptococcus mutans* and Related Streptococci

Alejandro Avilés-Reyes¹, James H. Miller², José A. Lemos^{1,*}, and Jacqueline Abranches^{1,*}

¹Department of Oral Biology, University of Florida, College of Dentistry, Gainesville, FL

²Department of Anesthesiology, University of Rochester School of Medicine and Dentistry, Rochester, NY

Summary

The ability of *Streptococcus mutans* to interact with collagen through the expression of collagen-binding proteins (CBPs) bestows this oral pathogen with an alternative to the sucrose-dependent mechanism of colonization classically attributed to caries development. Based on the abundance and distribution of collagen throughout the human body, stringent adherence to this molecule grants *S. mutans* with the opportunity to establish infection at different host sites. Surface proteins, such as SpaP, WapA, Cnm and Cbm, have been shown to bind collagen *in vitro*, and it has been suggested that these molecules play a role in colonization of oral and extra-oral tissues. However, robust collagen binding is not achieved by all strains of *S. mutans*, particularly those that lack Cnm or Cbm. These observations merit careful dissection of the contribution from these different CBPs towards tissue colonization and virulence. In this review, we will discuss the current understanding of mechanisms utilized by *S. mutans* and related streptococci to colonize collagenous tissues, and the possible contribution of CBPs to infections in different sites of the host.

Keywords

Streptococcus; collagen binding; adhesins

1. Introduction

Streptococcus mutans, a natural inhabitant of the oral cavity, is commonly associated with dental caries and occasionally with infective endocarditis (Harder *et al.*, 1974; Loesche, 1986; Nakano *et al.*, 2008; Dewhirst *et al.*, 2010; Han *et al.*, 2013). It encodes a small number of adhesins with strong and specific affinities to various components of the extracellular matrix (ECM), metabolic products, soluble host factors as well as other microorganisms (Banas, 2004; Bowen & Koo, 2011; Nobbs *et al.*, 2015). In the context of the oral cavity, *S. mutans* relies on both sucrose-dependent and -independent mechanisms for colonization and establishment of biofilms (Krzysciak *et al.*, 2014). In the presence of sucrose, glucosyltransferases (Gtfs) produce extracellular polysaccharides, i.e. glucans,

*Corresponding authors Mailing address: Department of Oral Biology, 1395 Center Drive, Box 100424, University of Florida, College of Dentistry, Gainesville, FL 32610, Tel: (352) 273-6672; Fax: (352) 273-8829, jlemos@dental.ufl.edu or jabranches@dental.ufl.edu.

which, in conjunction with glucan-binding proteins (Gbp), facilitate the generation of robust biofilms on the tooth surface. Biofilm accumulation restricts the diffusion of organic acid products derived from sugar metabolism and the flow of pH-buffering saliva, subjecting the enamel to repeated and prolonged exposure to acids thereby leading to demineralization and cavitation (for detailed reviews refer to Bowen and Koo, 2011 and Selwitz *et al.*, 2007). The sucrose-independent mechanism is directed by specific substrate recognition and adherence by surface adhesins that bind either to constituents of the salivary pellicle on the tooth surface, such as SpaP (also known as P1, PA, or antigen I/II) (Loimaranta *et al.*, 2005; Nobbs *et al.*, 2009), or to components of underlying tissues that become exposed due to demineralization of the enamel (e.g. collagen from dentin and roots) (Switalski *et al.*, 1993; Love *et al.*, 1997; Sciotti *et al.*, 1997). Additionally, *S. mutans* can colonize other parts of the body leading to extra-oral infections such as infective endocarditis (IE), and in rare cases, peritoneal abscesses and intra-amniotic infections (Romero *et al.*, 2008; Ioannidis *et al.*, 2011; Nakano *et al.*, 2011; Jung *et al.*, 2012; Kojima *et al.*, 2014). In extra-oral infections, the interaction of *S. mutans* with host components (i.e. platelets, macrophages, ECM proteins) can interfere with the normal function of the immune system and increase inflammation, resulting in retardation of tissue repair and homeostasis (Chia *et al.*, 2004; Nagata *et al.*, 2011; Nakano *et al.*, 2011). Initial adhesion to host tissues by bacterial pathogens is considered a prerequisite to establish colonization and subsequent pathogenesis (Nobbs *et al.*, 2015). Therefore, the identification and characterization of bacterial factors responsible for binding to host tissues affords a great opportunity to better understand the mechanisms from which disease germinates and to reveal new targets that can be exploited for infection prevention and treatment.

The ECM is a non-cellular component that is present in all tissues and organs, serving a multitude of functions that range from tissue development to tissue maintenance and repair (Hynes, 2009; Frantz *et al.*, 2010). These macromolecules are grouped into two categories: proteoglycans, which are composed of glycosaminoglycan chains covalently linked to a specific core, and fibrous proteins (Frantz *et al.*, 2010). Vertebrate genomes encode hundreds of different fibrous proteins, some of the major components being collagen, laminin, elastin, fibronectin, fibrillin and vitronectin (Singh *et al.*, 2012). The basement membrane (BM), which is present in every tissue of the human body, is a layer of specialized ECM molecules found basolateral to all epithelial and endothelial cells (Yurchenco, 2011). Collagen proteins, the most abundant type of ECM molecules in mammals, are essential structural components present in all connective tissue and are involved in many physiological processes (e.g. tissue integrity, repair and growth) (Ricard-Blum, 2011). Thus far, 28 different types of collagen have been identified in humans (Ricard-Blum, 2011). For instance, fibrils in bones, skin and cartilage are predominantly formed by collagen types I and II, whereas non-fibrillar collagens, such as types IV and VI, largely compose the flexible networks of BM alongside with laminin, nidogen/entactin and perlecan (LeBleu *et al.*, 2007; Ricard-Blum, 2011; Yurchenco, 2011; Singh *et al.*, 2012). Given its pervasiveness, it comes as no surprise that collagen lies at the interface of several malignancies ranging from autoimmune and developmental disorders (Di Lullo *et al.*, 2002; Ricard-Blum, 2011) to infectious diseases. In terms of bacterial infections, collagen serves as a target for adhesion by microorganisms through collagen binding proteins (CBPs). Bacterial CBPs mediate

highly specific and stringent binding to collagenous host tissues, often resulting in deep tissue and persistent infections (Singh *et al.*, 2012). Interaction with collagen is achieved by both Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* (Zong *et al.*, 2005), *Enterococcus faecalis* (Nallapareddy *et al.*, 2000), and *Yersinia enterocolitica* (Heise & Dersch, 2006) among others (Singh *et al.*, 2012). For this reason, the CBPs of human pathogens represent an important area of research with clear translational value as these virulence factors could be targeted for antimicrobial therapies and their mechanisms exploited for novel drug delivery molecules.

In 1985, Vercelotti and colleagues demonstrated that various bacterial species were able to bind and aggregate specific ECM proteins such as type IV collagen (Vercelotti *et al.*, 1985). This interaction has been further characterized in many classes of bacteria including Gammaproteobacteria, Actinobacteria and Firmicutes (Singh *et al.*, 2012). Early studies demonstrated that several streptococci were able to bind to collagen (Speziale *et al.*, 1987), although the specific CBPs mediating this process were not identified until later (Nobbs *et al.*, 2009). In UA159, the first sequenced strain of *S. mutans*, two surface proteins with collagen-binding properties were identified: the surface adhesin SpaP, and the cell-wall associated protein A (WapA) (Ajdic *et al.*, 2002). More recently, molecular and genomic analyses of *S. mutans* isolates have led to the identification of two additional CBPs, Cnm and Cbm (Sato *et al.*, 2004a & b; Nomura *et al.*, 2012; Cornejo *et al.*, 2013; Palmer *et al.*, 2013). Clinical and laboratory studies with Cnm⁺ and Cbm⁺ isolates strongly suggested that these CBPs are important virulence factors in systemic infections (Nomura *et al.*, 2009; Abranches *et al.*, 2011; Nakano *et al.*, 2011; Nomura *et al.*, 2013a; Avilés-Reyes *et al.*, 2014a) and, more recently, in caries severity (Miller *et al.*, 2015). The purpose of this review is to provide a comprehensive overview of the current literature on *S. mutans* CBPs, their association with disease development and progression, and to identify areas in need of further research. Findings from CBPs of related streptococci are also briefly discussed.

2. Identification of collagen binding proteins in streptococci

2a. SpaP and WapA in *S. mutans*

Early investigations of the cell wall surface components of *S. mutans* identified SpaP as a major surface adhesin that facilitates bacterial binding to components of the salivary pellicle on the tooth surface (Lee *et al.*, 1989). Subsequent studies identified SpaP homologues in other streptococci, and the characterization of their adhesive properties has implicated this surface molecule with multiple colonization strategies within the context of oral infection (Jenkinson & Demuth, 1997; Lamont *et al.*, 2002; Brady *et al.*, 2010). In *S. mutans*, SpaP is a 1556 amino acid polypeptide with four major domains: i) an N-terminal secretion signal, ii) an alanine-rich region, iii) a proline-rich region and, iv) the cell-wall anchoring LPXTG motif (Fig. 1A) (Brady *et al.*, 2010). SpaP specifically interacts with immobilized glycoprotein-340 (gp-340), an innate immunity scavenger receptor (Loimaranta *et al.*, 2005). In addition to interacting with salivary components, SpaP binds different types of ECM molecules, namely type I collagen, laminin and fibronectin (Sciotti *et al.*, 1997). Collagen binding by SpaP was first demonstrated through different binding activities of parent and *spaP* strains to immobilized collagen *in vitro* or to tooth fractions *ex vivo* (Love *et al.*,

1997). While binding forces between recombinant SpaP and type I collagen or laminin molecules have been described as fairly robust, long incubation periods and high levels of SpaP are required compared to what is necessary for optimal gp-340 binding (Love *et al.*, 1997; Sullan *et al.*, 2015). In UA159, a strain described as having relatively high level of SpaP surface expression (Crowley *et al.*, 2008), collagen-binding activity is virtually nonexistent *in vitro* (Avilés-Reyes *et al.*, 2014a). Nonetheless, the expression of SpaP by *S. mutans* strains harboring other CBPs, such as Cnm or Cbm, can result in diminished collagen binding compared to those that are Cnm⁺ or Cbm⁺ but SpaP⁻ (Nomura *et al.*, 2013b). It has been surmised that the expression of SpaP in Cnm⁺ or Cbm⁺ strains results in an alteration of the cellular hydrophobicity and zeta-potential, ultimately diminishing collagen-binding ability (Nomura *et al.*, 2013b; Lapirottanakul *et al.*, 2015).

In addition to SpaP, WapA is an LPXTG cell wall-anchored protein of *S. mutans* that mediates binding to collagen (Han *et al.*, 2006). Both SpaP and WapA were initially identified in a study analyzing the antigenic potential of SDS-extracted proteins of *S. mutans* Ingbritt in rabbits (Russell, 1979). From these studies, WapA, initially called antigen A, was considered a promising vaccine candidate for dental caries based on immunization studies using rodents, monkeys and human volunteers (Dao *et al.*, 1989). While vaccination efforts against dental caries using WapA were ultimately unfruitful, further analysis suggested that WapA affected both sucrose-dependent and sucrose-independent adherence mechanisms (Han *et al.*, 2006; Qian *et al.*, 1993). Direct collagen binding by WapA was observed using biotinylated cells that were co-incubated with immobilized type I collagen (Han *et al.*, 2006). This interaction was attributed to the presence of a collagen-binding domain (CBD) based on shared amino acid sequence with other CBPs of Gram-positive bacteria (Fig. 1A & B) (Han *et al.*, 2006). However, it appears that WapA does not contribute to significant levels of collagen binding *in vivo*, and the differences seen in the *wapA* mutant strain were likely the result of alterations on the bacterial cell envelope (Qian *et al.*, 1993). In conclusion, while the *S. mutans* SpaP and WapA proteins possess the capacity to bind collagen *in vitro*, additional *in vivo* studies are necessary to demonstrate whether these proteins are bona-fide CBPs.

2b. SpaP and WapA in other Streptococci

While WapA-like proteins are predicted to exist in four other species of streptococci (i.e. *S. rattii*, *S. devriesei*, *S. macacae* and *S. ferus*), no study thus far has examined the collagen-binding properties of these proteins. Conversely, orthologues of *S. mutans* SpaP can be found in other streptococci and although generally involved in similar functions, important functional differences have been described (Table 1) (Brady *et al.*, 2010). For instance, while most streptococcal species contain one copy of SpaP, *S. gordonii* and *S. criceti* harbor two copies (SspA/SspB and PAaA/PAaB, respectively). A common feature of SpaP orthologues is cell co-aggregation, yet the mechanism by which this is achieved appears to be species specific (Brady *et al.*, 2010). For example, the *S. gordonii* SspA and SspB not only interact with gp-340 and collagen, like the *S. mutans* SpaP, but can also mediate interactions between *S. gordonii* and other microorganisms such as *Candida albicans* (Holmes *et al.*, 1996), *P. gingivalis* (Love *et al.*, 2000) and *Actinomyces naeslundii* (Jakubovics *et al.*, 2005). On the other hand, the SpaP homologue of *S. intermedius* does not mediate binding to

saliva-coated glass and is unable to bind to either rat- or human-derived collagens *in vitro* (Petersen *et al.*, 2002). These differences are quite surprising considering the high levels of amino acid sequence homology and predicted functional domain structures observed among the SpaP orthologues (Brady *et al.*, 2010).

2c. Cnm and Cbm in *S. mutans*

As in many other bacterial species, genomic variability can be observed in *S. mutans* isolates as the core genome contains 1,373 genes, and the pan-genome at least 3,296 genes (Cornejo *et al.*, 2013; Song *et al.*, 2013). In this context, non-core genes can confer strains niche-specific physiological benefits. One such example is the Cnm protein (collagen binding protein of *S. mutans*), which has been identified in roughly 15% of all *S. mutans* isolates analyzed thus far (Abranches *et al.*, 2011; Cornejo *et al.*, 2013; Nakano *et al.*, 2010a). Cnm was initially identified as a cell wall component responsible for a cold agglutination phenotype and is now recognized as an important virulence factor of *S. mutans* (Sato *et al.*, 2004a; Abranches *et al.*, 2011; Miller *et al.*, 2015; Nakano *et al.*, 2011). Genetic analysis of Cnm revealed the presence of a N-terminal secretion signal, a collagen-binding domain (A domain) with over 50% identity to the A domain of the *S. aureus* Cna, a unique threonine-rich domain (B domain), and a C-terminal LPXTG cell wall-anchoring motif (Fig. 1A) (Sato *et al.*, 2004b; Avilés-Reyes *et al.*, 2014a). *In vitro* ECM-binding experiments demonstrated that a recombinant version of the Cnm A domain exhibited high affinities to both collagen and laminin (Sato *et al.*, 2004b). The ECM-binding phenotype conferred by Cnm was recapitulated in several additional Cnm⁺ isolates using immobilized collagen (types I, II, III and IV), laminin, as well as the collagenous sections of extracted human teeth (Abranches *et al.*, 2011 & unpublished; Avilés-Reyes *et al.*, 2014a; Nomura *et al.*, 2013a; Miller *et al.*, 2015). Accordingly, collagen-binding activity by isogenic *cnm* strains is negligible, indicating that surface expression of Cnm is essential for stringent collagen binding by *S. mutans* (Abranches *et al.*, 2011; Avilés-Reyes *et al.*, 2014a; Nomura *et al.*, 2013a). Moreover, expression of Cnm in the Cnm⁻ laboratory strain, UA159, yielded similar collagen and laminin binding affinities to those observed in the Cnm⁺ strains (Avilés-Reyes *et al.*, 2014a). Interestingly, the expression of Cnm endows *S. mutans* with the ability to invade non-phagocytic cells (Abranches *et al.*, 2009 & 2011; Miller *et al.*, 2015; Nomura *et al.*, 2012), a trait that is directly attributable to chronic and systemic infections [see Section 3.b].

The identification of Cnm and its association with both oral and extra-oral diseases [see Section 3], led to the characterization of the *cnm* locus within the *S. mutans* genome. Interestingly, two additional genes encoding putative surface CBPs, dubbed *cnaB* and *cbpA* (Palmer *et al.*, 2013; Avilés-Reyes *et al.*, 2014a) were located directly upstream to *cnm*. The *cnaB-cbpA-cnm* gene arrangement is conserved among all Cnm⁺ strains sequenced thus far and invariably located between the core genes SMU_2067 and SMU_2069. Like Cnm, both CnaB and CbpA possess a putative collagen binding domain (A domain), a unique threonine-rich region (B domain) and a C-terminal LPXTG motif (Fig. 1A). However, inactivation of either *cnaB* or *cbpA*, alone or in combination, in the Cnm⁺ OMZ175 strain, did not affect its collagen-binding activity or any other Cnm-related phenotypes (Avilés-Reyes *et al.*, 2014a). Furthermore, expression of either *cnaB* or *cbpA* in UA159 did not

enhance *S. mutans* collagen-binding activity, indicating that these genes neither appear to be involved in collagen-binding nor are they required for Cnm function (Avilés-Reyes *et al.*, 2014a). While collagen-binding capacity cannot be attributed to either CnaB or CbpA, it remains to be determined if purified CnaB and CbpA show ECM-binding activity *in vitro* as seen with SpaP and WapA.

Further characterization of *S. mutans* isolates with strong collagen-binding capacities has recently led to the discovery of yet another CBP termed Cbm (Nomura *et al.*, 2012). The collagen binding domains of Cnm and Cbm are 78% identical (Nomura *et al.*, 2012), which raises important questions as to their evolutionary relationship. Like Cnm, Cbm also possesses a threonine-rich B-domain of varying lengths among the different strains, although these are predominantly composed of tandem TTTT(AE/TE)P and subsequent TTTTE(GS/AS/TP/TS) regions compared to TTTEAP in Cnm (Nomura *et al.*, 2012). Interestingly, we recently found that the *cbm* gene is located at the same locus as *cnm*, i.e. between the core genes SMU_2067 and SMU_2069 (Avilés-Reyes *et al.*, unpublished). Sequence analysis between Cnm, Cbm, CnaB and CbpA shows similar arrangements between these proteins (Fig. 1B). Ribbon diagram models of the collagen binding domains of Cnm, Cbm, CnaB and CbpA reveal that only Cnm and Cbm possess similarity with the previously crystallized Cna from *S. aureus* (Fig. 1C).

2d. Cnm-like proteins in other streptococci

Both Cnm and Cbm belong to a group of CBPs with high amino acid sequence and structural similarities within their collagen binding segments (Kang *et al.*, 2013). Included in this group are the prototypical *S. aureus* and *E. faecalis* virulence factors Cna and Ace, respectively, as well as other streptococcal CBPs (Table 1) (van Wieringen *et al.*, 2010). In the animal pathogen *Streptococcus equi*, Cne collagen-binding activity was found to be equivalent to that of Cna (Lannergard *et al.*, 2003). In addition, the *S. gordonii* CbdA was shown to mediate binding to type I collagen as well as persistence in biofilms and instrumented *ex vivo* root canals (Moses *et al.*, 2013). Sequence-based searches using Basic Local Alignment Sequence Tool (BLAST) indicate that Cnm-like proteins are present in several other oral streptococci (e.g. *S. suis*, *S. ratti*, *S. parasanguinis* and *S. infantis*). However, it remains to be tested whether these genes encode bona-fide CBPs, or possess any evolutionary relationship with Cnm.

2e. Distribution of CBPs in *S. mutans*

While all strains of *S. mutans* sequenced thus far contain the *spaP* and *wapA* genes, the expression levels of these genes or proteins can vary widely. In the case of *spaP*, differential expression levels are due to either mutations in the coding and promoter regions (Sato *et al.*, 2011; Nomura *et al.*, 2013b; Lapiattanakul *et al.*, 2015;), or in the machinery involved in protein processing (e.g. sortase A) (Lee *et al.*, 2003). On the other hand, expression of *wapA* appears to be increased in the presence of sucrose (Zhu *et al.*, 2006).

Different than *spaP* and *wapA*, the *cnm* and *cbm* genes are limited to a subset of strains (Nakano *et al.*, 2010a; Abranches *et al.*, 2011; Nomura *et al.*, 2013a). Genetic analysis of *S. mutans* isolates including both recent clinical isolates as well as laboratory strains indicated

that the *cnm* locus (*cnaB-cbpA-cnm*) is present in approximately 15% percent of all isolates (Nakano *et al.*, 2010a; Abranches *et al.*, 2011; Cornejo *et al.*, 2013). The *cbm* gene is even rarer and has been found in less than 3% of isolates (Nomura *et al.*, 2012 & 2014). Combined, it appears that the prevalence of bona-fide CBPs with strong collagen binding activity in *S. mutans* isolates is nearly 20%. Interestingly, the distribution of Cnm and Cbm is uneven across the different *S. mutans* serotypes (Abranches *et al.*, 2011; Nomura *et al.*, 2013a; Lapirattanakul *et al.*, 2013). Strains of *S. mutans* are grouped into four serotypes (*c*, *e*, *f* and *k*) based on the chemical composition of the rhamnose-glucose polysaccharide (RGP) present in the cell envelope (Shibata *et al.*, 2003; Nakano *et al.*, 2007). In plaque, serotype *c* comprises over 75% of all isolates, followed by serotypes *e* and *f*, each comprising around 10%, while serotype *k* is rarely present (~ 5%) (Nakano *et al.*, 2007). Notably, an association between extra-oral infections and the presence of strains from less common serotypes (*e*, *f*, and *k*) has been described (Nakano *et al.*, 2008 & 2011; Nomura *et al.*, 2013). A similar trend has been observed for the distribution of Cnm and Cbm proteins across serotypes, where the majority of the strains expressing Cnm or Cbm belong to serotypes *e*, *f* and *k* (Abranches *et al.*, 2011; Lapirattanakul *et al.*, 2013; Nomura *et al.*, 2014).

3. Association of CBPs with streptococcal diseases

The classification of *S. mutans* as a human pathogen depends on the specific site of infection (Casadevall & Pirofski, 1999). For example, *S. mutans* in the oral cavity is classified as a class 2 pathogen meaning that it can “cause damage either in hosts with weak immune responses, or in the setting of normal immune responses” (Casadevall & Pirofski, 1999). In this context, *S. mutans* is able to infect and initiate dental caries in overall healthy individuals, albeit the development of carious lesions is exacerbated by host nutritional choices and other predispositions (e.g. high sucrose diet, xerostomia) (Selwitz *et al.*, 2007; Bowen, 2015). Conversely, when acting as a systemic pathogen, *S. mutans* is regarded as a class 1 pathogen generally requiring a weakened immune response or other pre-existing conditions (e.g. prosthetic heart valve, chronic heart disease, previously damaged endothelium). Regardless of the site of infection, the expression of CBPs bestows *S. mutans* with a capacity to attach to virtually any tissue site containing exposed collagen (like dentin and heart valves) and thus exemplifies the importance of these molecules in the development of oral and extra-oral diseases.

3a. CBPs and oral diseases

Upon physical trauma of the tooth, dentin-level carious lesions, or gingival recession, collagen-rich substrates such as dentin, root, cementum, and periodontal ligament fibers, become exposed and thus represent an ideal target for colonization by CBP⁺ *S. mutans* strains (Liu & Gibbons, 1990; Miller *et al.*, 2015). Furthermore, children infected with Cnm⁺ *S. mutans* displayed significantly increased rates of decayed teeth, extractions, and filled surfaces due to dental caries (defs, a metric of caries development assessing number of teeth) than children who were *S. mutans*⁺ Cnm⁻ (Nomura *et al.*, 2009). In the archetypical system used to assess cariogenicity, the rat caries model, decreased colonization and cariogenic potential were observed in a *cnm* mutant compared to the parental OMZ175

strain when using a high sucrose diet (Miller *et al.*, 2015). Scoring of carious lesions in animals infected with OMZ175 or its *cnm* counterpart did not show any significant difference in total number of lesions, yet significant differences in lesion severity were observed (Miller *et al.*, 2015). As suggested by the significantly lower numbers of total flora recovered from rats infected with the *cnm* strain, Cnm (and, perhaps, Cbm) may also be involved in interactions with the resident microbiota in addition to attachment to collagenous surfaces *in vivo* (Miller *et al.*, 2015). Thus, like WapA and SpaP, it is possible that Cnm modulates the bacterial biofilm by directly interacting with other members of the microbiome.

While the role of SpaP in the establishment and progression of dental caries under low sucrose conditions has been established (Crowley *et al.*, 1999), few studies have specifically looked at the collagen binding aspect of SpaP with regard to oral colonization. In a study by Love *et al.*, *in vitro* invasion of dentinal tubules was reported to be SpaP-dependent (Love *et al.*, 1997). The authors indicated that through invading the collagen-rich dentinal tubules, *S. mutans* might also be able to contribute to the development of root caries and pulpal infections (Love *et al.*, 1997). Similarly, the *S. gordonii* SpaP homologues, SspA/SspB, also appear to be required for invasion of dentinal tubules (Love *et al.*, 2000). Given that SspAB of *S. gordonii* also mediate co-aggregation with the periodontal pathogen *P. gingivalis*, it was shown that invasion of the dentinal tubules by *P. gingivalis* was SspAB dependent. While these results may indicate a role for SspAB in enhancing *P. gingivalis* pathogenic potential in root canal infections (Love *et al.*, 2000), such mechanism of infection is yet to be demonstrated *in vivo*.

3.b Collagen binding and systemic disease

The recognition and binding to collagen by streptococci has been implicated in systemic infections of various forms including respiratory (Bober *et al.*, 2010), cardiovascular (Switalski *et al.*, 1987; Nakano *et al.*, 2010b), and cerebral (Banerjee *et al.*, 2011; Nakano *et al.*, 2011), among others (Brandt & Spellerber, 2009; Singh *et al.*, 2012). Historically, *S. mutans* has been known to cause extra-oral infections such as bacteremia and infective endocarditis (IE) (Nakano *et al.*, 2008). The association of *S. mutans* with IE was first reported in 1928 (Abercrombie *et al.*, 1928) and, to this day, it remains a common cause of this life-threatening disease (Mylonakis & Calderwood, 2001; Nakano *et al.*, 2008; Rovey *et al.*, 2005; Simmon *et al.*, 2008). The pathogenesis of IE generally begins with the formation of sterile endocardial vegetations that originate from physical or inflammatory lesions that lead to the exposure of ECM proteins, production of pro-inflammatory cytokines, and deposition of fibrin and platelets (Que & Moreillon, 2011). These processes involved in vascular homeostasis are not exclusive to IE, and are employed in the repairing of other vascular injuries such as post-haemorrhagic stroke (Nakano *et al.*, 2011). Thus, the expression of CBPs may confer the organism an enhanced capacity to bind to the damaged collagen-rich endothelium and colonize extra-oral tissues (Fig. 2A). Under normal conditions, exposed collagen serves as a platform for platelets and other immune cells to bind, thus initiating tissue repair processes (Kerrigan & Cox, 2010). In this regard, CBP-mediated collagen binding not only facilitates bacterial colonization, but it could also

exacerbate the pathology of disease by blocking tissue repair mechanisms, thus causing further endothelial dysfunction (Fig. 2A).

Atherosclerotic coronary heart disease (ACHD) is a leading cause of death in the U.S. with over 600,000 fatal cases every year (Minino *et al.*, 2011). Epidemiological studies have demonstrated that chronic infection caused by periodontal bacteria (i.e. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*) contributes to the development of atherosclerotic inflammation (Beck & Offenbacher, 2001). Atherosclerotic plaque formation is a multifactorial event with defined stages of development: (i) cholesterol deposition, (ii) inflammation, (iii) ECM production and, (iv) thrombosis (Libby *et al.*, 2002). In a study screening for bacterial DNA in over 200 collected cardiovascular lesions from Japanese patients (including aortic, mitral and tricuspid valve regurgitation, aortic and mitral stenosis and, atherosclerotic plaque), a high prevalence of *S. mutans* DNA was detected in damaged heart valves and aneurysm wall specimens (42.7% and 62.8%, respectively) (Nakano *et al.*, 2009). Similarly, in cardiovascular samples from patients in Brazil, DNA from *S. mutans* was detected in all samples analyzed (100%) (Fernandes *et al.*, 2014; Oliveira *et al.*, 2015). Interestingly, the less common serotypes *e*, *f* and *k*, the serotypes in which Cnm or Cbm are predominantly found, showed a higher incidence in extra-oral/systemic infections (Nakano *et al.*, 2010b; Nomura *et al.*, 2013). Thus, a distinct role for specific *S. mutans* serotypes and the bona-fide CBPs Cnm or Cbm in the development of non-oral malignancies is strongly suggested.

Based on the observation that Cnm⁺ strains were frequently isolated from extra-oral sites, we hypothesized that Cnm may allow *S. mutans* to display an invasive behavior like *P. gingivalis*. This periodontal pathogen has been shown to invade and survive within non-phagocytic cells, leading to inflammatory (and thus, pro-thrombotic) responses (Roth *et al.*, 2009; Pollreis *et al.*, 2010; Reyes *et al.*, 2013). In an earlier study, we tested a panel of *S. mutans* strains for their ability to invade human coronary artery endothelial cells (HCAECs), and observed that all invasive strains in the panel were Cnm⁺. Inactivation of *cnm* in these strains abrogated collagen binding and HCAEC invasion (Abranches *et al.*, 2011). In a study from another group, a Cnm⁺ strain was shown to invade human aortic endothelial cells (HAECs) and induce production of the pro-inflammatory cytokines IL-6, IL-8 and MCP1 (Nagata *et al.*, 2011). Moreover, Cbm was shown to mediate invasion of human umbilical cord vein endothelial cells (HUVECs) and to contribute to increased vegetation size in a rat model for IE (Nomura *et al.*, 2012 & 2014). Finally, Kesavalu *et al.* demonstrated that systemic infection by the invasive OMZ175 (Cnm⁺) strain accelerated atherosclerotic plaque growth in pro-atherogenic ApoE^{null} mice (Kesavalu *et al.*, 2012). Thus, in the context of atherosclerosis, and similarly to *P. gingivalis*, intracellular invasion by Cnm⁺ *S. mutans* potentially contributes to the development and/or progression of disease. While a mechanistic understanding is still lacking, we speculate that Cnm mediates virulence in this setting in different, not mutually exclusive, manners: (i) by adhering to exposed collagen in damaged tissues, (ii) by evading immune surveillance through invasion of endothelial cells to avoid host immunity and antimicrobial exposure, and (iii) by triggering pro-inflammatory immune responses and further exacerbating tissue damage (Fig. 2B) (Beck & Offenbacher, 2001; Reyes *et al.*, 2013). Whereas atherogenesis is classically known to be driven by accumulation of cholesterol and oxidized low density lipoproteins (ox-LDL), which

promotes foam cell differentiation by macrophages (Libby *et al.*, 2002), bacterial infections can also accelerate foam cell formation (Qi *et al.*, 2003). While the contribution of *S. mutans* in this process has yet to be evaluated, the related organism *S. gordonii* was shown to stimulate foam cell formation by THP-1 cells (Okahashi *et al.*, 2011).

As eluded to above, invasion of endothelial cells could also lead to the development of chronic infections within the host by escaping immune and therapeutic challenges (Fig. 2C). Also, Cnm was shown to inhibit activation of the classical complement pathway through direct binding to the collagen-like domain of component C1q (Fig. 2C) (Kang *et al.*, 2013). Thus, it appears that expression of CBPs by *S. mutans* is an important and underestimated strategy contributing to the establishment and/or progression of chronic cardiovascular disease. Still, additional studies are warranted to confirm the role of these CBPs as mediators of cardiovascular infections.

4 . Cnm as a model for *S. mutans* protein O-glycosylation

Protein glycosylation is one of the most abundant post-translational modifications occurring in nature and has been found in all three domains of life (Abu-Qarn *et al.*, 2008). In bacteria, this process is ingrained in a variety of functions, including protein folding, cell adhesion, thermodynamic stability, immunomodulation and protection against proteases (Szymanski & Wren, 2005). The characterization of bacterial glycosylation machineries and substrates is an emerging field as these are considered potential therapeutic targets. Additionally, the development of synthetic pathways for heterologous expression of complex glycoproteins in bacterial hosts may provide new tools to produce medically relevant products (e.g. vaccines, human glycoproteins, etc.) (Szymanski & Wren, 2005).

In *S. mutans*, glycosylation has been primarily described in the context of production and localization of the RGP (Shibata *et al.*, 2003), with little attention given to the glycosylation of proteins. The exception being IDG-60, which was identified as an immune-dominant cell wall-associated glycoprotein involved in maintaining cell shape (Chia *et al.*, 2001) but the glycosylation machinery involved in IDG-60 modification has yet to be characterized. Upon detailed analysis of the *cnm* operon, we observed that *cnm* is co-transcribed with the core gene SMU_2067 encoding a putative glycosyltransferase. Inactivation of SMU_2067, dubbed *pgfS*, led to a decrease in Cnm-mediated phenotypes (i.e. collagen binding, HCAEC invasion and killing of *G. mellonella*), and rendered Cnm highly susceptible to proteolytic degradation (Avilés-Reyes *et al.*, 2014b). Moreover, western blot analysis revealed that the *pgfS* strain produced a Cnm variant of smaller size, suggesting that PgfS indeed modifies Cnm. *In silico* analysis of Cnm suggests that this modification is through O-glycosylation of the threonine-rich B-repeats of Cnm. Lectin binding analysis identified N-acetylglucosamine as one of the glycoconjugates associated with Cnm (Avilés-Reyes *et al.*, 2014b).

The use of Cnm to study protein glycosylation presents several clear advantages to this still underdeveloped field. First, carbohydrate metabolism and utilization is clearly linked to streptococcal virulence (Iyer *et al.*, 2005; Shelburne *et al.*, 2008; Abranches *et al.*, 2008; Kietzman & Caparon, 2010). Second, due to the involvement of Cnm in *S. mutans* virulence (Nakano *et al.*, 2011; Avilés-Reyes *et al.*, 2014b; Nomura *et al.*, 2014), a holistic

characterization of Cnm biogenesis may open new doors for the development of therapies to combat *S. mutans* infections, but will also advance our understanding of protein glycosylation in bacteria. Third, the co-occurrence of *cnm*-like and *pgfS* genes in other oral streptococci and related Gram-positive organisms indicates a conserved glycosylation pathway, which is yet to be effectively characterized (Inoue *et al.*, 2013; Avilés-Reyes *et al.*, 2014b). Finally, PgfS is a GT-A type glycosyltransferase predicted to have a single Rosmann fold with a conserved “DXD” metal binding motif (Coutinho *et al.*, 2003; Zhu *et al.*, 2015). Most glycosyltransferases characterized thus far belong to the GT-B family which is characterized by a twin Rosmann fold that is linked by a flexible active site so that it faces onto itself resulting in a cleft (Zhu *et al.*, 2011 & 2015). Thus, the characterization of PgfS-mediated glycosylation will provide relevant information applicable to other O-linked glycosylation pathways as well as other GT-A glycosyltransferases. Altogether, understanding the molecular mechanism by which PgfS glycosylates Cnm represents an important contribution to glycoproteomics research. Such information could potentially lead to the development of heterologous glycosylation machineries employing synthetic biology approaches for the production of recombinant proteins used for therapeutic purposes (Szymanski & Wren, 2005).

5. Additional streptococcal CBPs

Several other surface proteins with collagen-binding capacities have been identified and characterized in the genus *Streptococcus* (Table 1). For example, Cpa, from the human pathogen *S. pyogenes*, was identified as part of a highly recombinant region encoding the FCT pilus (Fibronectin- and Collagen-binding protein and T antigen) (Bessen & Kalia, 2002). Sequence and phylogenetic analysis of the FCT pathogenicity region identified 8 different FCT-types, and while the nucleotide content of this region may vary among strains, it typically contains a stand-alone regulator (RofA/Nra), a *srtC* gene for the pilus assembly, the backbone proteins (BP) and ancillary proteins (AP1, often referred to as Cpa, and AP2) (Falugi *et al.*, 2008). Cpa is located at the pilus tip and has been shown to directly interact with type I collagen *in vitro* (Kreikemeyer *et al.*, 2005). Other APs often associated with the FCT are F1/SfbI and F2/PrtF2 proteins, which are two major fibronectin adhesins that indirectly interact with type IV collagen through pre-bound fibronectin (Dinkla *et al.*, 2003a).

CBPs have also been shown to remodel host immune responses, as is the case with the M proteins encoded by the *emm* gene in streptococci (Fischetti, 1989). Thus far, over 200 variations of M protein have been identified, and are generally regarded as key anti-phagocytic factors with only a handful exerting collagen-binding activity (e.g. M1, M3, M31 and M55) (Dinkla *et al.*, 2003b; Reissmann *et al.*, 2012). The interaction of M proteins with collagen is mediated by its N-terminal hypervariable region, referred to as the PARF motif (peptide associated with rheumatic fever) (Dinkla *et al.*, 2007; Reissmann *et al.*, 2012). As the acronym suggests, subjects exposed to these M protein variants, either through infection or vaccination, are at risk of developing collagen autoimmunity due to an altered presentation of collagen molecules upon M protein binding (Dinkla *et al.*, 2007 & 2009). It is also worth mentioning that strains of the closely related *S. dysgalactiae* subsp. *equisimilis* present similar collagen-binding strategies, specifically through M protein 31.5

(Reissmann *et al.*, 2012). As in *S. pyogenes*, direct collagen binding by *S. equisimilis* M proteins is associated with post-infection sequelae such as glomerulonephritis and acute rheumatic fever (Reid *et al.*, 1985; Haidan *et al.*, 2000; Brandt & Spellerberg, 2009). Finally, direct binding to type I collagen by the *S. pyogenes* membrane-bound leucine-rich repeat (LRR) lipoprotein Slr, adds to its previously known role in mediating resistance to neutrophil phagocytosis (Reid *et al.*, 2003; Bober *et al.*, 2011).

Like *S. pyogenes*, *S. pneumoniae* and *S. agalactiae* are able to bind to collagen through pili-mediated mechanisms. In *S. pneumoniae*, the RrgABC pilus possesses significant capacity to bind several ECMs *in vitro*, including type I collagen (Hilleringmann *et al.*, 2008). RrgA, the component necessary for ECM attachment, is also required for phagocytic uptake and intracellular survival through interaction with complement receptor 3 (CR3) (Orrskog *et al.*, 2012). While the interaction was clearly important for infection *in vivo*, the presence of collagen neither inhibited nor enhanced CR3 binding, suggesting that the RrgA-collagen interaction might not directly contribute to *S. pneumoniae* virulence (Orrskog *et al.*, 2012). On the other hand, strains of *S. agalactiae*, often encode up to two distinct pili, with a genetic organization consisting of three genes that encode LPXTG motif-carrying proteins (PilABC) along with two sortase genes involved in pilus assembly (Nobbs *et al.*, 2008). The pilus tip adhesin, PilA, has been shown to mediate binding to the endothelium of the blood-brain barrier in addition to collagen adherence, resulting in downstream activation of host pro-inflammatory responses (Banerjee *et al.*, 2011). However, the contribution of collagen binding by *S. agalactiae* is not clear, as clinical isolates taken from cases of invasive neonatal disease, appear to lack collagen-binding activity (Dramsi *et al.*, 2012). In *S. gallolyticus* subspecies *gallolyticus*, the ability to bind to collagen and other ECM components by endocarditis-derived isolates is thought to play a role in the development of systemic infections (Sillanpa *et al.*, 2008). This is accomplished by Acb, a CBP required for attachment and colonization of heart valves, and shown to play an important role in IE (Sillanpa *et al.*, 2009; Danne *et al.*, 2011). Notably, the Pil1 locus containing the *acb* gene, along with the pilus subunit protein *sbs7* and its sortase *srtC1* genes, is not present in all strains of *S. gallolyticus* subsp. *gallolyticus* but is present in ~ 90% of IE isolates (Sillanpa *et al.*, 2008; Danne *et al.*, 2011). Interestingly, certain strains of *S. gallolyticus* subsp. *gallolyticus* that do not encode *acb* are also able to bind to type I collagen, suggesting that this species encodes additional and uncharacterized CBPs (Sillanpa *et al.*, 2008).

6. Current knowledge and potential gaps

Over the past two decades, studies on the biogenesis, function and biology of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as many CBPs has been greatly expanded (Patti *et al.*, 1994; Schneewind & Missiakas, 2012). The best characterized CBP found in GPB is the *S. aureus* Cna, which binds to collagen via the collagen hug mechanism (Zong *et al.*, 2005). In this interaction, the A domain (composed of subdomains N1N2) is reoriented to accommodate a monomeric triple helix of collagen which is then wrapped and locked in the N1N2 interdomain “hole” of Cnn (Zong *et al.*, 2005). It has been suggested that the *S. mutans* Cnm and Cbm use a similar mechanism to bind collagen (Kang *et al.*, 2013), although the crystal structure for either protein has yet to be resolved. The B domain of Cna, composed of 1–4 repeating subunits of 23 kDa, does not

alter the capacity of the A domain to interact with collagen (Rich *et al.*, 1998; Snodgrass *et al.*, 1999). While the Cna B-domain does not appear to be glycosylated, the B domain of Cnm undergoes *O*-glycosylation, a process that was shown to confer proteolytic stability and, possibly, function (Avilés-Reyes *et al.*, 2014b). This recently discovered aspect of Cnm biology should be further explored focusing on the mechanism of Cnm glycosylation, the amino acid residues targeted for post-translational modifications, and the effects glycosylation has on protein folding, function and stability.

Another important aspect of Cnm is its ability to mediate invasion of non-professional phagocytic cells. Thus far, Cnm was shown to mediate invasion to HCAEC (Abranches *et al.*, 2009), HUVEC (Nomura *et al.*, 2012) and human gingival fibroblasts (HGF) (Miller *et al.*, 2015), yet no study has looked at the host cell surface receptor interacting with Cnm. This event can be effectively inhibited using either anti-Cnm antibodies or cytochalasin D (Abranches *et al.*, 2011; Avilés-Reyes *et al.*, 2014b), indicating that both Cnm binding and host uptake mechanisms are important in the invasion process. Future studies should focus on characterizing the mechanism of Cnm-mediated invasion by using antibodies against collagen, laminin, integrins, caveolin and other host cell surface receptors. Additionally, host cell lines lacking specific surface receptors, or co-immunoprecipitation of host surface proteins with Cnm, can be used to identify new players in this interaction. Furthermore, the ability to invade must be accompanied with the capacity to withstand any intracellular antibacterial challenges. While macrophages readily eliminate *S. mutans* (Chen *et al.*, 2008), it appears that Cnm⁺ *S. mutans* can escape the vesicles and live freely in the cytoplasm of endothelial cells where a less hostile environment enables bacterial survival for prolonged periods of time (Abranches *et al.*, 2009). To further understand the contribution of Cnm towards the establishment and development of chronic infections, it would be interesting to investigate *S. mutans* intracellular survival within non-phagocytic cell types, such as smooth muscle cells, fibroblasts and cardiomyocytes, as well as the host responses (Stones & Krachler, 2015).

As noted before, Cnm and Cbm are highly homologous proteins with identical functions (Nomura *et al.*, 2012, 2013a & 2014). We recently found that, like *cnm*, the *cbm* gene is located immediately upstream of *pgfS* (Avilés-Reyes *et al.*, unpublished). These observations raise important evolutionary questions regarding the origin of *cnm/cbm*, their distribution amongst *S. mutans* isolates, and their association with other core and non-core genes such as *pgfS*, *cnaB* and *cbpA*. Moreover, the presence of CBP-coding genes genetically linked to *pgfS* homologues in other species of *Streptococcus* indicates that glycosylated CBPs with high-affinity to collagen are not restricted to *S. mutans* (Avilés-Reyes *et al.*, 2014b).

A better understanding of the biology of CBPs can ultimately facilitate the development of new approaches to prevent and combat streptococcal infections originated from CBP⁺ strains. For instance, screening for Cnm⁺ strains in patients undergoing major dental procedures may help identify subjects that are at a greater risk to develop extra-oral infections. While a questionable strategy, these subjects can be advised to receive antibiotic prophylaxis (Biswas *et al.*, 2010; Diz-Dios, 2014; Duval & Hoen, 2014). Although *S. mutans* infections can be effectively treated with penicillin (Baddour *et al.*, 2015), invasion

of non-professional phagocytic cells may represent a novel strategy by which *S. mutans* can evade antibiotic treatment (Knoll *et al.*, 2007; Jung *et al.*, 2012; Foster *et al.*, 2014). This ability to invade the intracellular compartment of non-phagocytic cells and “hide” from host-derived or prescribed antibiotics could potentially lead to treatment failure and recurrent infections, a rare yet real problem in IE (Gauduchon *et al.*, 2000; Mansur *et al.*, 2001; Thuny *et al.*, 2012). In terms of the applicability of CBPs as vaccine candidates, it has been shown that passive immunization with anti-Cnm antibodies significantly increase the survival of the *G. mellonella* larvae systemically infected with Cnm⁺ *S. mutans* (Avilés-Reyes *et al.*, 2014a). Likewise, vaccination with the A domain of the *E. faecalis* Ace led to significant protection against enterococcal IE, supporting the potential of these molecules as vaccine candidates (Singh *et al.*, 2010). It remains to be determined whether active or passive immunizations using, respectively, Cnm antigens or anti-Cnm antibodies can prevent the development of invasive infections in mammals. Finally, tissue colonization could be prevented at the CBP-collagen interface using various strategies to block this interaction (Krachler & Orth, 2013).

While the collagen-binding mechanism of Cnm can be extrapolated from the *S. aureus* Cna model, binding to collagen by other CBPs is much less understood. For example, AgI/II family proteins do not contain a collagen-binding domain akin to Cna, Ace or Cnm (Brady *et al.*, 2010). Similarly, the exact mechanism of collagen binding by the *S. pyogenes* LRR lipoprotein, Slr, is yet to be determined as Slr strongly binds to both native and denatured forms of type I collagen (Bober *et al.*, 2011). While these findings indicate that such interaction is not dependent on primary and secondary structures of type I collagen (Bober *et al.*, 2011), little is known about the recognition region on collagen and the domain of Slr mediating such interaction. On the other hand, binding of streptococcal M proteins to the CB3-region of type IV collagen involves the octapeptide motif **A***X***YLZZLN**, where *X* and *Z* represent variable amino acids (Dinkla *et al.*, 2007). Furthermore, heterologous expression of M3-protein in *S. gordonii* significantly increased the ability of this organism to bind to type IV collagen (Dinkla *et al.*, 2009). Thus, it is clear that streptococci have developed multiple strategies to interact with collagen proteins warranting further investigations into how these events occur.

In conclusion, the identification of bona-fide CBPs, like Cnm and Cbm, has unraveled a previously underestimated facet of *S. mutans* as they contribute to disease. Direct binding to collagen grants *S. mutans* with the ability to colonize both soft and hard tissues as well as to invade non-professional phagocytic cells. Moreover, expression of CBPs allows *S. mutans* to evade immune surveillance and antimicrobial treatments. The presence of Cnm/Cbm orthologs in other species suggests that this could be a common trait of streptococcal bacteria. Despite some progress over the past several years, thorough mechanistic studies are still necessary to define the precise contribution of streptococcal CBPs to host colonization, inflammation and possibly to chronic diseases.

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Figure 1. Amino acid sequence analysis of CBPs identified in *S. mutans*

(A) Conserved predicted domains identified in SpaP, WapA, Cnm, Cbm, CnaB and CbpA. (SS) secretion signal, (Ala) alanine-rich repeats, (V) variable region, (P) proline-rich repeats, (A) collagen binding A domain, (B) threonine-rich repeats (B domain), LPXTG cell-wall anchor motif. (B) Sequence alignment between the collagen-binding region of SpaP, WapA, Cnm and Cbm with the prototypical Cna from *S. aureus*. Amino acids in the shaded box represent the linker region between sub-domains N1 and N2 known to play a crucial role in the collagen-binding affinity. (C) Ribbon diagrams of the collagen-binding domains of Cnm, Cbm and WapA based on available crystal structures from Cna (swissmodel.expasy.org). Areas of high homology are represented in blue low homology regions appear in orange.

Figure 2. Proposed mechanisms of virulence mediated by *S. mutans* CBPs

(A) The presence of CBPs on the surface of *S. mutans* mediate direct attachment to collagen exposed after endothelial injury. This promotes bacterial adherence preventing the activity of host immune factors involved in tissue repair and homeostasis. (B) Systemic infection by *S. mutans* during atherosclerotic plaque growth may lead to an exacerbated immune response through: (i) recruitment of monocytes, and (ii) subsequent lipid digestion leading to formation of foamy macrophages that contribute to the development of atherosclerosis. (iii) Foamy macrophages and (iv) bacterial invasion of endothelial cells increase pro-inflammatory responses while smooth muscle cells produce ECM proteins (e.g. collagen), leading to (v) platelet deposition and activation. This, in turn, results in the development of a fibrin clot which provides the substrates recognized by circulating bacteria, leading to pathogen colonization as well as further immune activation. (C) CBP⁺ *S. mutans* can employ two distinct strategies to evade immune surveillance and antimicrobial challenges: i) through direct binding to the collagen-like domain of C1q, thus preventing complement activation, and ii) through invasion of endothelial cells that serve as a reservoir where bacteria can escape host-derived, antibacterial factors (e.g. antimicrobial peptides and complement) and antibiotic treatments. BM, basement membrane; ox-LDL, oxidized Low Density Lipoprotein; MΦ, macrophage.

Table 1

List of streptococcal surface proteins displaying collagen-binding properties.

Protein group	Protein	Species	Reference
Ag I/II family	SpaP	<i>S. mutans</i>	Sciotti <i>et al.</i> , 1997
	SspAB	<i>S. gordonii</i>	Love <i>et al.</i> , 2000
Cell wall anchored (LPXTG)	WapA	<i>S. mutans</i>	Han <i>et al.</i> , 2006
	Cnm	<i>S. mutans</i>	Sato <i>et al.</i> , 2004b & Abranches <i>et al.</i> , 2011
	Cbm	<i>S. mutans</i>	Nomura <i>et al.</i> , 2012
	CbdA	<i>S. gordonii</i>	Moses <i>et al.</i> , 2013
	Cne	<i>S. equis</i>	Lannegard <i>et al.</i> , 2003
Pili	Cpa	<i>S. pyogenes</i>	Kreikemeyer <i>et al.</i> , 2005
	F1/SfbI	<i>S. pyogenes</i>	Dinkla <i>et al.</i> , 2003a
	F2/PrtF2	<i>S. pyogenes</i>	Dinkla <i>et al.</i> , 2003a
	Acb	<i>S. gallolyticus</i>	Sillanpa <i>et al.</i> , 2009
	RrgA	<i>S. pneumoniae</i>	Hilleringmann <i>et al.</i> , 2008
Lipoprotein	Slr	<i>S. pyogenes</i>	Bober <i>et al.</i> , 2011
M proteins	M1, M3, M31 and M55	<i>S. pyogenes</i>	Dinkla <i>et al.</i> , 2003b & Reismann <i>et al.</i> , 2012
	M31.5	<i>S. dysgalactiae</i>	Reismann <i>et al.</i> , 2012