Infective endocarditis caused by the Mitis group Streptococci: what turns a normal commensal into a potential killer?

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# Table of Contents

Abstract ................................................................................................................................. 3  
Acknowledgment .................................................................................................................. 4  
1. Introduction ....................................................................................................................... 5  
  1.1 Problem formulation .................................................................................................... 6  
2. General background ......................................................................................................... 7  
  2.1 Streptococcus genus ................................................................................................... 7  
  2.2 Viridans Streptococci ................................................................................................. 9  
2.3 Streptococcus mitis Group .......................................................................................... 10  
3. Modulation of the immune system ................................................................................ 11  
4. Colonization of the oropharynx by Streptococcus mitis ............................................... 14  
  4.1 Colonization Attributes ............................................................................................ 14  
5. Infective endocarditis ....................................................................................................... 16  
6 Molecular methods of some virulence factors presents in S.mitis ...................................... 18  
  6.1 Phage proteins PblA and PblB ................................................................................. 18  
    6.1.1 Individuation of genetic loci that mediate binding to human platelet in S. mitis strain SF100 .................................................................................................................. 18  
    6.1.2 PblA and PblB are encoded by a lysogenic bacterophage of SF100 .................. 19  
    6.1.3 Mechanism of cell surface expression of the Streptococcus mitis platelet binding proteins PblA and PblB ......................................................................................... 23  
      6.1.3.1 The role of holin and lysin in PblA and PblB surface expression .......... 24  
      6.1.3.2 The loss of PblA and PblB expression is associated with a decrease on platelet binding in vitro ................................................................................................. 25  
      6.1.3.3 The loss of PblA and PblB expression is associated with a decrease in the expression of virulence ......................................................................................... 26  
      6.1.3.4 PblA and PblB interact with choline residues in the cell wall .......... 26  
    6.2 Immunoglobulin A1 degradation ............................................................................. 29  
    6.2.1 Bacterium-induced IgA1 degradation .................................................................. 29  
    6.2.2 Amino acid sequence of S. mitis required as a substrate for the streptococcal IgA1 protease .......................................................... 31  
    6.2.3 Phylogenetic tree based on the distribution of the zinc metalloprotease genes .. 35  
7. Discussion ......................................................................................................................... 37  
8. Conclusion ......................................................................................................................... 41  
9. Perspective for the future ................................................................................................. 41  
10. Glossary and abbreviations .......................................................................................... 43  
References ............................................................................................................................ 49
Abstract

Infective endocarditis (IE) is an infection of the bloodstream with a successive colonization of bacteria in the lining of the heart’s chamber, a heart valve or a blood vessel. Despite the new developments in medicine and health care, there is a significant mortality as high as 40% per year. One of the precursor of IE is \textit{S. mitis}, an oral commensal bacterium located in our oropharynx that turns into a pathogen. Since very little is known about the relation between \textit{S. mitis} and IE, most of the researchers' effort are focused on important findings: the individuation of genetic loci that mediate binding to human platelet in \textit{S. mitis} strain SF100, the evidence that PblA and PblB are encoded by SM1 phage, and the relation between the loss of PblA and PblB expression associated with a decrease on platelet binding in vitro. Moreover, studies revealed that the molecular mechanisms by which \textit{S. mitis} degrades immunoglobulin A (IgA1) occurs through cleavage of immunoglobulin A protease. Finally, the identification of amino acid sequences, required as a substrate for the streptococcal IgA1 protease in the hinge region of human IgA1, was investigated. These results have been considered of particular importance as a first step toward the development of specific enzyme inhibitors.

Terms elaborated on in the glossary are marked with (*) throughout the text.


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1. Introduction

Infective endocarditis (IE) is an endovascular microbial inflammation facing the heart and valves with a growth of a large septic thrombus on one of the cardiac valves. The more the thrombus grows, the more the valve fails producing a septic embolus that, if located in the brain, heart or lung, causes high mortality and morbidity (Kerrigan & Cox 2012).

The incidence of IE ranges between 1.7 and 6.2 cases per 1,000,000 patient years. Studies revealed that males are more affected than females in a ratio 2:1, and the probability of getting IE increases with age. Even with aggressive therapy composed of a 4-6 weeks long antibiotic treatment and/or surgery with implementation of a prosthetic valve, there is a significant mortality as high as 40% per year. (Kerrigan & Cox 2012, Beynon et al. 2006).

One of the precursor of IE is *S. mitis*, an oral commensal bacterium located in our oropharynx that turns into a pathogen. Very little is known about how exactly *S. mitis* causes IE although it is believed that the direct binding of *S. mitis* to platelet in human bloodstream plays a crucial role in the pathogenesis of IE; the purpose of this project is to give an overview of the strategies by means of *S. mitis* used to colonize the human oropharynx. In addition, a key factor to understand the transition from a commensal to a pathogenic state is to look into the bacterial genome as well as its virulence factors trying so to figure out possibilities to reduce the risk of IE (Mitchell 2011).
1.1 Problem formulation

The purpose of this report is to observe how *S. mitis*, a normal commensal, can turn into a pathogen and of what extend. A deep examination of the bacterial genome, its virulence factors as well as an understanding of how *S. mitis* interact with the host's immune system is indispensable to, hopefully, answer the main question of this project which is:

- How a normal oral commensal with few identified toxins and virulence factors turns into a potential killer in the human host?

A number of sub-questions can help to find an answer to the main problem:

- What is the physiology of *S. mitis* and which are the identified virulence factors of *S. mitis*?

- How *S. mitis* colonizes the human oropharynx?

- Which molecular methods concerning the virulence factors of *S. mitis* have been used so far?
2. General background

2.1 Streptococcus genus

The term *Streptococcus* derives from the Greek *strepto* (twisted) and *coccus* (spherical), it consist of spherical gram positive bacterium which typically is arranged in pairs or chains (*Figure 1*). There are more than 100 recognized species of *Streptococcus* and many of them are pathogenic for humans and animals. (Nobbs et al. 2009, Spellerberg and Brandt 2011, Hardie et al. 1997).

The earliest differentiation of streptococci has been made by using blood agar to distinguish strains that were beta-hemolytic from those that were not (Nobbs et al. 2009, Facklam 2002). The term Hemolysis comes from the Greek *hemo* (blood) and *lysis* (loosing) and consist on the breakdown of red blood cells (erythrocytes); the substance which causes hemolysis is called hemolysin, an exotoxin released by streptococci. There are three different types of hemolysis found in cocci: α hemolysis, β hemolysis and γ hemolysis (also referred as non-hemolytic). α hemolysis occurs when there is a partial destruction of red blood cells present in the blood agar plate. This partial destruction is caused by the production of hydrogen peroxide released by the bacterium which, oxidizing hemoglobin to green methemoglobin, lead to a greenish agar under the colony. β hemolysis is the complete lysis of erythrocytes in the agar plate where the area appears yellow and transparent. γ hemolysis refers to bacteria which do not cause hemolysis; the agar under and around the colony is unchanged (Spellerberg and Brandt 2011).
In 1933, thanks to Lancefield, the classification of streptococci evolved. She developed a technique to group streptococcal β hemolytic strains according to the carbohydrate composition of cell wall antigens’ (known as C substances which consist of polysaccharides) (Nobbs et al. 2009, Facklam 2002). Such discovery was a great breakthrough since, before 1933, the only tests used for differentiating many of the streptococci were fermentation and tolerance tests. In 1937 Sherman proposed a scheme which collected all the previous tests to organize streptococci into four categories. These can be listed as follows: the pyogenic division (which included the beta-hemolytic strains with group specific antigen A, B, C, E, F, and G), the viridans division (consisting of non beta-hemolytic, non high pH tolerant at growth conditions, non salt tolerant, and which did not grow at 10°C), the lactic division (which includes those streptococci that can grow at 10°C but not at 45°C, being non beta-hemolytic, and that fail to grow in the presence of 6.5% NaCl) and finally the enterococci (which includes those streptococci that are not into the other three divisions due to slightly different behaviors) (Nobbs et al. 2009, Facklam 2002).

From a genetic point of view, *Streptococcus* genus may also be classified differently into six groups based on 16S rRNA gene sequencing (Figure 2) which provide information regarding the genetic relationship of a broad range of *Streptococcus* genus through homology studies and comparative oligonucleotide catalogues; others genetic tests for identify all *Streptococcus* species can be DNA-DNA base pairing reassociation procedures, useful to discover genetic relationship within and between small groups of species or within single species, and pulsed gel electrophoresis (PFGE)* (Spellerberg and Brandt 2011, Hardie et al. 1997).
2.2 Viridans Streptococci

The aim of this project is to shed more light into the *Streptococcus mitis* group which belongs to the viridans streptococci. All the viridans streptococci share common features such as being catalase-negative, gram-positive arranged in chains, leucine aminopeptidase positive, pyrrolidonylarylamidase negative, and no growth in 6.5% NaCl broth. Nearly all species, except those in the salivarious group, are bile-esculin negative. Many viridans streptococci refer as the oral streptococci whereas some of them originate from gastrointestinal, vaginal, and dairy product sources (Facklam 2002).
2.3 Streptococcus mitis Group

*S. mitis* is an oral commensal of the human oropharynx and belongs to the viridans streptococci group. Nevertheless, *S. mitis* can cause a variety of invasive disease in humans, changing from the commensalism to pathogen; infective complications include infective endocarditis, bacteremia*, septicaemia*, eye infections, pneumonia* as well as bloodstream infections in neutropenic* and immune-compromised patients and in those which are subject to cytotoxic* anti-cancer chemotherapy (Mitchell 2011).

*S. mitis* cells grow in pairs or short chains and they present an arrow-headed shape with a length of 0.5µm (Mitchell 2011). The sequence of the *S. mitis* genome has 2.146.611 base pairs with an average GC content of 39.98% (Denapaite et al. 2010). As gram-positive bacteria* *S. mitis* cells possess one or two surface polysaccharides in their cell wall because they create a barrier between the bacterial cells and the surrounding environment. The cell wall of *S. mitis* biovar 1 strain SK137 possesses the C-polysaccharide (a ribitol teichoic acid with identical structure of the phosphocholine disubstituted form of pneumococcal C-polysaccharide), which was referred as the common antigen being present also in the closely related species *S. pneumonia*. Moreover, its cell wall has a teichoic acid-like polysaccharide, called capsular polysaccharide, with an unique structure considered as the type-specific antigen* (Bergström et al. 2000).

Since the physiology of *S. mitis* is very close to those of the major human pathogen *S. pneumoniae*, it has been hypothesized that *S. mitis* has evolved from a pathogenic colony, leading to the loss of virulence factors. Was also demonstrated that the components of the Mitis group are susceptible to transformations; this is probably due to the special feature, that most of them have in common, consisting in the harboring of a large number of mobile elements and repeat elements within insertion sequences (IS) (Denapaite et al. 2010). On the other hand it seems that sometimes *S. mitis* can escape from commensalism, becoming a potential pathogen. In order to investigate how related the two species are, Denapaite et al. analyzed the genome sequence of *S. mitis* B6 and compared it with that of *S. pneumoniae* R6. The most exciting results show the presence of 22 choline-binding proteins (CBPs)* in *S. mitis* B6 which are well known in *S. pneumoniae* to be implicated in virulence and interaction with host cells and bacteria cell physiology (Denapaite et al. 2010, Hakenbeck et al. 2009). Another important virulence feature of *S. pneumoniae* is the presence of cell wall bound proteins containing the LPXTG motif*; by now 18 cell wall associated surface
proteins bearing LPXTG motif have been identified, which cover almost the 7% of the *S. pneumoniae* coding sequence (Denapaite et al. 2010).

Combining ACT program* with the silico genomic comparison it has been possible to test that the majority of virulence factors involved in colonization and adherence in *S. pneumoniae* R6 are also present in *S. mitis* B6. However, in *S. pneumoniae* R6 there are three choline binding proteins (such as PcpA, PspA and PspC) and three gene cluster encoding the hyaluronidase genes, *ply* and *lytA*, and the capsular gene which are absent in *S. mitis* B6, confirming their importance as virulent factors for the pathogenicity of *S. pneumoniae* (Denapaite et al. 2010).

3. Modulation of the immune system

Some bacteria, have the ability to start the infection at the mucosal surface of the body through secretion of proteolytic enzymes named IgA1 proteases. Among streptococci, only *S. pneumoniae*, *S. oralis*, *S. sanguinis* and certain strains of *S. mitis*, have the ability to produce IgA1 proteases, consisting in metalloproteinases* that cleave the Pro 227-Thr 228 peptide bond in the IgA1 hinge region. However, it is still unknown which amino acid sequence is required as potential target site of the cleavage (Batten et al. 2003). These enzymes are responsible of the specific cleavage of human IgA1, the immunoglobulin that ensure antibody defense of mucosal surfaces. Once initiated the cleavage at a single peptide bond in the hinge region of the protective IgA1, two fragments are generated, one named Fab whereas the other is named Fc (Senior and Woof 2005). This proteolytic division of IgA1 impairs its function, especially because of the masking of epitopes* on the pathogens by the Fabs formed. By doing so, other immunoglobulins do not recognize epitopes and the result is an enhancement of the adherence of *S. pneumoniae*, *S. oralis*, *S. sanguinis* and *S. mitis* to host cells (Plaut 1983).

The synthesis of IgA is caused by plasma cells lying within the mucus membrane and occurs under the epithelium lining in the respiratory and genital tract as well as in the intestine. Moreover, IgA synthesis also occurs in organs such as the lactating breast, salivary glands, and lacrimal glands (Plaut 1983). IgA is present in both serum and secretions of human beings which, in turn, contains two subclasses of IgA named respectively IgA1 and IgA2. IgA1 predominates in serum (~80%), while IgA2 is found mostly in secretions (ranging between 35 and 50%). Serum IgA is monomeric with a molecular weight of 160,000 Da and it has two light and two heavy polypeptide chains. On the other hand, secretory IgA is dimeric, composed of polymers of 2-4 IgA monomers linked by two
additional chains with a molecular weight of 380,000 Da. Although there is a significant difference in subunit and polypeptide chain assembly in serum and secretory IgA, the primary structure of the two subclasses of IgAs are very similar, with exception for the amino acid sequence in the hinge region of the heavy polypeptide chain (See Figure 3). This region is considered of great importance because the hydrolysis of IgA1 protease occurs in this segment. It has been shown that the human secretory IgA1 is cleaved at the same hinge region peptide bond as in serum IgA1. Because the hinge peptide is not present in IgA2 molecules, both serum and secretory IgA2 proteins are IgA1 protease-resistant (Senior and Woof 2005, Plaut 1983, Batten et al. 2003).

![Diagram](image)

**Figure 3**: Proteolytic cleavage of serum IgA1 by IgA1 protease. The red arrows show the possible IgA1 protease target sites on the IgA1 heavy chain (designed as H whereas L stands for light). Note that V=Variable region and C=Constant region (Merino et al. 2001).

Usually the infection begins with the interaction of the pathogen with host epithelial membranes; being the mucosal defense IgA-mediated, it seems that the IgA1 protease plays an important role as virulence factor for the immune system (Plaut 1983). Additionally, it has been observed that the amount of enzyme secreted is directly related to the aggressiveness of the pathogen. Some decades ago, it has been discovered a valuable screening method for the detection of IgA1 proteases activity.
Figure 4 schematizes step by step the technique used for the detection of IgA1 proteases in bacterial colonies. (Plaut 1983).

Figure 4: Detection of IgA1 protease in bacteria: 1) Pouring of IgA beads in overlay agar over bacterial colonies. 2) Binding of Fab fragments released from protease positive colonies to nitrocellulose. 3) Autoradiography of the membrane reveals the location of the positive colonies (Plaut 1983).
4. Colonization of the oropharynx by Streptococcus mitis

4.1 Colonization Attributes

Many streptococci colonizes mammals by forming biofilm communities which are strongly dependent on adherence of cells to a surface. As the Figure 5 illustrates, the adherence and colonization by streptococci can be summarized into four steps: the first two steps involve (A) the long range interaction adherence with suitable surface molecules through pili bacteria\(^*\), and (B) the short range adherence of some pioneers bacteria which forms stronger bonds with the surface molecules leading to multiple adhesins. During the third step (C), the intermicrobial signalling (designed as stars in the figure), the formation of extracellular polymeric substance (EPS)\(^*\) and the nutritional adaption, give rise to a society produced by cell division and multiplication. In the last step (D) the incorporation of other microorganisms together with cell-cell signaling, intergenic coaggregation, metabolic synergy and genetic exchange, leads to the formation of a community (Nobbs et al. 2009).

Figure 5: Adherence and colonization by streptococci subdivided into four steps: A. Pioneers, B. Settlers, C. Society and D. Community (Nobbs et al. 2009).

The development of the community is dependent on many factors including adherence, signaling, nutritional adaption, and host modulation. In addition, environmental conditions such as pH, temperature, oxygen availability, organic metabolites etc. may influence Streptococcus colonization. Figure 6 schematizes the processes of adherence, biofilm formation, environmental sensing and virulence, it also indicates the secreted proteins that are involved. Adhesins include cell wall-anchored polypeptides such SfbI\(^*\), as well as anchorless proteins such as Eno\(^*\). GtfBC\(^*\) secreted
proteins produces polysaccharides, whereas SpeB degrades host proteins supplying additional nutrients. EPS and capsular materials (respectively blue and purple shading) contribute for the development of the community. TCSS is a two-component signal transduction system. ScaABCD transporters ensure nutritional homeostasis and may regulate adherence. Note that cell wall-linked proteins (south-east quadrant) may also contribute to virulence, while transporters (north-west quadrant) may contribute to adherence (Nobbs et al. 2009).

Figure 6: Processes of adherence, biofilm formation, environmental sensing and virulence with the involved secreted proteins (see the glossary for more information) (Nobbs et al. 2009).
5. Infective endocarditis

Infective endocarditis (also called bacterial endocarditis) is an infection of the bloodstream with a successive colonization of bacteria in the lining of the heart’s chamber (named endocardium), a heart valve or a blood vessel (*Figure 7*) (Texas Heart Institute 2012).

![Healthy heart valve](image1)

*Figure 7: Infection caused by viridians streptococci in the heart chambers or valves. The figure shows an healthy heat valve (upper right) in comparison to a damaged valve (on the bottom right side) (Mayo Clinic 2012).*

In most of the cases IE affects individuals with previous heart disease who manifest bacteraemia as source of infection which can be caused by dental, gastrointestinal, genitourinary, respiratory or cardiac procedures (Ramsdale et al. 2004). The majority of native valve IE (consisting in acute that causes progressive illness or sub-acute which is more indolent and can extend over many months) and late prosthetic valve endocarditis (which occurs some months after valve surgery) is due to viridians streptococci (50-70%), *Staphylococcus aureus* (25%) and enterococci (10%). If untreated quickly with antibiotics, surgery is often needed to replace or repair damaged valves (Prendergast 2006, Brusch et al. 2012). Moreover, IE can cause other complications such as blood clot (embolism*), arrhythmia and congestive heart failure (CHF)* (Texas Heart Institute 2012). The process by which bacteria adhere and colonizes injured endothelium during bacteraemia can be summarized in *Figure 8*. Two different mechanisms can occur depending if the epithelium is
damaged (Figure 8A) or if the valve tissues are inflamed (Figure 8B). The second mechanism can provide a reason by which patients without pre-existent valve disease can develop IE as well as IE patients with previous heart complications (Prendergast 2006).

Figure 8: Step by step in bacterial valve colonization. (A) Colonization of damaged epithelium: Stromal cells and extracellular matrix proteins activates the deposition of fibrin-platelet clots which in turn bind to the circulating streptococci. The binding of fibrin and streptococci pull in monocytes and stimulate them to produce tissue factor activity (TFA) and cytokines; these mediators cause an activation of the coagulation cascade as well as an attraction and activation of blood platelets, and induce the production of cytokine, integrin and TFA to the neighboring endothelial cells, increasing bacterial colonization. (B) Colonization of inflamed valve tissues: When a local inflammation occur damaged endothelial cells release β1 integrins which bind to plasma fibronectin that in turn adhere by bacteria through wall attached fibronectin binding proteins. As consequence endothelial cells produce TFA and cytokines activating blood clotting and an increase of the inflammation, enhancing so bacteria colonization; the bacteria finally lyse endothelial cells by releasing haemolysins (Prendergast 2006, Moreillon and Que 2004)
Consequent tissue damage may result in abscess formation and septic emboli which can spread in the brain, spleen and kidney with catastrophic consequences. Despite the new developments in medicine and health care (such as improved antimicrobial chemotherapy and curative surgery) IE still has high mortality and morbidity (Prendergast 2006).

6 Molecular methods of some virulence factors presents in *S.mitis*

6.1 Phage proteins PblA and PblB

The binding of *S. mitis* to human platelets is considered to play an important role for what may concern the pathogenesis of infective endocarditis. In order to understand this process it has been decided to focus mostly in researches based on three important findings:

- Individuation of genetic loci that mediate binding to human platelet in *S. mitis* strain SF100 (Bensing et al. 2001a).
- The encoding of PblA and PblB into a lysogenic bacteriophage of SF100 (Bensing et al. 2001b).
- The loss of PblA and PblB expression with the decreasing of platelet binding in vitro (Mitchell et al. 2007).

Such methods of analysis will be briefly described with a focus on their results.

6.1.1 *Individuation of genetic loci that mediate binding to human platelet in* *S. mitis* *strain SF100*

The possibility of the beginning of IE due to a direct binding between the streptococci and the human platelets was confirmed by in *vitro* experiments. As a result of that, they can mediate the development of mature vegetations within a progressive accumulation of platelets and bacteria. The purpose of the investigation made by Bensing et al. was to identify bacterial components that are involved in the enhancement to platelet binding by strain SF100 (a streptomycin-resistant variant of *S. mitis* 12021, isolated from blood from a patient with IE). For this purpose, several techniques were used, whereas, the most interesting are listed as follows: quantitative assay for binding to platelets, transposon mutagenesis* and selection of low-binding variants, DNA sequence analysis and complementation tests* (Bensing et al. 2001a). As first step the platelet binding by SF100 strain
was tested and the results showed an increase of the phenomena from 1.4 to 3.7% of the applied inoculum after 2 h of incubation. Interestingly, trypsination of bacteria reduced platelet binding compared to untreated microorganisms. This result suggested that the platelet binding by SF100 strain was selective and strongly correlated with particular surface proteins (Bensing et al. 2001a). To test this hypothesis, transposon mutagenesis and selection of low-binding variants were attempted. Two mutant strains, PS101 and PS116 were generated and then processed by DNA sequence analysis of the two loci. In the case of PS101 locus, an OFR encoding a protein of 399 amino acids with a molecular mass of 43 kDa was sequenced. The first 31 amino acids could include a signal peptide, whereas the remaining are hydrophobic with 12 potential transmembrane-spanning regions which may indicate an integral membrane protein likely to be a member of small molecule transporters. The gene has been called \textit{pblT} which stands for “platelet binding locus transporter” (Bensing et al. 2001a) since quantitative assay revealed that PblT contributes to platelet binding. For the PS116 locus two cell wall-associated surface proteins were found encoded by \textit{pblA} and \textit{pblB} genes. PblA protein consist of 71 amino acids terminal signal peptide and has a molecular mass of 107 kDa with tryptophan-rich repeats; these repeats could serve to anchor the protein within the cell wall. \textit{pblB}, on the other hand, encodes a protein with 121 kDa where, the C-terminal repeats, suggests similarity with a tail fiber protein. Gene complementation and functional mapping by insertion-duplication mutagenesis suggests that PblB may function as direct platelet adhesin; moreover, the expression of PblB may be linked to that of PblA since it seems to affect the surface of PblB (Bensing et al. 2001a, Bensing et al. 2001b).

6.1.2 PblA and PblB are encoded by a lysogenic bacterophage of SF100

One of the hypothesis in the pathogenesis of IE is the ability of bacteria, such as \textit{S. mitis}, to adhere in the bloodstream to human platelets on the damaged endocardial surface, allowing the colonization of the cardiac valves. As suggested by Bensing et al. the two surface proteins PblB and PblA may be linked to the platelet binding of \textit{S. mitis}. These two proteins share some features which are atypical for surface proteins since they resemble the structural components of bacteriophages; the amino-terminal half of PblA and the carboxy-terminal half of PblB resemble respectively the protein region of the \textit{Lactococcus lactis} phage r1t and the tail fiber protein of the \textit{Streptococcus thermophilus} phage 01205 (Bensing et al. 2001b). Moreover, the carboxy-terminal half of PblB resembles the host attachment proteins of various temperate and lytic phage. The
similarity in pblAB locus to phage genes led Bensing et al. to investigate whether phage production could be induced* from S. mitis strain SF100. In order to test if the pblAB locus constitute an encoded segment of a prophage, they induced the expression of pblAB locus by mitomycin C and UV light; these reagents are known to be DNA damaging agents used especially to induce mutations in the lytic cycle of temperate phages (Bensing et al. 2001b). This process was monitored by the incorporation of lacZ reporter (a structural gene of the lac operon which encodes β-galactosidase (LacZ), an intracellular enzyme that cleaves lactose into glucose and galactose) into pbla, generating strain PS291 (forward orientation) and strain PS294 (reverse orientation). LacZ gene was used to monitor the transcription of these genes, as a promoter is necessary to start the transcription. Afterward, cultures carrying LacZ were respectively used as controls and exposed to mitomycin C and UV light. Results showed that PS291 cultures exposed to mitomycin C and UV light reveals a significant increase in β-galactosidase activity (See Figure 9). Conversely, strain PS294 (consisting of incorporation of lacZ reporter into Pbla in the opposite orientation), showed no increase in β-galactosidase activity after exposure to mitomycin C and UV light (Bensing et al. 2001b). An increase in β-galactosidase activity for PS291 strain reveals that in the forward orientation there is a natural promoter. Conversely, if the transcription does not take place there will be no β-galactosidase activity. It was investigated whether the transcription takes place in forward orientation or not, and in this case it occurs in the forward orientation. Bensing et al. investigated whether the genes need to be transcribed only in concert with agents that induced the production of phage SM1 (Bensing et al. 2001b).
Figure 9: Induction of the expression of pblAB locus by mitomycin C and UV light monitored by the incorporation of lacZ reporter into pblA, generating strain PS291 (forward orientation) and strain PS294 (reverse orientation) to measure the β-galactosidase activity. The asterisk denotes significant different value (P< 0.001) from untreated cultures (control) (Bensing et al. 2001b).

To test if the increased transcription is related to the increased expression of PblA and PblB, Western blot analysis was attempted in order to examine the effect of inducing agents on the expression of PblA and PblB (Bensing et al. 2001b). The Figure 10 illustrates the PblA and PblB production in both, uninduced and induced culture. Proteins extracted from uninduced cultures of the PblA- and PblB- deletion mutant PS344 are shown in lane 1, whereas proteins extracted from SF100 are shown in lane 2, and proteins from SF100 treated with mitomycin C and UV light results in lane 3 and 4 respectively. Blots were probed with anti-PblA serum (A) as well as anti-PblB serum (B) (Bensing et al. 2001b).

Interestingly, results showed that anti-PblA serum reacts with a protein that migrated at 120 kDa and with minor 110 and 85 kDa proteins (predicted size of pblA is 107 kDa) (Figure 10 A, lane 2). Nor anti-PblA serum nor anti-PblB serum reacted with proteins extracted from PS344, (Figure 10 AB, lane 1); these results confirm the specificity of both, anti-PblA serum and anti-PblB serum.

The anti-PblB serum reacted with a protein that migrated at 110 kDa and with a minor 120 kDa protein (predicted size of pblB is 120 kDa) (Figure 10 B, lane 2) (Bensing et al. 2001b).
Proteins from SF100 treated with mitomycin C and UV light showed an increase in amount in both PblA and PblB (Bensing et al. 2001b).

Although results from induction of pblA transcription by mitomycin C and UV light showed that pblAB locus constitute an encoded segment of a prophage, the medium of induced cultures of SF100 was examined using electron microscopy. In the supernatant, there was detected a small isomeric headed phage, named SM1. The heads are roughly 60 nm in diameter and the noncontractile tails were approximately 150 nm x 8 nm. Taxonomically, SM1 belongs to the Siphoviridae class of bacteriophages (Figure 11 A) (Bensing et al. 2001b).

Afterward, Bensing et al. investigated whether the pblAB deletion PS344 was defective in phage production. Electron microscopy was used to reveals that the supernatant of mitomycin C induced culture PS344 had phage particles; the phage, named SM1'AB, present similar head compare to SM1 but lacked tail (Figure 11 B).
6.1.3 Mechanism of cell surface expression of the *Streptococcus mitis* platelet binding proteins PblA and PblB

From the results obtained above, data indicates that PblA and PblB are two surface proteins encoded by a lysogenic bacteriophage (SM1) which, in turn, mediate binding to human platelet. However, since they do not contain signal sequences or cell wall sorting motifs, the mechanism by which these proteins bind to the host surface and how they are exported and assembled onto the bacterial surface is still unknown. For this purpose, Mitchell et al. investigated whether the surface expression of PblA and PblB was linked to the lytic cycle of SM1 phage (Mitchell et al. 2007).

*Figure 11:* Electron microscope image of SM1 phage (A) and SM1 AB phage (B). Of particular interest the lacked tails in SM1 AB phages. Magnitude, 100nm. (Bensing et al. 2001b).
6.1.3.1 The role of holin and lysin in PblA and PblB surface expression

To investigate the role of holin and lysin in PblA and PblB surface expression, the genes encoding the two proteins surface were substituted with a chloramphenicol resistance gene and the mutation was verified by the use of polymerase chain reaction (PCR) and Western blot analysis using polyclonal anti-PblA antibodies (αPblA) or anti-PblB (αPblB) antisera. The resulted isogenic variants were induced with UV light to test for PblA and PblB expression (Mitchell et al. 2007). (See Figure 12).

The Figure 12 (lane 1) shows the exposure of strain SF100 to UV light which result in the expression of PblA and PblB in either supernatant, cell wall and protoplasts. On the other hand, the disruption of genes encoding holin (generating PS1005 strain) and lysin (generating PS1006 strain), respectively, shows the loss of both PblA and PblB in the supernatant and in the cell wall (Figure 12 lane 3 and lane 4).
6.1.3.2 The loss of PblA and PblB expression is associated with a decrease on platelet binding in vitro

To test if the deleted holin and lysin genes led to significant decrease in platelet binding, it has been made a comparison between strain PS344 (containing deletion of \( pblA \) and \( pblB \) loci), strain SF100, and, respectively, the holin mutant (strain PS1005) and the lysin mutant (strain PS1006) for their ability to bind human platelets in vitro (Figure 13) (Mitchell et al. 2007).

The histogram shows that strain PS344 has 44 ± 11% (\( P^*<0.05 \)) reduction in platelet binding, whereas PS1005 strain has 51 ± 6.4% (\( P<0.002 \)) reduction in platelet binding. Interestingly, PS1006 has shown the most significant decrease in platelet binding (83 ± 3.1% reduction (\( P<0.0001 \)))(Mitchell et al. 2007).
6.1.3.3 The loss of PblA and PblB expression is associated with a decrease in the expression of virulence

To test if the deleted pblA and pblB genes result in a decrease of the expression of virulence, rabbits were used as cavia to infect intravenously with SF100 and its mutant counterpart, PS344, in a 50:50 ratio. After 24h the mutant strain showed a significant reduction in its ability to propagate the infection. In order to prove the equally growth of the strains, samples were monitored every hour. The results showed no differences in both the strains. Thus, it seems that PblA and PblB contribute to virulence in the pathogenesis of IE (Mitchell et al. 2007).

6.1.3.4 PblA and PblB interact with choline residues in the cell wall

In order to understand the mechanism by which PblA and PblB are exported and assembled onto the bacterial surface, the amino acid composition of the two proteins surface was investigated: results suggested that PblA and PblB contain regular repeats of glycine and tryptophan rich motif. These amino acids are comparable in composition to the choline binding domain of the pneumococcal autolysin LytA*. Figure 14A shows the location of the predicted choline-binding glycine and tryptophan rich motif of respectively PblA and PblB (hatched boxes) (Mitchell et al. 2007).

To test if PblA and PblB bind to the bacterial surface by interaction with choline residues in the S. mitis cell wall, the two proteins surface were purified with choline and its structural analogue ethanolamine. Figure 14D shows the chemical structure of choline and its chemical analogue ethanolamine. In most of the cases the choline binding domains of other streptococci have higher affinity for tertiary amines, hence, Mitchell et al. analyzed the binding of PblA and PblB applied to diethylaminoethanol (DEAE) cellulose*. Proteins were separated by SDS-PAGE*, sodium dodecyl sulfate polyacrylamide gel electrophoresis, a technique used to separate proteins according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge) and silver stained* (Figure 14B lane 1 and 2) (Mitchell et al. 2007). Finally, Western blot analysis was used to detect PblA and PblB proteins probed with respectively anti-PblA sera and anti-PblB sera (Figure 14B lane 3 and 4) (Mitchell et al. 2007). Figure 14B lane 1 shows the supernatants of PblA and PblB taken from cultures of S. mitis SF100 cells applied to DEAE cellulose column. Figure 14B lane 2 shows proteins eluted from DEAE column with 2% choline chloride. Figure 14B lane 3 and 4 shows the Western immunoblot of eluted protein probed with respectively anti-PblA sera and
anti-PblB sera. Under these conditions both PblA and PblB were found to bind to DEAE, and could be eluted with choline (Mitchell et al. 2007).

To investigate whether PblA and PblB could be shift from the bacterial surface, the whole bacteria was exposed to choline or ethanolamine for 30 minutes at room temperature and subsequently there was performed Western immunoblot by the use of respectively anti-PblA sera and anti-PblB sera. Figure 14C shows PblA and PblB eluted from washed *S. mitis* SF100 cells which were incubated in the presence of 0.02% to 20% ethanolamine in PBS* (phosphate buffered saline), 20% choline in PBS, 20% NaCl in PBS or PBS. Results demonstrated that it was possible to elute PblA from the surface of whole SF100 cells using 0.02% of ethanolamine as well as 20% of choline, whereas PblB could be eluted only using 20% ethanolamine. These discovers suggest that PblA and PblB bind the cell wall by interacting independently with choline residues. Moreover, PblB seems to have higher affinity for the choline-binding than PblA. This last observation coincide to the supposed function of PblB, as being tail fiber protein of SM1 phage (Mitchell et al. 2007).
Figure 14: The PblA and PblB choline binding in the S. mitis SF100 cell wall. A: location of the predicted choline-binding glycine and tryptophan rich motif of respectively PblA and PblB (hatched boxes). B PblA and PblB were separated by SDS-PAGE (lane 1) and purified by choline affinity purification (lane 2). Afterwards, PblA and PblB were analyzed through Western Immunoblot and probed with anti-PblA sera (lane 3) and anti-PblB sera (lane 4). C Washed S. mitis SF100 cells were incubated in the presence of 0.02% to 20% ethanolamine in PBS, 20% choline in PBS, 20% NaCl in PBS or PBS. D Chemical structure of choline and its chemical analogue ethanolamine (Mitchell et al. 2007).
6.2 Immunoglobulin A1 degradation

The successful colonization by *S. mitis* might be explained not only considering adherent factors and binding property to platelet, but also by means of the ability to escape local defense mechanisms, such as secretory immunoglobulin A and serum immunoglobulin A. These antibodies have the ability to neutralize toxins and prevent the penetration of pathogenic substances. Moreover, secretory immunoglobulin A (S-IgA), protects the mucosal surface by reducing microbial colonization. For this purpose, it has been investigated that the molecular mechanisms by which some oral streptococci, including *S. mitis*, degrade immunoglobulin A through cleavage of immunoglobulin A protease (Reinholdt et al. 1990). Additionally, it has been identified the amino acid sequence required as a substrate for the streptococcal IgA1 protease in the hinge region of human IgA1 (Batten et al. 2003). Finally, a phylogenetic tree, based on the distribution of the zinc metalloprotease genes, (such as iga, zmpB, zmpC and zmpD genes) has been studied in *S. pneumoniae* and related commensal species in respect to a better understanding of the functions played by these proteases (Bek et al. 2012).

6.2.1 Bacterium-induced IgA1 degradation

In order to test the possible degradation of S-IgA1 caused by IgA1 protease, 143 strains representing the seven species *S. sanguinis*, *S. gordonii*, *S. oralis*, *S. mitis*, *S. salivarius*, *S. anginosus*, and *S. mutans* were incubated with human myeloma IgA1. IgA1 degradation was revealed by SDS-PAGE and immunoelectrophoresis (IEP) consisting in a separation and characterization proteins method based on electrophoresis and reaction with antibodies (in this case the antiserum used was rabbit anti S-IgA) (Reinholdt et al. 1990). Figure 15 shows three types of changes caused by 62 strains (Reinholdt et al. 1990). It is possible to note that patterns 1 (consisting of IEP product after incubation of myeloma IgA1 with *S. sanguinis* ATCC 10556) and 2 (consisting of IEP product after incubation of myeloma IgA1 with *S. oralis* ATCC 10557) are characterized by the presence of two distinct precipitation lines. They may indicate that the total cleavage of the substate into Fab and Fc fragments happened for their reactivity with anti S-IgA. However, pattern 1 is characterized by Fab and Fc with a slightly different electrophoretic mobilities, whereas, pattern 2 shows the two fragments with similar electrophoretic mobilities (It has further elucidated that this difference was due to a different mobility of Fc rather than Fab fragments). Differently, the third pattern (consisting of IEP product
after incubation of myeloma IgAl with *S. mitis* biovar 2 strain SK96) shows only one precipitation line, which had moved in the cathode direction rather than intact IgA1 substrate (int), used as control (Reinholdt et al. 1990).

![Figure 15: Three IEP product patterns representing different changes in IgA1 (Fri) after induction with IgA1 protease from the following strains: S. sanguinis ATCC 10556; 2, S. oralis ATCC 10557; 3, S. mitis biovar 2 strain SK96. Note: Int, Intact IgA1 used as control (Reinholdt et al. 1990).](image)

In order to confirm the identity of the fragments obtained by IEP, SDS-PAGE analysis was attempted (see Figure 16) (Reinholdt et al. 1990).

All the strains representing the degradation patterns 1 and 2, gave rise to bands of different molecular weight, in which, the Fab fragment demonstrated to be 40 kDa (hence resulting pattern 1), whereas Fc fragment resulted to be 36 kDa (resulting pattern 2). Interestingly, the molecular weight of Fc fragment may change slightly depending on the IgA1 substrate used, as myeloma IgA1(Fri) monomeric, myeloma IgA1(Mor) monomeric and myeloma IgA1(Kah) dimeric (not shown). As expected, the change in the IgA1 substrate resulted to be note only through a decrease in the molecular weight, as pattern 3 confirms (Reinholdt et al. 1990).
6.2.2 amino acid sequence of *S. mitis* required as a substrate for the streptococcal IgA1 protease

From previous researches made by Kilian et al. 1980, it has been individuated that the cleavage site in the hinge region of IgA1 (being Pro227-Thr228) is necessary for IgA1 protease to cleave the protein into two segments. However, informations about the exact amino acid sequence required to cleave IgA1 by IgA1 protease of streptococci that differ from *Haemophilus* and *Neisseria* species, have not yet been identified. For this reason Batten et al. examined the site requirement in IgA1 for cleavage by different streptococcal IgA1 proteases by constructing mutated IgA1s with amino acid substitutions at some point of relevant interest. *Figure 17* illustrates the amino acid sequences of human IgA1 in the hinge region of the α chain (wildtype IgA1) and the four IgA1 mutants in which the amino acid on the left side has been substituted with the amino acid on the right side (for instance mutant antibody P227T, where Pro227 has been substituted with Thr227) (Batten et al. 2003).
All the bacteria showed their protease activity after 16 hours of incubation with IgA1, obtaining Fab and Fc fragments instead of IgA1. To confirm this, the cleavage of IgA1 has been inhibited by the presence of 25mM EDTA. On the other hand, IgA2 showed resistance to cleavage even after 72 hours (Batten et al. 2003).

Western blot analysis was performed in order to test whether the cleavage by IgA1 proteases from strains of different species of streptococci occurred in the mutant antibodies*. By doing so it has then been possible to outline the precise amino acid sequences necessary for the cleavage of *S. mitis* IgA1 protease (Batten et al. 2003). To note, in this project it has been reported only informations concerning *S. mitis*, avoiding so all the other streptococci tests performed by Batten et al. 2003.

_Figure 18_ shows the results obtained after digestion run under reduced conditions and probed with an anti-human IgA α chain-specific peroxidase conjugate that bound to epitopes in the Fab region: lane 1 shows P227T untreated and lane 5 is the result of digestion between mutant antibody P227T with IgA1 proteases of *S. mitis* SK 564. Antibody P227T was resistant to cleavage by the IgA1 proteases of *S. mitis* SK564 (Batten et al. 2003). Note that the resulted lanes obtained from other streptococci are not explained in this report.
Figure 19 shows the results obtained after digestion run under reducing conditions and probed with an anti-human IgA α chain-specific alkaline phosphatase-conjugated antibody specific for epitopes in the Fc region of uncleaved IgA. Lane 1 shows P227T and T228P antibodies untreated, lane 2 results in the digestion between wildtype IgA1 and IgA1 proteases of *S. mitis* SK597; lane 3 is the result of digestion between mutant antibody P227T with IgA1 proteases of *S. mitis* SK597, lane 4 results from the digestion between mutant antibody T228P with IgA1 proteases of *S. mitis* SK597. Moreover, lane 5, lane 6 and lane 7 are the results of digestion between wildtype IgA1, mutant antibody P227T and mutant antibody T228P, respectively, with IgA1 proteases of *S. mitis* SK597. The ability of IgA1 protease to cleave the IgA1 different substrates (mutated antibodies) changed in regards to the different strains of *S. mitis*. IgA1 protease of *S. mitis* strain SK599 did not cleave nor mutant antibody P227, nor mutant antibody T228P. In contrast, IgA1 protease of *S. mitis* strain SK597 cleaved mutant antibody T228P but not mutant antibody P227 (Batten et al. 2003).
Figure 20 shows the results obtained after digestion run under reduced conditions and probed with an anti-human IgA α chain-specific peroxidase conjugate that bound to epitopes in the Fab region: lane 1 shows T228V untreated and lane 5 is the result of digestion between mutant antibody T228V with IgA1 proteases of *S. mitis* SK564. Antibody T228V was partially cleaved by the IgA1 proteases of *S. mitis* SK564, giving two fragments as result (Batten et al. 2003). Note that the resulted lanes obtained from other streptococci are not explained in this report.

Figure 21 shows the results obtained after digestion run under reducing conditions and probed with an anti-human IgA α chain-specific peroxidase conjugate that bound to epitopes in the Fab region: lane 1 shows T228/236V untreated and lane 5 is the result of digestion between mutant antibody T228/236V with IgA1 proteases of *S. mitis* SK564. Antibody T228/236V was cleaved by the IgA1 proteases of *S. mitis* SK564, giving two fragments as result (Batten et al. 2003). Note that the resulted lanes obtained from other streptococci are not explained in this report.

From Figure 18 to Figure 21 are shown the molecular mass markers on the left and the unit is kD (kilodalton) (Batten et al. 2003).

Figure 20: Western blot analysis performed to test the action of IgA1 protease on mutant antibody T228V (Batten et al. 2003)

Figure 21: Western blot analysis performed to test the action of IgA1 protease on mutant antibodies T228/236V (Batten et al. 2003)
6.2.3 Phylogenetic tree based on the distribution of the zinc metalloprotease genes


The function and substrate specificity of ZmpB and ZmpD are still unknown, even if ZmpB had shown to increase the proinflammatory cytokine TNF-α in the lower respiratory tract, contributing to inflammation. However, the mechanism by which ZmpB changes the level of TNF-α is still under investigation (Blue CE et al. 2003).

It has been reported that Pneumococcal ZmpC was able to activate human matrix metalloprotease 9 (MMP-9), to cause shedding of syndecan-1 and to induce ectodomain shedding of mucin 16 (muc16). The consequences lead to an increased possibility for inflammation of the human host (Bek et al. 2012).

The Figure below (Figure 22) reports the phylogenetic tree of *S. pneumoniae* and related commensal species, obtained through BLAST searches using representatives of each gene as the query sequence (Bek et al. 2012). This analysis provided evidence of frequent intraspecies transfer of entire genes and combination of genes. Recently, Bek et al. tested whether the iga, zmpB, zmpC, zmpD genes were present or not (Bek et al. 2012). Note that the type strains of individual species are indicated on the left within the species name. Moreover, the presence of iga (A), zmpB (B), zmpC (C) and zmpD (D) genes are indicated by the letter (red uppercase letters and black lowercase letters indicates respectively its presence and absence); finally the values shown as percentages at the nodes of the tree are based on 500 replications, where the bar on the left bottom indicates the genetic distance (number of substitutions per nucleotide) (Bek et al. 2012).
Figure 22: Phylogenetic tree based on the distribution of the zinc metalloprotease genes (such as iga, zmpB, zmpC and zmpD genes) studied in S. pneumoniae and related commensal species (Bek et al. 2012).
7. Discussion

In this session I decided to discuss results reported in Chapter 6 in regard to PblA and PblB surface proteins and IgA1 protease, keeping in mind that the purpose of the investigation has been to have an elucidation of the possibility for these molecules to be candidate as virulence factors, contributing to the pathogenesis of IE.

From the results obtained in Chapter 6.1, the two surface proteins PblB and PblA may be linked to the platelet binding of S. mitis, whereas an integral membrane protein designed PblT is more likely to be a member of small molecule transporters with still unclear function. Bensing et al. Demonstrated that pblAB locus may belongs to a lysogenic phage, SM1; induction of the transcription of S. mitis SF100 by the use of UV light and mitomycin C shown an increased activity of β-galactosidase in the strain PS291 (forward orientation) compared to the control (uninduced strain PS291) (Bensing et al. 2001b). LacZ has been incorporated into pblA gene in order to study the regulation of gene expression during the transcriptional fusion (also called operon fusion); the measure of the β-galactosidase activity should be proportional to the rate of transcription of the gene of interest cloned upstream of lacZ (Pressi G. et al. 2001). A significant difference has been statistically observed for PS291 treated with UV light and mitomycin C by means of the calculation of the P value (P<0.001). Figure 10 shows the Western blot analysis results, these evidenced the effect of the inducing agent which is directly correlated with the expression of PblA and PblB. The absence of PblA and PblB proteins extracted from uninduced cultures of PblA- and PblB- deletion mutant PS344 probed with respectively anti-PblA serum (A) and anti-PblB serum (B), demonstrated the specificity of both, anti-PblA serum and anti-PblB serum (lane 1). Conversely, lane 2 shows that anti-PblA serum and anti-PblB serum reacted with proteins which have been predicted to be PblA and PblB due to their molecular weight (Bensing et al. 2001b).

Lane 3 and lane 4 show cultures of S. mitis SF100 strain induced, respectively, with mitomycin C and UV light which demonstrated an increase in the amount of both PblA and PblB. These results suggest that the transcription of the pblAB locus is induced with mitomycin C and UV light. Moreover, an increase in the transcription is directly related to an increase in the expression of PblA and PblB cell wall proteins (Bensing et al. 2001b).

Finally, in order to confirm that pblAB locus constitute an encoded segment of a prophage, Bensing
et al. examined, by the use of an electron microscope, the supernatant of induced cultures of *S. mitis* SF100 strain. Moreover, it has been examined whether the *pblAB* deletion PS344 was defective in phage production through observation under electron microscope of supernatant of induced culture PS344 (which consist on those culture with deleted *pblA* and *pblB* genes). *Figure 11A* and *Figure 11B* show, respectively, the presence of a phage designated SM1 and the presence of a phage with lacked tail. This observation suggests that the deleted region correspond to the tail assembly without affecting other mechanisms of the lytic cycle such as head assembly, DNA packaging, and particle release (Bensing et al. 2001b).

Results from Mitchell et al. investigations gave two important outlines: the disruption of the genes encoding the two surface proteins gave a significant decrease in platelet binding in *vitro* as well as significant decrease in virulence, measured through a rabbit model of IE. Secondly, the expression of PblA and PblB on the bacterial surface depends on the activity of holin and lysin, two proteins encoded in the SM1 bacteriophage, that permeabilize the cell envelope, which, in turn, allow the transport of the surface proteins to the cell wall bacteria where, finally, they bind to choline residues (Mitchell et al. 2007).

*Figure 12* shows results obtained to investigate the role of holin and lysin in PblA and PblB surface expression by disruption of genes encoding holin (generating PS1005 strain) and lysin (generating PS1006 strain). Interestingly, these mutations did not interfere with the expression of the two proteins in the protoplasts, demonstrating that their synthesis occurs even if the abrogation of the phage lytic cycle prevent their expression on the cell surface (Mitchell et al. 2007).

From the results obtained in *Figure 13* it is possible to confirm that PblA and PblB plays a significant role in *S. mitis* SF100 strain for what concern the platelet binding. Additionally, holin and lysin seems to be indispensable to reach a necessary level of PblA and PblB for surface expression and platelet binding (Mitchell et al. 2007).

Results obtained from Chapter 6.2 demonstrated that some oral streptococci, including *S. mitis*, degrade immunoglobulin A through cleavage of immunoglobulin A protease. IgA1 degradation was revealed by SDS-PAGE and immunoelectrophoresis (IEP) (See respectively *Figure 15* and *Figure 16*) (Reinholdt et al. 1990).

Moreover, the identification of the amino acid sequence required as substrate for the streptococcal IgA1 protease in the hinge region of human IgA1 has been identified in Chapter 6.2.2 (Batten et al. 2003). After a series of Western blot analysis, it was possible to give some important outlines: IgA1
protease of *Streptococcus mitis* required proline at position 227 but not threonine at position 228, which could be substituted by valine. *Figure 19* revealed that IgA1 proteases of different strains of *S. mitis* may have different amino acid requirements for cleavage (Batten et al. 2003).

For what may concern the methods used during the investigations in chapter 6, I think it is important to discuss about their advantages and disadvantages. The most interesting and used methods are listed as follows:

- **Transposon mutagenesis.** The advantages related to this technique are mainly related to the ability to create mutation phenotypes through transposon elements and the stability of transposon-generated mutations because once inserted, the frequency of further movements of transposons is low (Karcher 1995). On the other hand the size of transposable elements results in the impossibility to perform fine detail analysis (single nucleotides), moreover, transposons have some preferred site for integration and their mutagenesis of a chromosome can be non-random as it should be. In most of the cases transposon-generated mutations causes gene knockouts which deactivate the site in the gene (Paustian and Kurtz 1994).

- **DNA sequencing.** This technique uses dideoxynucleotide (ddNTP) to determine the sequence of nucleotides (such as A, G, C, T). The advantage of the method is that elongation can be terminated at specific positions through the use of an inhibitor which stops the action of DNA polymerase I at the particular base which it is supposed to represent. Since its invention, the basic procedure has not changed. However, important features such as simplicity, speed and reliability need to be improved (Techcouncil 2011, Metzker 2005). An alternative technique which can substitute Sanger's method is next generation sequencing (NGS). It offer benefits in performing genetic analysis, especially for large-scale projects. On the other hand one limit of using this technique is the short sequencing read lengths that it provides (Hert et al. 2008, Metzker 2005). Moreover, the method can not guarantee that the sample (in our case the bacterium) expresses all the genes as proteins only because they have been sequenced and found. For this purpose it is possible to use Western blot analysis.

- **Western blot analysis.** The benefits of Western blot analysis are essentially two, being sensitivity and specificity: sensitivity is due to its ability to detect proteins in a range of 0.1 ng. Consequently, an high sensitivity need few antibodies for testing, reducing the cost
significantly. Specificity occurs through gel electrophoresis, which separate proteins in accordance to size, charge and conformation giving informations about the size of the protein of interest. Another factor that contributes to specificity is antibody-antigen interaction, which shows high affinity for a specific protein, even in a mixture of 300.000 different proteins. Sometimes it is possible to obtain erroneous results leading to false-positive or false-negative detection. False-positive results can occur when the antibody reacts with a non intended protein, whereas false-negative results occur in presence of larger proteins when they do not have enough time to transfer properly to the membrane. Moreover, Western blot analysis is a nonquantitative analysis and the cost of antibodies is high (Moore 2009). An alternative technique to western blot analysis is the immunoassay ELISA (enzyme-linked immunosorbent assays), which results to be more sensitive than western blot analysis and quantify the protein concentration in the sample (Bio supply UK n.d).

• IEP. This technique is an important tool which combines the sample separation, via an electric current, with the individuation of a specific protein, produced by the immune system, by means of antibodies detection. It is highly sensitive and specific, however, a disadvantage consist in the difficult results' interpretation (Levinson 2009).

• Proteins separation by SDS-PAGE. The advantage of the technique is its high sensitivity, it requires proteins as little as 2 pmol. On the other hand the technique is not effective for carbohydrate-rich or membrane proteins and it is less effective for proteins of similar molecular weight (The Ohio State University 2012).

• BLAST searches. BLAST search has three important advantages, such as its speed, its sensitivity and a possibility to switch the analysis from nucleotides searches to amino acids by translating a nucleotide sequence into six frames and search for a protein database. A disadvantage is the low sensitivity for some weak DNA hits (Boston university 2012).
8. Conclusion

The purpose of this project was to give an overview of the possible strategies that *S. mitis* can use to colonize the human oropharynx, changing its state from commensal microorganism into pathogen. As introduced previously, the majority of native valve IE and late prosthetic valve endocarditis is caused by viridians streptococci (50-70%), where, *S. mitis* seems to play an important role as mediator in the colonization of the damaged endocardial surface with a progressive accumulation of platelets and bacteria. Despite improvements in medicine and health care, IE has still high mortality and morbidity; these data suggest to research how *S. mitis* turns into a potential killer.

During this project a lot of knowledge has been gathered about *S. mitis* colonization and its virulence factors. An interesting hypothesis could raise from the pathogen *S. pneumoniae*, a possible ancestor of *S. mitis* which evolved during generations into *S. mitis*, losing some of its virulence factors and becoming mostly commensal.

Probably the switching between pathogen and commensal is the increased expression of one of the possible virulent factors (such as PblA and PblB surface proteins), with subsequently increased platelet binding. Moreover, the expression of holin and lysin seems to play an indispensable role in order to reach a necessary level of PblA and PblB for surface expression and platelet binding. Additionally, the cleavage of IgA1 protease in the hinge region degrades IgA1 and it need a specific amino acid sequence as cleavage site.

Yet, no one of the virulence factors studied in this project should be supported as the most reliable and responsible for the progression of IE disease. The theories are more likely inter-related and to some extent all contribute to developing *S. mitis* as pathogen.

9. Perspective for the future

In my opinion future research should be devoted to investigate deeply the interaction of *S. mitis* with human cells as well as the factors which lead to its transition from commensal to potential killer. Highest focus should be directed towards finding out the cascade of processes happening during the colonization of *S. mitis*. This would make it easier to find the causes and factors that definitely contribute to the pathogenesis of IE, which in turn would make it possible to treat tempestively the disease and cure the patient.

Finally, I think that molecular methods such as NGS can be challenges tools in respect to the
efficiency of the sequencing compared to the traditional Sanger's method. This new technique allows to obtain until one milliard of bases in one day at low costs, giving the whole genome sequence of the sample under analysis. With NGS it is easier to investigate a lot of *S. mitis* and screen them for virulence genes and resistance genes in order to understand better their behaviors.
10. Glossary and abbreviations

ACT program: Artemis Comparison Tool to carry out pair-wise genome comparison.

Antigen: any substance that causes your immune system to produce antibodies against it.

Antibody: protein secreted as a result of the antigen provoked immune response.

Antiserum: human or animal or bacterial serum containing antibodies that are specific for one or more antigens.

Bacteraemia: an invasion of the bloodstream by bacteria.

BLAST: Basic Local Alignment Search Tool, finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to those with similar sequences in common.

Blood agar plate: growth media containing mammalian blood (usually sheep or horse) to detect hemolytic activity.

Catalase: an enzyme exposed to oxygen which catalyzes the decomposition of hydrogen peroxide to water and oxygen.

CBPs: Choline-Binding Proteins.

Complementation test: used to test the relationship between two different strains of an organism which both have homozygous recessive mutations that produce the same phenotype but which do not reside on the same gene.

CHF: Congestive Heart Failure.

Cytokines: proteins, peptides, or glycoproteins working as signaling molecules used in cellular communication.
Cytotoxic: any substance with a particular feature of being toxic to cells.

DEAE: diethylaminoethanol cellulose.

Embolism: any obstruction in a blood vessel due to a blood clot or other foreign matter that gets stuck while traveling through the bloodstream.

Endothelial cells: cells lining the inner walls of the blood vessels.

Exotoxin: a toxin formed and excreted by the bacterial cell.

Extracellular matrix proteins: proteins produced by cells and secreted into the environment in which the cells are embedded.

Epitope: is the part of an antigen that is recognized by the immune system, specifically by antibodies.

EPS: Extracellular Polymeric Substance.

Fermentation test: test designed to differentiate bacteria on the basis of fermentative metabolism of carbohydrates.

Fibrin: fibrous, non-globular protein involved in the clotting of blood.

Gram-positive bacteria: those bacteria that are stained dark blue or violet by gram staining; they have an high amount of peptidoglycan in the cell wall and typically lack the outer membrane found in gram-negative bacteria.

HEXXH...E motif: many metallopeptidases bind a tetrahedally-coordinated atom of zinc, or rarely another metal, by use of the sequence motif: His-Glu-Xaa-Xaa-His.

IEP: Immunelecrophoresis: two-stage process; electrophoresis is conducted in the first stage and
immunoprecipitation using antibodies against specific proteins in the second stage without removing the proteins from the separation media (usually agarose).

Induction: the production of a specific morphogenetic effect by use of appropriate agents.

Integrin: a large group of cell adhesion molecule which consist on two subunits both essential in promoting cell adhesion.

LPXTG motif containing region: found at the C terminus of many surface proteins of Streptococcus and Streptomyces species.

Metalloproteinase: a protease enzyme with a catalytic mechanism which involve a metal.

MMP-9: matrix metalloprotease 9, Proteins of the matrix metalloproteinase (MMP) family involved in the breakdown of extracellular matrix in normal physiological processes as well as in disease processes.

Monocytes: a type of white blood cell which play a role in immune system function.

muc16 : mucin 16, a member of the mucin family glycoproteins. It has found application as a tumor marker.

Myeloma: accumulation of malfunctioning or "cancerous" plasma cells.

Neutropenic patient: those more susceptible to bacterial infections characterized by an abnormally low number of neutrophils that serve as the primary defense against infections by bacteria in the blood.

NGS: Next Generation Sequencing.

Oropharynx: the pharynx between the soft palate and the epiglottis.
OFR: Open Reading Frame.

P value: is the probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true. When the \( p \)-value is less than the significance level \( \alpha \), which is often 0.05 or 0.01, then the null hypothesis is rejected and the result is said to be statistically significant.

PBS: Phosphate Buffered Saline, a water-based salt solution containing sodium chloride, sodium phosphate, and, in some cases potassium chloride and potassium phosphate. PBS groups help to maintain constant the pH.

Pili bacteria: hairlike appendages found on the surface of many bacteria.

Pneumococcal autolysin LytA: virulence factor involved in autolysis consisting in the destruction of a cell through the action of its own enzymes.

Pneumonia: is an infection of the lungs that is caused by bacteria, viruses, fungi, or parasite.

PFGE: Pulsified Gel Electrophoresis.

proinflammatory cytokine TNF-\( \alpha \): Tumor necrosis factor-alpha (TNF-\( \alpha \)) cytokine, consisting of small cell-signaling protein molecules

phylogenetic tree: branching diagram showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics.

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, separates proteins according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge).

Septicaemia: caused when certain bacteria get into the bloodstream.
Sequence motif: a sequence pattern of nucleotides in a DNA sequence or amino acids in a protein.

Silver stain: used to detect proteins after electrophoretic separation on polyacrylamide gels.

Stromal cells: connective tissue cells of an organ found in the loose connective tissue.

Syndecan-1: The protein encoded by this gene is a member of the syndecan proteoglycan family. The syndecans mediate cell binding, cell signaling, and cytoskeletal organization.

TFA: Tissue factor activity.

Tolerance tests: is a test to check how bacteria breaks down glucose.

Transposon mutagenesis: a biological process that transfer genes to a host organism's chromosome causing mutation.

Trypsination: is the process of using trypsin, a proteolytic enzyme which breaks down proteins enabling the cells to adhere to the vessel.

Western blot analysis: analytical technique used to detect specific proteins in the given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins that are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein.

Glossary of Figure 6

Emm6: an M6 protein protects the bacterium from the phagocytosis by regulating the complement activation on the bacterial surface.

Eno: magnesium ion binding protein.

GbpA: Glucan-binding protein A.
GtfBC: Glycosyltransferase GtfB.

Lmb: Laminin-binding surface protein Lmb.

SfbI: Fibronectin-binding protein I.

SpeB: important streptococcal virulence factor which cleaves human fibronectin and degrades vitronectin. Also cleaves human IL1B precursor to form biologically active IL1B. Can induce apoptosis in human monocytes and epithelial cells in vitro, and reduces phagocytic activity in monocytic cells.
References


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