Investigations into the role of mPIP, the mouse homologue of hPIP/GCDFP-15, in innate host defense

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

mPIP is a mouse homologue of human PIP/GCDFP-15 which is an established marker of both malignant and benign pathological conditions of the mammary gland. mPIP gene expression has been identified in both lacrimal and salivary glands of healthy mice and the mPIP protein has been detected in saliva. The mPIP protein has been found to bind oral bacteria, showing the highest affinity for streptococci, suggesting a potential function of mPIP in the non-immune host defense in the mouse oral cavity. Since the exact functions of mPIP are still unknown, we examined the roles of mPIP through both *in vitro* and *in vivo* studies, specifically to address the possible role of this protein in non-immune host response through modulating the oral flora.

The *in vitro* studies were primarily focused on elucidation of the consequences of interaction between mPIP and oral bacteria, in particular to examine whether mPIP plays a role in bacterial aggregation. The *in vivo* studies addressed the roles of mPIP through the analysis of an mPIP knockout mouse model generated in our laboratory. Following confirmation of the null mutation, the delineating the phenotype of this model was pursued through morphopathological analysis as well as examination of the impact of the lack of mPIP on the mouse oral flora.

The null mutation in the mPIP knockout mice was confirmed by both the gene and protein analysis. Histological analysis revealed lymphocytic proliferation in both the submaxillary and prostate glands of the mPIP knockout mice. In addition, both quantitative and composition differences in the oral flora of mPIP knockout mice were identified when compared with wild-type controls. Specifically, a higher proportion of the oral bacteria of mPIP knockout mice were found to belong to genus *Streptococcus* and certain genera were found to be absent from the oral cavity of these mice. The effect of knockout mouse saliva, which lacks mPIP, on the aggregation of oral bacteria was compared to wild-type mouse saliva. Our data suggests that mPIP contributes to saliva-induced bacterial aggregation.

While oral flora has multiple functions, including protection against infection, mPIP might play a role in the non-innate host defense through modulating the resident oral flora in the mouse. The identification of lymphocytic proliferation in submaxillary and prostate glands of mPIP knockout mice suggests that mPIP might also interfere with lymphocyte activity, playing a possible immunomodulatory role.

ACKNOWLEDGEMENTS

This work would not have been possible without the continuous support and mentorship of my supervisor, Dr. Yvonne Myal. Thank you very much for offering me the opportunity to experience basic sciences research and to attain a graduate degree. Also thank you for encouraging me to experience other aspects of the graduate student life such as teaching, participating in student's association activities, attending and presenting at seminars and last but not least, attending aerobic classes during the lunch hour.

I would like to thank my advisory committee members, Dr. Jeff Wigle and Dr. Steve Pind for their advice on my project; special thanks to Dr. Steve Pind for offering both his time his bench space during my protein purification studies.

I express my sincere gratitude to Dr. George Bowden, Professor Emeritus in the Department of Oral Biology, for his support and dedication throughout my bacteriology studies.

I would like to extend my appreciation to other faculty members for their valuable input into my project: Dr. Thomas Klonisch, Dr. Jiuyong Xie, Dr. John Wilkins and Dr. Robert Shiu; special thanks for Dr. Shiu for keeping his door open all the time for suggestions and advice not only for me but also for all the other students.

My graduate studies would not have been the same without the other members of the Myal lab whom I would like to thank. First, I want to thank Anne Blanchard for her support during my entire project, especially for helping me with the animal work and for showing me how to use a fire extinguisher. Thanks to Tamara and Nellie who made my last summer in the lab an unforgettable one. Also, thanks to Barbara for being there whenever I needed help.

I would like to acknowledge the National Sciences and Engineering Research Council of Canada for funding this project and also the University of Manitoba and the Health Sciences Centre Foundation who offered me funding in the form of scholarships.

I would also like to acknowledge my friends, which are too many to mention, who kept asking "When will you get it done?", therefore encouraging me to bring this thesis to its end.

Finally, I would like to thank my life partner and best friend Laurentiu for standing by me during the ups and downs of my life as a graduate student. I was happy to walk with him toward the same goal and to see how our common journey towards a graduate degree comes to an end.

Last but not least, I would like to thank my parents, Laura and Cristian, for their encouragement during my masters program and for their continuous support regardless of the choices I have made. To them I dedicate this thesis.

Most of all, I thank God for the wisdom and strength He has given me throughout these years.

ABBREVIATIONS

α	Alpha
µg	microgram
µl	microlitre
µm	micrometer
ºC	degrees Celsius
1 X	one time
4 X	four times
BCA	bicinchoninic acid
BSA	bovine serum albumine
DNA	deoxyribonucleic acid
cDNA	complementary deoxyribonucleic acid
CFUs	colony forming units
ddH_2O	double-distilled water
DMP	dimethyl pimelimidate
DTT	dithiothreitol
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ES cells	embryonic stem cells
GCDFP-15	gross cystic disease fluid protein-15
IgG	immunoglobulin G
hPIP	human prolactin-inducible protein
HRP	horseradish peroxidase
IPTG	isopropyl β-D-1-thiogalactopyranoside
KO	knock-out
LB	Luria Berthani
mM	milimolar
M	molar
mPIP	mouse prolactin-inducible protein
mRNA	messenger ribonucleic acid
OD	optical density
OPD	o-Phenylenediamine Dihydrochloride
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIP	prolactin-inducible protein
PBS	phosphate-buffered saline
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SMGP	submaxillary gland protein
TBS	tris buffered saline
TBST	tris buffered saline with Tween 20
tRNA	transfer ribonucleic acid
WT	wild-type

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I. BACKGROUND

1. The prolactin-inducible protein

1.1. Human PIP/GCDFP-15 in the context of breast cancer

Worldwide, breast cancer is the fifth most common cause of cancer death (www.who.int). Breast cancer is the most common cancer among Canadian women (www.cancer.ca) and it is the second leading cause of cancer-related deaths in women (after lung cancer).

Tumor markers are molecules, usually proteins, produced by the cancer tissue or by the body in response to cancer growth (Hayes et al., 2001). Tumor markers can be used for diagnostic purposes, to assess prognosis, to predict sensitivity or resistance of tumors to different methods of therapy or to determine the responsiveness to treatment. Currently, the only validated predictive breast cancer markers are the estrogen receptor (ER) and progesterone receptor (PR), which are used to select tumors which may benefit from adjuvant hormonal therapy, and HER-2, which is used for identifying breast cancer patients who may benefit from trastuzumab (Cinieri et al., 2007; Duffy, 2005). Several associations between the gene expression profiles of breast tumours and responsiveness to chemotherapy have been found (Lonning et al., 2007).

PIP/GCDFP-15 (prolactin-inducible protein/gross cystic disease fluid protein-15) is considered as a marker that is associated with both benign and malignant pathological conditions of the mammary gland. PIP was first identified as an abundant component of the breast gross cystic disease fluid (Haagensen, Jr. et al., 1979), as well as a protein secreted by the breast cancer cell line T-47D when treated with prolactin (Shiu and Iwasiow, 1985).

PIP/GCDFP-15 is regarded as a functional marker of apocrine cells (Mazoujian et al., 1983; Mazoujian et al., 1989). However, apocrine carcinomas exhibit a reduction in GCDFP-15 expression in larger and lymph node-positive tumours suggesting that GCDFP-15 expression is a transient characteristic of apocrine carcinomas which is lost upon tumor growth (Honma et al., 2005).

Expression of the PIP gene was detected in several human breast cancer lines and it was found to be modulated by different hormones, such as prolactin, androgen and human growth hormone (Murphy et al., 1987b). PIP mRNA expression was detected by RT-PCR analysis in 92% of primary breast tumors and the mRNA levels correlated well with PIP protein levels determined by immunohistochemistry (Clark et al., 1999).

In a larger study, immunohistochemical analysis revealed expression of PIP in 72% of breast carcinoma samples (Wick et al., 1989). PIP protein expression was also used in the differential diagnosis of metastatic breast cancer versus other primary origin carcinomas (Matsuoka et al., 2003; Tohnosu et al., 2006; Tornos et al., 2005). The presence of PIP protein in the serum of some breast cancer patients has been detected by Western blot analysis (Murphy et al., 1987a) and circulating anti-PIP antibodies have been identified in sera of patients with both malignant and benign breast disease (Pasquinelli et al., 1999).

PIP mRNA was undetectable in a sample of normal breast tissue obtained from reduction mammoplasty (Myal and Shiu, 2000). However, few isolated epithelial cells of normal breast tissue showed positive immunohistochemical staining for PIP (Haagensen, 1986). Also, low levels of PIP mRNA expression were detected by PCR in 15% of normal epithelial breast tissue samples (Clark et al., 1999).

1.2. Other characteristics and possible roles of PIP/GCDFP-15

In healthy individuals, PIP has been found at privileged immunological sites (Pasquinelli et al., 1999) including the submucosal glands of the lung, sweat, salivary and lacrimal glands (Mazoujian et al., 1983), as well as being secreted in saliva, breast milk, amniotic fluid and seminal plasma (Autiero et al., 1991; Autiero et al., 1995; Murphy et al., 1987b; Myal et al., 1994; Myal and Shiu, 2000).

While a function for PIP has not yet been defined (de Longueville et al., 2005), several findings suggest a potential role for this protein in immunomodulation and host defense mechanisms. Human seminal plasmatic PIP has been shown to bind immunoglobulin G (Chiu and Chamley, 2003). PIP is down-regulated in the nasal lavage fluid of allergic individuals (Bryborn et al., 2005) and in the tears of blepharitis patients (Koo et al., 2005). Human PIP also binds to certain strains of bacteria isolated from the human mouth, ear canal and skin (Schenkels et al., 1993; Schenkels et al., 1997).

In addition, gp17, a glycoprotein demonstrated to be identical to PIP/GCDFP-15 (Autiero et al., 1995; Gaubin et al., 1999), was isolated from human seminal plasma and was found to bind $CD4^+$ T lymphocytes (Autiero et al., 1991). This binding causes inhibition of $CD4^+$ T lymphocyte apoptosis concomitantly with a moderate up-regulation of Bcl-2 expression, emphasizing that gp17 may have a functional relevance in tumor pathology (Gaubin et al., 1999). Also, it was proposed that as a CD4-binding factor, PIP may provide protection from HIV infection by interfering with the HIV envelope / CD4 binding (Autiero et al., 1997).

PIP was also identified in the epithelium of the normal prostate, its expression being enhanced in prostate carcinomas, suggesting that testing for PIP expression may make a useful contribution as a diagnostic index of malignant transformation in the prostate (Tian et al., 2004).

1.3. The mouse prolactin-inducible protein (mPIP)

The mPIP/mSMGP (mouse prolactin-inducible protein/mouse submaxillary gland protein) is now the established murine homologue of human PIP/GCDFP-15, exhibiting 46.6% identity at the amino-acid level (Figure 1). Mouse SMGP cDNA was first cloned from the adult mouse submaxillary gland (Windass et al., 1984). However, the homology with human PIP was only later shown when a search for a mouse homologue was initiated using cDNA from mouse submaxillary gland and PCR techniques and the mouse and human amino-acid sequences were first aligned (Myal et al., 1994).

The mouse PIP gene is localized on chromosome 6q34 consecutive with four other genes sharing a common structure, SVA (seminal vesicle autoantigen) and SVAL (SVA-like) 1-3 (Osawa et al., 2004). These members also share several amino acids (SVA, SVAL1 and SVAL2 protein exhibit 25-30% amino-acid identity with mPIP) suggesting that this cluster of genes formed during evolution as a result of gene duplications and translocations, events specific to the mouse genome while in other mammals PIP appears to be the only family member (Osawa et al., 2004). Expression of these mouse genes has been detected by RT-PCR analysis in several tissues; mPIP expression is limited to lacrimal and all major salivary glands whereas the other members of the family showed different patterns of expression, including SVA which is found in the seminal vesicles and SVAL2 in the lactating mammary gland (Osawa et al., 2004).

Northern Blot analysis of eighteen different mouse tissues using mPIP cDNA as a probe revealed that mPIP expression was limited to the lacrimal and submaxillary glands (Myal et al., 1994). Expression was detected in the mouse submaxillary gland as early as embryonic day 14, a period that coincides with the initiation of submaxillary gland development in the embryo (Lee et al., 2003). *In situ* hybridization studies showed that mPIP expression within the mouse submaxillary gland is localized to the acinar cells (Lee, 2000). The mPIP gene was also found to be expressed in the mouse prostate

Figure 1. Amino-acid sequence alignment of mouse and human PIP.
There is 46.6% identity at the amino-acid level between mouse and human PIP.

during early postnatal development, however its expression was turned off by 10 weeks of age, suggesting an androgen regulated gene expression pattern (Lee et al., 2003). However, castration followed by re-administration of exogenous androgen failed to induce mPIP gene expression in mice (Lee et al., 2003).

The mPIP protein has been detected in adult mouse lacrimal gland tissue (Lee, 2000) and the protein has also been identified secreted in mouse saliva (Lee et al., 2002).

1.4. The functions of mPIP

The presence of mPIP gene expression in the mouse submaxillary gland during embryonic development, suggests that mPIP may play a functional role in the developing gland (Lee et al., 2003). Like its human counterpart, mPIP was found to bind several oral bacterial strains specifically, showing the highest affinity for streptococci (Lee et al., 2002). This observation suggests that mPIP may have a role in the non-immune host response by modulating the flora of the mouse oral cavity (Lee et al., 2002). However, like its human homologue, the exact roles of mPIP are currently not known.

1.5. Other homologues of PIP

The rat prolactin-inducible protein has 56% identity at the amino-acid level with mPIP (Mirels et al., 1998). Expression of the rat PIP protein was localized to the developing and mature acinar cell of the submaxillary and parotid glands (Mirels et al., 1998). In the rat lacrimal gland, expression of PIP was found to be drastically inhibited by androgens *in vivo* (Myal et al., 1994). In addition, castration stimulated rat PIP gene expression in the lacrimal gland of adult rats and re-administration of exogenous androgen completely

abolished PIP gene expression in the castrated animals (Myal et al., 1994). No alterations of PIP gene expression were detected in female rats undergoing ovariectomy followed by estrogen replacement (Myal et al., 1994). However, modulation of rat PIP expression by hormones is tissue specific, as androgen had no effect on rat PIP expression in the submaxillary gland (Myal et al., 1994).

Other homologues of human PIP were identified by Southern blot analysis of genomic DNA retrieved from different species: monkey, dog, cow, rabbit and chicken (Myal and Shiu, 2000). Full length PIP cDNAs were isolated from the salivary glands of several mammals, such as guinea pig, rabbit, cow, chimpanzee and macaque. The corresponding proteins exhibiting 58-75% amino acid identity with human PIP and 44-50% identity with mouse PIP (Osawa et al., 2004). Rabbit and guinea pig PIP expression was detected by RT-PCR in lacrimal and salivary glands, and the rabbit PIP was also present in the colon (Osawa et al., 2004).

2. The structure and main functions of the mouse salivary glands

Like humans and the other mammals, mice possess three pairs of major salivary glands: submandibular (also known in the mouse as submaxillary), parotid and sublingual. Salivary glands consist of two main types of cells: acinar cells (secretory cells that produce saliva) and ductal cells. The acinar cells are either mucous cells, secreting large glycoproteins called mucins, or serous cells producing other types of proteins.

In mice, the parotid gland consists of serous acinar cells, the sublingual gland is composed of mainly mucus cells and the submaxillary gland is a mixed gland with both serous and mucous acinar cells (Tucker, 2007). Secretions of the acinar cells are drained in the intercalated ducts that lead into striated excretory ducts which open into the mouth. In the rodent submandibular glands, granular convoluted tubules are situated between the intercalated and striated ducts. The cells of these tubules produce

enzymes present in the cells as granules and possess receptors for several hormones such as testosterone, thyroid hormone and glucocorticosteroids which are able to control the secretory activity of these cells (Arancibia and Assenmacher, 1985; Shafer and Muhler, 1960).

The activity of the mouse major salivary glands differs between males and females (Tucker, 2007). Sexual dimorphism of the mouse submandibular gland has been well-recognized and widely studied (Pinkstaff, 1998; Shafer and Muhler, 1960). As an example, the rapid development of the granular convoluted tubule is evident only in male individuals between four and six weeks of age, a period coinciding to an increase in plasma testosterone levels (Jayasinghe et al., 1990). In addition, numerous differences in gene expression have been identified between the male and female mouse salivary glands. These mainly involve up-regulation in the male individuals, with many of these differences being tissue-specific (Treister et al., 2005).

Exocrine secretions of the salivary glands, together with the gingival crevicular fluid constitute the whole saliva (Edgar, 1992) which plays multiple roles in the homeostasis of the oral cavity. Saliva lubricates and hydrates the soft tissues of the mouth and plays a role in the food bolus formation, thus facilitating digestion (Tabak, 2006). Salivary proteins have different functions in modulating the resident oral bacteria, such as promoting aggregation or adhesion of oral bacteria to oral tissues or direct antibacterial activity (Mandel, 1987; Tabak, 2006). Saliva also contributes to raising the pH of the dental plaque (Tabak, 2006) and helps to neutralize the reflux acids in the esophagus (Mandel, 1987).

In addition, an endocrine function has been attributed to the rodent submaxillary glands, which were found to synthesize several hormonal factors: renin (Bing et al., 1980), nerve growth factor (NGF), epidermal growth factor (EGF), transforming growth

factor beta and kallikreins, all of which are secreted into the saliva (Arancibia and Assenmacher, 1985; Sabbadini and Berczi, 1995).

In summary, the mouse salivary glands exhibit both endocrine and exocrine functions (Arancibia and Assenmacher, 1985), similar to other components of the digestive tract such as the stomach liver and pancreas.

3. Physiological determination of PIP concentrations *in vivo*

3.1. Methods for quantification of proteins in body fluids

The ability to measure the content of specific proteins in body fluids is important in order to establish the normal values and to detect any variations in these values. The ultimate goal is to identify possible roles for each protein considering that the concentration of a protein might be increased or decreased, or new proteins might appear as a consequence of a certain disease.

Radioimmunoassay (RIA) was among the first modern methods used to assay the concentration of a specific circulating protein. The technique was introduced as an assay to determine the concentration of insulin in human plasma (Yalow and Berson, 1960), showing for the first time that hormone levels can be detected in the blood by an *in vitro* method. RIA consists of mixing known quantities of radioactive labeled protein of interest (antigen) with an antibody to that antigen, then adding unlabeled antigen and measuring the amount of labeled antigen that was displaced. The discovery of the RIA was one of the major accomplishments of medical research in the 20th century and Rosalyn Sussman Yalow received the 1977 Nobel Prize in Medicine for the development of the RIA for insulin (Kahn and Roth, 2004).

Lately, the use of RIA has been largely replaced by ELISA (Enzyme-Linked ImmunoSorbent Assay), also known as EIA (enzyme immunoassay), in which the antigen-antibody reaction is measured by a colorimetric signal instead of radioactivity. ELISA was first used to determine the concentration of IgG in rabbit serum using alkaline phosphatase as a reporter enzyme (Engvall and Perlman, 1971), followed by quantification of the human chorionic gonadotropine in urine using horseradish peroxidase coupled with glutaraldehyde as a reporter (Van Weemen and Schuurs, 1971).

Different variants of ELISA are used, the classification of the methods varying among different authors. Three main systems form the basis to all ELISA techniques, direct, indirect and sandwich ELISA, and each of them can be used for competition or inhibition ELISAs (Crowther, 2001). Another way of categorizing the ELISA methods is by grouping them into antibody capture assays, antigen capture assays and two-antibody sandwich assays, each of which can be performed in antibody or antigen excess or as an antigen or antibody competition, thus yielding to a total of 12 possible combinations; however, not every combination leads to a useful assay (Harlow and Lane, 1988).

There are currently over 120 000 articles, including almost 3000 reviews, listed in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez, accessed August 2007) when searching the terms “ELISA” or “EIA”. The large numbers of analytical and clinical investigations that rely on these measurement procedures worldwide demonstrates that the development of EIA/ELISA methods had a significant impact on clinical diagnosis and healthcare in general (Lequin, 2005).

In addition to RIA and ELISA, immunoblotting techniques can be used for semi-quantitative analysis of proteins. Although classical Western blotting is considered a qualitative method for the detection of proteins in biological samples, it can be used as a quantitative method providing that specific controls are included and it is usually used

when no ELISA is available or when certain components in the sample interfere with an ELISA (Mathrubutham and Vattem, 2005). Another technique that may be used for quantitative analysis of proteins is dot blotting, also known as spot/slot blotting, differing from the classical Western by the fact that protein samples are not separated by electrophoresis but are spotted directly onto a membrane support followed by detection with a specific antibody. Slot blotting has been reported to have comparable sensitivity and accuracy with ELISA methods (Zhu et al., 2005), providing that specific antibodies and appropriate controls and standards were available. Quantification of proteins by Western or slot blotting is performed by computer-assisted technology using image analysis software systems which permit pixel quantification of electronic images (Vierck et al., 2000).

Recently, proteomic tools for the quantification of proteins and peptides in biological samples have been developed. Conventional proteomics relies on 2-D gel electrophoresis integrated with mass spectrometry analysis and it is mainly used to compare protein samples quantitatively, resulting in a relative protein quantitation (Lill, 2003). However, the absolute quantitation of proteins by mass spectrometry using standard curves has been attempted (Oda et al., 1999). Although absolute quantification of proteins using mass spectrometry can be achieved, immunoassays such as ELISA are still considered to produce a more precise quantitation at low protein concentration, providing that an antibody is available (Lill, 2003).

3.2. Quantification of PIP in body fluids

Quantification of the secreted PIP in human body fluids has been performed to determine the normal values as well as any variations from the normal range in order to identify a possible role for this protein.

Previously, radioimmunoassay measurements were used to determine the levels of hPIP in human plasma, saliva, gross cystic disease fluid and in the culture media of neoplastic epithelial breast cancer cells (Haagensen, Jr. et al., 1977; Haagensen, Jr. et al., 1979). More recently, enzyme-linked immunosorbent assay (ELISA) methods were developed to quantify PIP in culture media of breast cancer cells (Revillion-Carette et al., 1988), nipple aspirate fluid (Alexander et al., 2004) and human seminal plasma (Chiu and Chamley, 2003; Osawa et al., 1996). ELISA was also used to detect circulating anti-PIP antibodies in sera of patients with both malignant and benign breast disease (Pasquinelli et al., 1999).

Novel proteomic tools such as 2-dimensional gel electophoresis and mass spectrometry have been used to detect variations of PIP levels, specifically the down-regulation of PIP in nasal lavage fluid of allergic individuals (Bryborn et al., 2005) and in the tears of blepharitis patients (Koo et al., 2005).

Although mouse PIP has been found to be secreted in mouse saliva (Lee et al., 2002), no attempts have been made to determine the physiological levels of this protein in mouse body fluids.

4. Oral commensal flora

4.1. Components of the mammalian oral resident flora

The oral resident organisms are diverse and consist of a wide range of species of, bacteria, viruses, mycoplasma, yeast and even, on occasion, protozoa. This diversity is due to the fact that the mouth is composed of several different habitats (Marsh and Martin, 1999). However, bacteria are the predominant components of the commensal oral flora.

4.2. Bacteria in the human oral cavity

The human oral cavity *in utero* is normally sterile (Marsh and Martin, 1999). The establishment of an indigenous oral flora begins to occur within the first days of life when the neonate oral cavity comes into contact with microbes from a variety of external environmental sources (Liljemark and Bloomquist, 1996). The indigenous microbial flora of the human oral cavity develops slowly into a complex community, reaching its most complex state when the adult microhabitats such as teeth and gingival crevices are formed (Liljemark and Bloomquist, 1996).

While different species of organisms are found to colonize specific habitats within the mouth, streptococci have been isolated from all sites in the mouth and comprise a large proportion of the resident oral flora of humans (Marsh and Martin, 1999), being also the most extensively studied oral bacteria. For example, *Streptococcus mutans* and *Streptococcus sanguis* preferentially colonize hard surfaces like teeth, whereas *S. salivarius* is recovered predominantly from the oral mucosa (Marsh and Martin, 1999).

More than 700 bacterial species or phylotypes have been detected in the oral cavity (Aas et al., 2005). Some of these may be readily cultured and identified whereas others can be cultured only with difficulty or not cultured at all (Ofek and Royle, 1994). Recently, culture-independent molecular techniques have been utilized to extend the knowledge on the breadth of bacterial diversity in the healthy human oral cavity, including not-yet-cultivated phylotypes (Aas et al., 2005).

The normal oral flora benefits the host in different ways. First, it provides protection from similar but more pathogenic species. Humans possess low levels of circulating antibodies specific to their indigenous flora which may cross-react with external pathogens, thus playing a role in elevating the hosts immunity to virulent strains (for example, antibodies to *Neisseria meningitidis*, a common causative agent of meningitis, have been found in individuals colonized with the normal resident *Neisseria lactamicus*,

providing partial protection of the host against all types of *N.meningitidis*) (Liljemark and Bloomquist, 1996). Second, the indigenous oral flora forms a barrier to colonization by more virulent bacteria through spatial inhibition of adhesion sites (it is known that in the human oral cavity bacteria must first adhere to a surface in order to be able to colonize), by competing for limited available nutrients or by producing inhibitory substances (Liljemark and Bloomquist, 1996).

Although the normal oral flora usually protects against disease, indigenous species may become pathogenic when they leave their usual habitat and multiply in a new microhabitat where their by-products cause disease or when they reproduce under certain conditions and become a larger proportion of the bacterial population (Liljemark and Bloomquist, 1996). Oral disorders such as dental caries and periodontal disease result from complex interactions between diet, the normal flora and the host and appear following disequilibrium among the bacterial populations of the oral cavity (Trudel et al., 1986). Organisms strongly associated with caries and periodontal disease may be considered components of the resident flora and may be present, sometimes in very low numbers, in the absence of disease (Bowden and Hamilton, 1998).

Therefore, the indigenous oral flora is able to cause oral infections. This is particularly important since the oral cavity can act as the site of origin for dissemination of pathogenic organisms to distant body sites, causing systemic diseases (Li et al., 2000), due to the fact that that the tooth-tissue interface represents an unique site in the body for micro-organisms to enter the bloodstream (Marsh and Martin, 1999). The oral cavity has been recognized for a long time as an origin for a variety of diseases including brain abscess, pulmonary and gastric problems and several systemic infections (Miller, 1891).

Oral commensal bacteria, most commonly streptococci belonging to the viridans group, are the most common causative agents of infectious endocarditis (Hahn et al.,

2005), an inflammation of the heart valves. Recently, a relationship between periodontal disease in pregnant women and preterm birth, accompanied by all the complications associated with low birth weight, has been established (Offenbacher et al., 2006). Improvement of the pregnancy outcome following treatment of the periodontal disease in the pregnant woman has been suggested but not yet proven (Offenbacher et al., 2006). In addition, periodontal disease has been identified more frequently in patients with cardiovascular disease, although a causal relationship hasn't been established (Demmer and Desvarieux, 2006). Also, patients with diabetes who have periodontitis are at greater risk of developing poor glycemic control (Mealey and Oates, 2006).

As oral pathology is related to several systemic inflammatory processes, as well as pre-term deliveries and low birth weight, there is sufficient evidence to support the benefits of identifying and controlling oral pathogens (Costerton and Keller, 2007).

4.3. Similarities between oral and intestinal resident flora of humans

A similar function has been attributed to the commensal flora of the intestinal tract, which plays a role in intestinal mucosal homeostasis (Xavier and Podolsky, 2005) but can also initiate immune-mediated intestinal inflammation therefore playing a role in the development of inflammatory bowel disease (Kim et al., 2005). In addition, the intestinal commensal flora contributes to the pathophysiology of obesity. Differences between the distal gut microbiota of obese versus lean humans have been reported and the relative abundance of certain bacterial species increase with weight loss of obese individuals (Turnbaugh et al., 2006). Differences between the normal intestinal bacteria of obese and lean mice have also been identified (Bajzer and Seeley, 2006).

Therefore, similarly to the oral commensal bacteria, the intestinal resident flora exhibits a "wolf in sheep's clothing" type of behaviour by playing roles in the innate

immunity mechanisms but also being able to trigger diseases (Xavier and Podolsky, 2005).

4.4. Bacteria in the mouse oral cavity

As in humans, bacteria are normally present in biofilms on the hard and soft surfaces in the oral cavity of mice. However, only a limited amount of literature addressing the composition of mouse oral flora in available.

Several studies addressed the bacteriology of the oral cavity of BALB/c mice because this model possesses a restricted oral microbiota, which facilitates the study of its variation (Coulombe and Lavoie, 1995). A detailed examination of the BALB/c mice oral flora resulted in the identification of only 18 different species of bacteria, *Lactobacillus murinus* and *Staphylococcus aureus* accounting for nearly 75% of the total cultivable flora (Trudel et al., 1986). The aforementioned study used (as methods for bacterial isolation and identification) unstimulated saliva aspiration and selective media culturing followed by characterization using conventional microbiology techniques, therefore only the cultivable flora was detected. The results of a more recent study (Rodrigue et al., 1989) indicate that swabbing is the superior sampling method of the mouse oral microbiota and that the immuno-colony-blot assay is a more suitable technique for the detection of the different bacterial species present in the samples.

As in humans, the indigenous oral bacterial flora of mice varies with age (Wolff et al., 1985). It has also been shown that environmental factors affect the mouse oral microbiota masking any impact of genetical background of the mouse strains (Gadbois et al., 1993). In addition, even in similar conditions, there is some variation in the oral flora of mice from one animal to another (Trudel et al., 1986). Moreover, the sampling and bacterial identification techniques play an important role in the evaluation of the oral microbiota (Rodrigue et al., 1989).

Taking all these factors into account, it is important that studies addressing variations in the oral flora of mice are performed in carefully controlled environments maintaining as many constant conditions as possible.

4.5. Interactions of oral bacteria with salivary components

Salivary components impact the colonization of the oral cavity with bacteria through four different mechanisms: binding to microorganisms to facilitate their clearance from the oral cavity; serving as receptors in oral surface pellicles for microbial adhesion to host surfaces; antibacterial activity (inhibiting microbial growth or mediating microbial killing); and serving as microbial nutritional substrates (Scannapieco, 1994).

Bacterial aggregation is thought to promote the clearance of microorganisms from the oral cavity by inhibiting bacterial adherence (Mandel, 1979) and factors promoting this phenomenon which are present in saliva are considered components of oral defense mechanisms (Ericson et al., 1975). However, co-aggregation is another phenomenon where oral bacteria bind to microorganisms already adherent to the saliva-coated enamel, thus promoting dental plaque formation (Marsh and Martin, 1999).

Different methods have been used to study saliva-induced bacterial aggregation (Koop et al., 1989b) and several factors influencing saliva-induced bacterial aggregation have been evaluated, such as bacterial concentration or the presence or absence of calcium ions and the culture medium (Koop et al., 1989a). However, bacterial aggregation can be strongly strain-dependent (Koop et al., 1989a).

Saliva-induced bacterial aggregation has been studied most often by using oral streptococci, as this group is one of the common microorganisms in the oral cavity that preferentially colonizes tooth surfaces and is a significant component of human dental plaque (Yamaguchi, 2004). The effect of different components of saliva on the aggregation of oral streptococci has also been explored, such as the influence of the

blood group reactive substances in saliva on the aggregation of *Streptococcus rattus* (Ligtenberg et al., 1992) or the relationship between salivary Ig A saliva-induced aggregation of *Streptococcus intermedius* (Yamaguchi, 2004). Although numerous studies have addressed the human-saliva induced aggregation (Koop et al., 1989a; Koop et al., 1989b; Ligtenberg et al., 1992; Yamaguchi, 2004), less is known about the effects of saliva of other species, such as laboratory animal models like mice or rats, on bacterial aggregation.

5. Protein purification

5.1. General strategies used in protein purification

Protein purification is the process of isolating a single type of protein from a complex mixture and is necessary in order to determine the function, structure and interactions of the protein of interest. Initially limited to native sources, the process of protein purification now usually relies on recombinant DNA technology, not only overcoming problems of source availability and source safety, but also facilitating targeted modifications of the protein's amino-acid sequence (Walsh, 2002).

Before attempting any protein purification, several factors need to be taken into account, such as defining the purpose for which the protein is produced, assessing the need of retaining the biological activity, and knowing whether purification of that specific protein or of a related molecule has been reported before (Cutler, 2004).

An essential pre-requisite to the purification and characterization of any protein is the ability to detect and quantify the total protein concentration and the target protein level in the source (Walsh, 2002). Isolating a protein from a mixture relies on exploiting as much as possible the differences between the protein of interest and the other components of

the source. Proteins can be separated taking into account their size (by chromatography, ultrafiltration or gel electrophoresis), solubility under particular conditions (by precipitation), charge (by ion-exchange chromatography), specific binding ability (such as hormone-receptor, enzyme-substrate, antigen-antibody, or binding to metal ions) and other special properties (like stability at extreme pH or heat) (Cutler, 2004). Both native and recombinant proteins are usually purified using a similar overall approach.

5.2. Production of recombinant proteins in bacteria

The gene or cDNA coding sequence of any protein can be isolated and introduced into a variety of expression systems in order to produce recombinant proteins and a large number of proteins are being routinely produced by recombinant means for both academic and applied purposes (Walsh, 2002).

Several host systems are available for producing recombinant proteins including phage, bacteria, yeast, plants, filamentous fungi, insect or mammalian cells grown in culture, and transgenic animals. Choosing the host depends upon the specific requirements and applications for the recombinant protein.

Bacterial systems are commonly used for overexpression of recombinant proteins because they are able to produce higher yields at lower costs when compared with other expression systems; however, frequent problems are encountered in when attempting to recover soluble functionally active protein (Cutler, 2004).

Escherichia coli, a Gram negative bacterium, is the most common bacterial system used for the production of heterologous proteins. Using *E. coli* for recombinant protein production seems attractive because it is able to grow rapidly and at high density on inexpensive substrates, it is well characterized genetically and an increasing number of mutant strains and cloning vectors is available (Baneyx, 1999).

The usual process of producing recombinant proteins in *E. coli* includes a sequence of events (Figure 2.A). First, the heterologous cDNA is cloned into a plasmid (vector) able to replicated inside the bacterial cell. Then the plasmid is transferred into the cells, a process called bacterial transformation, followed by culturing the microorganisms in a suitable media where both cellular growth and induction of protein transcription can be controlled (Balbas, 2001). Finally, the protein of interest is detected, extracted from the bacterial cell and purified. Each of these basic components is specific to the system used, however, there are several generally-accepted steps incorporated in the design of most systems in order to overcome the most encountered problems.

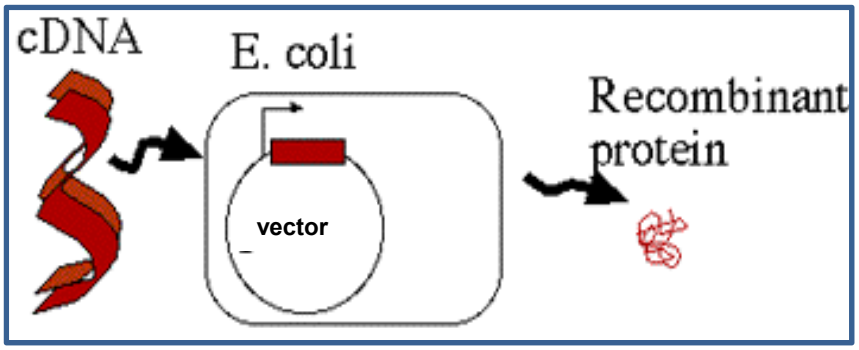
As an example, a number of strategies have been developed to ensure that plasmid-free cells will not overtake the culture. The usual technique is the use of plasmid-encoded antibiotic resistance marker genes so that supplementing the growth medium with antibiotics results in killing the plasmid-free cells (Baneyx, 1999).

Another generally accepted strategy is that recombinant proteins are often produced in *E. coli* as fusion proteins, using fusion partners in order to facilitate protein purification and immobilization. Those molecules may also improve the solubility of the protein, thus preventing inclusion body formation (Balbas, 2001; Baneyx, 1999). The two most commonly used fusion protein tags are glutathione S-transferase (GST tag) and 6 x histidine residues ($(His)_6$ tag).

The most frequently occurring problem of using *E. coli* for protein production is that overexpression of the recombinant protein is accompanied by misfolding and formation of insoluble protein aggregates known as inclusion bodies (Baneyx, 1999; Cutler, 2004). Extra procedures have to be used to extract the recombinant from the inclusion bodies. This frequently means solubilization by denaturing, followed by *in vitro* refolding of the protein, an inefficient process which does not guarantee a biologically active product (Baneyx, 1999).

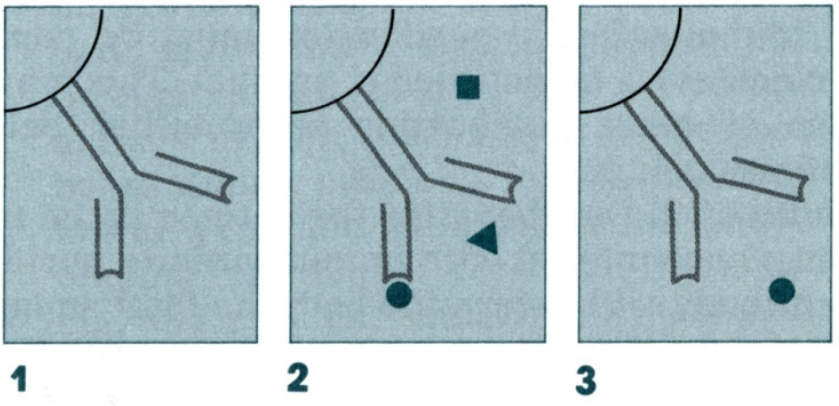
Figure 2. Protein purification.

A. Production of recombinant proteins in *E. coli*



B. The main steps of immunoaffinity purification of proteins

(1) preparing the antibody matrix
(2) binding the antigen to the antibody matrix
(3) elution of the antigen
(from Harlow and Lane, 1998)



Another drawback for the use of *E. coli* as a preferred host for the production of human proteins is the inability of *E. coli* to perform posttranslational modifications of proteins (Balbas, 2001). Although this type of modification, such as glycosylation, will probably remain beyond the reach of *E. coli*, bacterial systems are still considered suitable for the cost effective production of a variety of eukaryotic proteins (Baneyx, 1999).

5.3. Immunoaffinity purification of proteins

Immunoaffinity purification is one of the most powerful techniques for the isolation of proteins and it is based on the specific interaction between antigen and antibody, where the antibody is attached to a solid matrix (Harlow and Lane, 1988). The process depends on the availability of an antibody specific to the antigen and is divided into 3 main steps (Figure 2.B): preparation of the antibody matrix, binding the antigen to the antibody matrix and elution of the antigen. The success of the immunoaffinity purification relies on the availability of a suitable antibody, purity of the antigen, the affinity of the antibody for the antigen and the ease with which the antibody-antigen bond can be broken in order to elute the antigen (Harlow and Lane, 1988).

6. Functional determination of mPIP

6.1. Knockout mice as tools for addressing protein functions

The laboratory mouse (*Mus muculus*) offers particular advantages for the study of human biology and diseases compared with other model organisms initially used for producing induced genetic mutations (such as worms, flies or zebrafish). Most (99%) mouse genes have homologs in humans, and targeted mutagenesis by homologous

recombination is possible in the mouse, allowing genes to be precisely and efficiently altered (Austin et al., 2004).

Observing the characteristics of knockout mice, that have one or more of their genes artificially inactivated, helps in understanding how specific homologue genes can contribute to disease in humans. Currently, the literature describes knockout mice that include approximately 10% of the known mouse genes (Austin et al., 2004). However, the research community is trying to develop a genome-wide project, tentatively named the Knockout Mouse Project (KOMP), consisting of publicly available mutant embryonic stem cell lines, each with a different gene knocked out and a database with the corresponding phenotypic data (Austin et al., 2004).

Some knockout mice are murine models of human genetic diseases and have demonstrated that a single gene defect is capable of causing disease (Tymms and Kola, 2001). A barrier encountered in the knockout mouse based research is the developmental lethality of approximately 15% of the gene knockouts, limiting the study of the specific genes essential to embryonic development. However, advanced technology has overcome the fetal lethal phenotypes by the development of systems, such as the *Cre/loxP*, which enabled the generation of “conditional” knockouts, allowing the analysis of adult mice with targeted mutations of the genes of interest (Tymms and Kola, 2001). Another limitation of the use of knockout mice is encountered when knocking out a gene fails to produce an observable change in a mouse or the change differs from the predictable phenotype based on previous clinical data (Tymms and Kola, 2001). An example of a predictable phenotype is represented by the knockout mouse for the p53, a tumor suppressor gene whose mutation was found to be the most common genetic lesion of human cancers; these mice developed different neoplasms at a young age (Donehower et al., 1992). However, this finding also proved that the oncogenic mutant of p53 gene is not required for the development of some tumors (Donehower et al., 1992).

Models like this which do not reproduce the exact phenotypes of human diseases are valuable by contributing to understanding interspecies differences (Thyagarajan et al., 2003).

In addition to knockout mice, other approaches have been used to study the loss of gene and protein function such as the gene expression silencing via RNA interference (using small hairpin RNA) or generation of dominant negative mutations that generate gene products which may competitively inhibit the functions of the wild-type gene products.

6.2. Methods for generating knockout mice

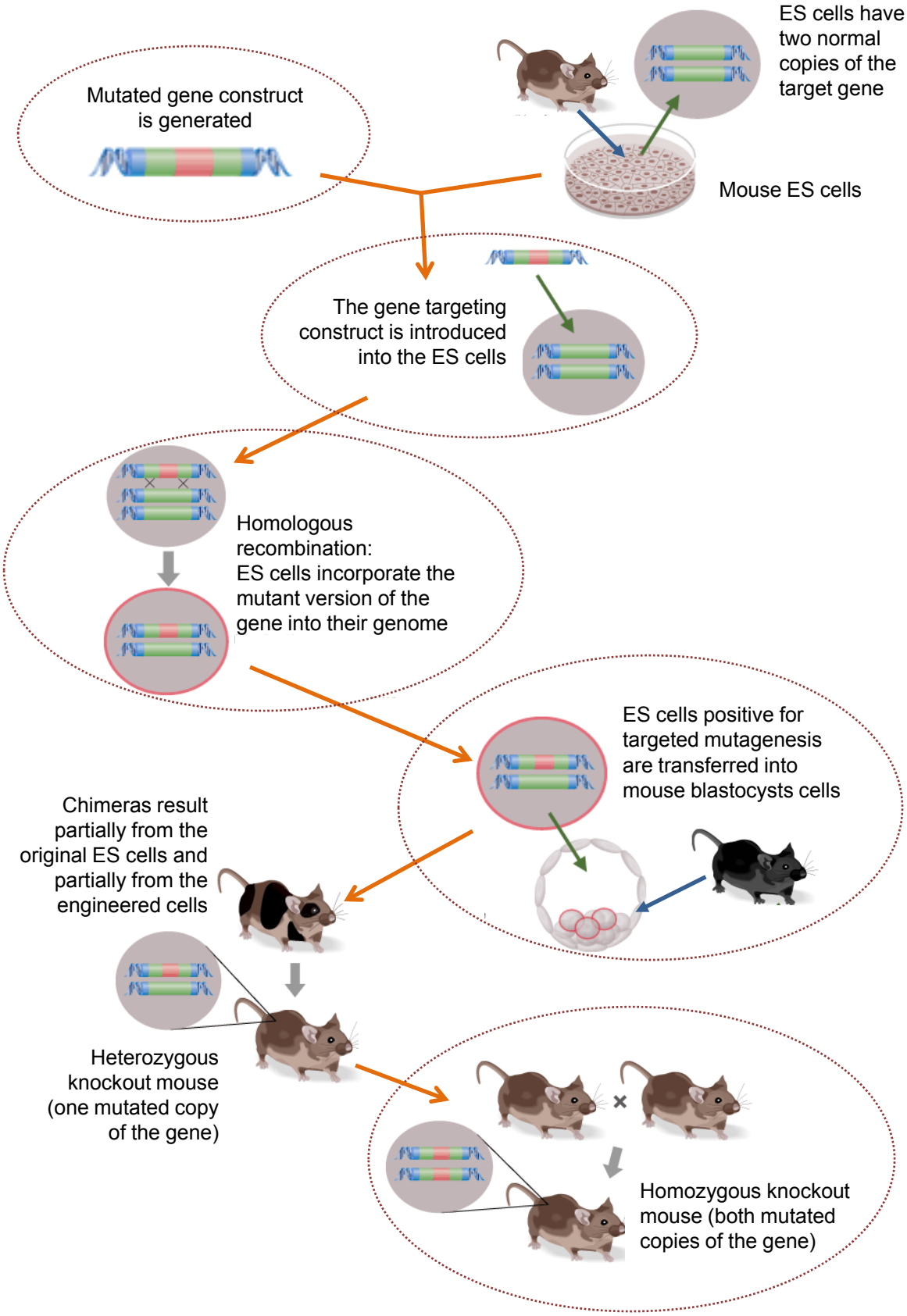
The main technique used to generate knockout mice is based on the concept of gene targeting by homologous recombination (Galli-Taliadoros et al., 1995; Tymms and Kola, 2001).

The usual protocol for generating knockout mice consists of several basic steps (Figure 3), although slight variations from this technique have been used by different researchers.

First, a gene targeting construct is developed, consisting of a mutated version of the gene of interest that makes it inoperable. This implies the availability of a genomic clone containing the gene of interest (Galli-Taliadoros et al., 1995), which is usually isolated from a mouse DNA library. The engineered construct has to be composed of at least three essential DNA elements: a marker gene that allows for the positive selection of the mutated genes (usually an antibiotic resistance gene, such as neomycin) and two homologous flanking sequences, one located upstream and the other downstream of the mutated gene, which enable targeted insertion into the genome.

Embryonic stem (ES) cells are isolated from mouse blastocysts and cultured *in vitro* under special conditions so that they are able to proliferate without undergoing

Figure 3. Generation of knockout mice: basic steps.



differentiation, enabling the ES cells to maintain their totipotent state (Tymms and Kola, 2001). The gene targeting construct is then introduced into the ES cells by electroporation, a process relying on electric current used to transport the DNA across the cell membrane. Some of the ES cells undergo homologous recombination, incorporating the mutated version of the gene into their genome in place of their wild-type gene. These ES cells can be separated from the ones that did not incorporate the construct through the use of the positive selection marker included in the mutated gene, usually by adding the corresponding antibiotic to the culturing media of the ES cells. In addition, negative selections markers can be used, such as the PGK-DTA (diphtheria toxin A-fragment gene) cassette which was shown to increase the recombination efficiency within the targeted locus (Yagi et al., 1990; Yu et al., 2000). Then, the ES cells are screened to differentiate between the actual gene targeting event and a background of random insertion of the mutated gene. The screening strategies often use PCR, Southern Blotting or a combination of both methods (Galli-Taliadoros et al., 1995).

In the next step, the ES cells positive for targeted mutagenesis are transferred into mouse blastocysts cells originating most commonly from mice with a different coat color than the line from which the ES cells were originally harvested, in order to allow the mutant gene to be followed. The resulting blastocysts contain two types of stem cells, the original ones and the newly mutated, each of them carrying genetic information for a different coat color. These blastocysts are inserted into the uterus of pseudo pregnant female mice that give birth, if successful, to chimeras, which are mice that partially result from the original stem cells and partially from the engineered stem cells and whose coats shows patches of both coat colors.

If the ES cells with the mutant gene contribute to the germline, the next generation of mice has one non-functional copy of the gene (heterozygote knockouts). A rate limiting step in producing a gene-knockout mouse strain is generation of chimeras that transmit

the desired gene mutation to subsequent generations (Galli-Taliadoros et al., 1995). Inbreeding the heterozygote knockout mice results in generation of mice possessing both mutated copies of the gene, known as homozygous knockouts or simply “knockout mice”.

These genetic engineered animal models are able to provide insights into the molecular mechanisms that underlie different diseases and may be also used for developing therapeutic approaches (Thyagarajan et al., 2003).

II. RATIONALE AND HYPOTHESIS

The PIP gene sequence is conserved across species (Mirels et al., 1998; Myal et al., 1994; Myal and Shiu, 2000; Osawa et al., 2004). As well, tissue specificity also appears to be generally conserved across species. Therefore, it has been hypothesized that the function of PIP protein is also conserved (Lee, 2000; Myal and Shiu, 2000).

Mouse PIP belongs to an intrachromosomal cluster of five active genes (also including SVA and SVAL1-3) sharing a common structure, whereas in non-rodent animals PIP seems to be a single functional gene (Osawa et al., 2004). Therefore, mPIP may display some unique characteristics when compared with its human homologue.

In healthy individuals, human PIP was identified at several ports of entry to the body, in saliva, tears, seminal plasma, amniotic fluid, submucosal glands of the lungs and sweat glands (Autiero et al., 1991; Mazoujian et al., 1983; Murphy et al., 1987b; Myal and Shiu, 2000), representing privileged immunological sites (Pasquinelli et al., 1999). In addition, it was found to bind to IgG and CD4 in human seminal plasma (Autiero et al., 1991; Chiu and Chamley, 2003) and to interfere with the interaction between the human immunodeficiency virus and lymphocytes, also inhibiting CD4+ T lymphocyte apoptosis (Gaubin et al., 1999). Human PIP binds to bacteria isolated from the human mouth, ear canal and skin (Schenkels et al., 1993; Schenkels et al., 1997). Therefore, possible immunomodulatory and host defense roles for this protein have been suggested.

Mouse PIP has been found to bind to oral bacteria; therefore a role of this protein in the non-immune host response though modulating the flora of the mouse oral cavity has been proposed (Lee et al., 2002). However, the consequences of this interaction have not been determined yet.

While the exact roles of mPIP, as like hPIP, are currently not known, we address the functions of this protein focusing on the **hypothesis that mPIP plays a role in a non-immune host defense mechanism, specifically through modulating the mouse resident oral flora.**

III. RESEARCH OBJECTIVES

The objectives of this study are to further the function of mPIP through both *in vivo* and *in vitro* studies, focusing on the possible role of this protein in non-immune host response through modulating the oral flora.

1. To investigate mPIP functions *in vivo*

1.1. To confirm the null mutation in a mPIP knockout mouse model, previously generated in our laboratory

1.2. To delineate the phenotype of the mPIP knockout mice, focusing on two particular specific aims and always using wild-type mice for comparison

a. To analyze the knockout mice from a morphopathological perspective

b. To examine the impact of the absence of mPIP on the mouse oral flora

2. To investigate mPIP functions *in vitro*

2.1. To obtain purified and biologically active mPIP in order to use it for further *in vitro* studies

2.2. To determine the physiological concentration of mPIP in mouse saliva in order to establish the amount of protein necessary for the protein-bacteria interaction studies

2.3. To determine the consequences of mPIP interaction with oral bacteria, specifically to demonstrate whether mPIP plays any role in bacterial aggregation

IV. MATERIALS AND METHODS

1. Animal housing

Female and male wild-type CD1 mice were purchased from the University of Manitoba Animal Breeding Facility. The animals were housed at the University of Manitoba Animal Care Facility, where all procedures were sanctioned in a protocol approved by the Animal Care Committee, under Canadian Council for Animal Care Guidelines. The mice were kept in plastic cages with wood-chip bedding and fed rodent chow and water *ad libitum*.

2. Generation of the mPIP knockout mouse model

A gene-targeting construct was prepared in which one of the four exons of the mPIP gene (exon 2) was replaced with the neomycin resistant gene, PGK-NEO cassette (Figure 4). The PGK-DTA (diphtheria toxin A-fragment gene) cassette was used as a negative selection marker. This construct was then transfected into mouse embryonic stem (ES) cells which were screened by Southern Blot hybridization using a 5' specific probe. ES cells positive for targeted disruption of the mPIP gene were expanded into mouse blastocysts that were implanted into properly prepared pseudopregnant female recipients, yielding chimeric mice. The chimeric mice demonstrating germline transmission were then used to produce heterozygotes and homozygotes for the mPIP gene mutation. A polymerase chain reaction (PCR) with mouse genomic DNA was used to routinely genotype KO mice using primers yielding different product sizes in the presence of the wild-type (395 bp) and mutant (289 bp) versions of the mPIP gene. The wild-type mPIP allele was detected using a forward primer (5'-ATCTTAGGTGACCCCTGTGA-3') located 5' to the deletion site of exon 2 and reverse

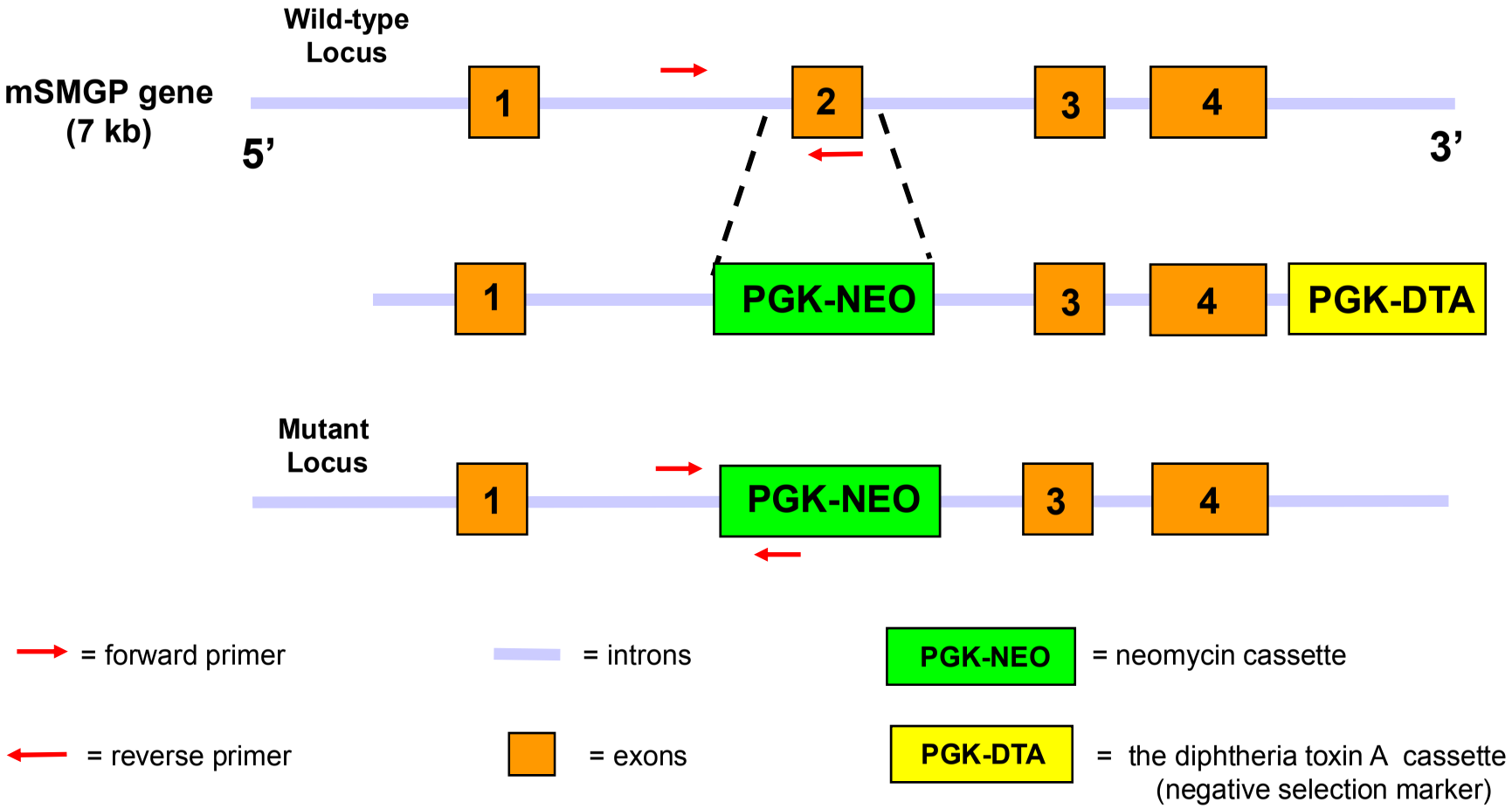


Figure 4. Generation and PCR detection strategy of the mPIP knockout mouse.
Exon 2 of the mPIP gene was replaced with the neomycin resistance cassette PCR was used to differentiate between the wild-type and mutant allele. Red arrows indicate the positions of forward and reverse primers. Expected product size: wild-type 395 bp, mutant 289 bp.

primer (5'-CCTGTGCTGTTGATGGAACA-3') located inside the region of exon 2. The mutant mPIP allele was detected using the same forward primer as the wild-type allele and a different reverse primer (5'-TAAAGCGCATGCTCCAGACT-3') located inside the neomycin gene.

Transfection of the construct into the ES cells, aggregation of mutated ES cells with blastocysts and generation of chimeric mice were done at the University of Connecticut, CT, USA. Generation of the gene targeting construct, screening for mutated ES clones, mouse genotyping by PCR and breeding of the chimeric mice were performed in our laboratory by the senior technician Ms. Anne Blanchard.

3. Mouse saliva and lacrimal fluid collection

The mice were anesthetized by intraperitoneal injection of 0.01-0.02 ml of 2.5% avertin per gram of body weight. The anesthetized mice were then injected subcutaneously with 0.01 ml of pilocarpine solution (0.5-1 mg/ml in PBS) per gram of body weight. After both drugs were administered, the mice were placed in a custom multi-animal holder (Figure 5). Mouse saliva was collected in ice-chilled tubes. Saliva samples were vortexed for 1 minute to reduce viscosity and centrifuged (10,000 X g) at 4ºC for 10 min to remove any debris. The supernatant was transferred into another tube and immediately stored at -20ºC until further analysis. In order to produce sufficient samples, repeated saliva collections were performed on the same animals, whenever possible.

During the saliva collection procedure, lacrimal fluid accumulated in the internal angle of the eyes of most the animals. This fluid was collected by careful suction using a pipette, designated as mouse tears and stored into plastic eppendorf tubes at -20ºC until further analysis.

Figure 5. Collection of mouse saliva using the multi-animal holder. Anaesthetized mice were injected with Pilocarpine to stimulate saliva secretion and placed on an approximately 45º inclined surface in a multi-animal holder, as shown. Mice were secured by the tails with tape. Mouse saliva was collected in ice-chilled glass tubes.

4. Tissue collection

Adult mice were sacrificed and selected tissues dissected out. Tissues used for protein extraction were placed into microcentrifuge tubes, immediately frozen and stored at -70°C. Tissues used for paraffin embedding were fixed overnight in 10% buffered formalin phosphate (Fisher Scientific, ON, Canada) and then stored in 70% ethanol until they were sent to Manitoba Breast Tumor Bank (Manitoba Institute of Cell Biology, Winnipeg, Manitoba) for embedding into paraffin blocks and histology.

5. Protein extraction from tissues

Frozen mouse tissue samples were weighed, cut into small pieces on aluminum foil covered dry ice using a scalpel and placed into 2ml microcentrifuge tubes on ice. Sample isolation buffer (SIB) containing 5% SDS, 50mM Tris-HCl (pH 6.8), 20mM EDTA, 5mM β-glycerophosphate and one Complete™ Mini protease inhibitor cocktail tablet (Roche Diagnostics, QC, Canada) per 10ml buffer was added to the sample. Between 200-300µl SIB was used for 50mg of tissue. Tissue samples were homogenized in the SIB buffer for 30 seconds on ice, followed by sonication twice for 15 seconds. The lysates were then centrifuged for 15 minutes at 13,000 x g at room temperature. The clear supernatants were collected into clean microcentrifuge tubes and stored at -20°C for further use.

6. Determination of protein concentration

The protein concentrations of the mouse tissue samples were determined by the BCA (bicinchoninic acid) protein assay using a Micro BCA™ Protein Assay Kit (Pierce, IL, USA) according to the manufacturer's instructions. Briefly, 500 µl of the Micro BCA™ working reagent was mixed in microcentrifuge tubes with 1 µl of diluted sample (1:500 in

double-distilled water). Different dilutions of bovine serum albumin (BSA) were prepared (range 0-20µg/ml) fresh each time from the BSA ampoules provided in the kit, mixed with Micro BCA™ working reagent at the same time as the samples and used as protein standards. The microcentrifuge tubes containing the mixtures of BCA reagent and samples or standards were incubated for 1h in a 60°C waterbath and then cooled to room temperature. 200µl of each sample and protein standard was then transferred in duplicate to a 96-well microplate and the absorbance was read at a wavelength of 562nm using a spectrophotometer connected to a computer equipped with SoftMax Pro software. The standard curve was generated and the concentration of each sample was calculated using the SoftMax Pro software.

7. The anti-mPIP rabbit polyclonal antibody

7.1. Generation and reconstitution of the "SMGP peptide"

Peptides composed of 20 amino-acids each were selected from the complete protein sequence of mPIP based upon the analyses of their predicted antigenicity, hydrophilicity, and surface accessibility in order to be used for antibody production. The specificity of the peptides was confirmed using the BLAST online program (http://www.ncbi.nlm.nih.gov/blast/). The peptide MAVVPITANRYYTYNTVRMN (position 127-146 aminoacids in mPIP sequence, gi: 46577671) was chosen as the most suitable for antibody production and designated as the "SMGP peptide". Both the peptide and the rabbit polyclonal anti-peptide antibody were commercially generated by Alpha Diagnostics Intl. Inc. (San Antonio, TX). A cysteine was added at the NH_2-terminus for coupling to carrier proteins via the free -SH group (small peptides must be coupled to a carrier protein in order to elicit high titer antibodies). The lyophilized peptide was 75%

pure. It was reconstituted in double-distilled sterile water supplemented with 1M NaOH, as directed by the manufacturer, until completely dissolved (final concentration 20mM NaOH). The 0.5mg/ml peptide solution was aliquoted and stored frozen at -20°C until further use.

7.2. Generation of the anti-mPIP polyclonal antibody

The peptide CMAVVPITANRYYTYNTVRMN, which will be referred to as the SMGP peptide, was generated by Diagnostics Intl. Inc. (San Antonio, TX) and used to immunize 2 rabbits in order to produce anti-mPIP polyclonal antibody. Fifteen ml of the pooled rabbit antiserum was subjected to affinity purification on a synthetic peptide coupled agarose column.

The affinity-purified antibody was supplied by Alpha Diagnostics Intl. Inc. (San Antonio, TX). This antibody was designated as mPIP Ab-2 and was used in this study for Western blot analysis and ELISA.

8. Western blot analysis

Frozen mouse tissue protein or mouse saliva samples were thawed and the desired amount of each sample was mixed 3:1 with 4 X SDS sample buffer into 0.5 ml microcentrifuge tubes and heated for 5 minutes at 100°C. The 4 X SDS sample buffer contained 500 mM Tris (pH 6.8), 40% glycerol, 8% SDS, 0.04% (w/v) bromophenol blue and 0.4M dithiothreitol (DTT). The standard size protein marker used was Precision Plus Protein Standard Dual Color (Bio-Rad Laboratories Inc., ON, Canada) containing proteins ranging between 10-250kDa.

The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12 or 15% separating gels (Table 2) under reducing

conditions (DTT, final concentration 0.1M) and the Tris-Glycine buffer system (Table 1), using a Mini-PROTEAN® 3 electrophoresis cell (Bio-Rad Laboratories Inc., ON, Canada). The proteins were transferred onto 0.45 µm nitrocellulose membrane (Bio-Rad) using a Mini-Trans-Blot cell (Bio-Rad). Following blocking the non-specific binding by incubating for 1h at room temperature or overnight at 4ºC in blocking solution consisting of 5% nonfat dry milk in TBST (Table 1), the blots were incubated for 1 h with mPIP Ab-2 diluted 1:4000 in blocking solution and washed 3 X 10 minutes with TBST. The blots were then incubated for 1 h with Goat anti-rabbit IgG HRP (horseradish peroxidase)-Conjugate (Bio-Rad Laboratories Inc., ON, Canada) at 1:10000 in blocking solution and washed again with 3 X 10 minutes TBST. Finally, the Western blots were developed utilizing the Pico Chemiluminescence substrate (Pierce Biotechnology, Rockford, IL) and exposed to photographic film (Kodak Biomax MR, Baie d'Urfe, QC).

9. Immunohistochemical analysis of mPIP

Paraffin embedded mouse submaxillary and lacrimal gland tissue was used for immunohistochemistry. Five µm sections were processed using the automated Discovery Staining Module at Manitoba Institute of Cell Biology, University of Manitoba. Briefly, a standard protocol was used and tissues were incubated for 60 minutes with the mPIP Ab-2 antibody at 1:4000 dilution followed by incubating 30 minutes with goat-anti rabbit immunoglobulin (Cedarlane, Hornby, ON) at 1:400 dilution. Positive staining for mPIP was assessed by light microscopy. H&E (hematoxylin and eosin) histological staining was also performed on serial sections of the mouse tissue in order to clearly identify the structures positive for mPIP staining.

Table 1. Solutions used for SDS-PAGE Western blot analysis.

STEP OF EXPERIMENT	SOLUTION	COMPOSITION	PREPARATION
Casting gels	4 X Separating Gel Buffer	1.5 M Tris 0.4% SDS pH 8.8	27.26g Tris Base 0.6g SDS Adjust pH to 8.8 and make up to 150ml with ddH_2O.
	4 X Stacking Gel Buffer	0.5 M Tris 0.4% SDS pH 6.8	6.07g Tris Base 0.4g SDS Adjust pH to 6.8 and make up to 100ml with ddH_2O.
Electrophoresis	5 X SDS-PAGE Running Buffer	125 mM Tris 960 mM Glycine 0.5 % SDS pH ~ 8.3	15g Tris Base 72g Glycine 5 g SDS Make up to 1L with ddH_2O and store at RT/4°C. Dilute 1:5 with ddH_2O and use in both electrophoresis cell chambers as 1 X SDS-PAGE Running Buffer.
Transfer	10 X Transfer Buffer	250 mM Tris 1.92 M Glycine pH ~ 8.3	60.6g Tris Base 288g Glycine Make up to 2L with ddH_2O and store at RT.
	1 X Transfer Buffer	25 mM Tris 19.2 M Glycine 20% Methanol pH ~ 8.3	100ml 10 X Transfer Buffer 200ml Methanol 700ml ddH_2O Store at 4°C and use cold.
Washing and diluting antibodies	10 X TBS	200mM Tris 1.37M HaCl pH 7.6	48.4g Tris Base 160g NaCl Make up to 2L with ddH_2O and store at RT.
	1 X TBST	20 mM Tris 137 mM NaCl 0.5% Tween 20 pH 7.6	200ml 10 X TBS 1 ml Tween 20 Make up to 2L with ddH_2O and store at RT.

SDS = sodium dodecyl sulfate
ddH_2O = double-distilled water
RT = room temperature
TBS = Tris-buffered saline
TBST = TBS Tween 20

Table 2. Protocol used for preparing different concentrations of protein electrophoresis gels.

Quantities are calculated for two 0.75mm gels.
APS = ammonium persulphate (10% made fresh every week and stored at 4°C)
Separating and Stacking Gel Buffers: see Table 1

SEPARATING (RESOLVING) GEL				STACKING GEL	
	10%	**12%**	**15%**	**4%**	
4X Separating Gel Buffer	2.25 ml	2.25 ml	2.25 ml	4X Stacking Gel Buffer	750 µl
30% Polyacrylamide	3.0 ml	3.6 ml	4.5 ml	30% Polyacrylamide	400 µl
ddH2O	3.7 ml	3.1 ml	2.2 ml	ddH2O	1832 µl
10% APS	45 µl	45 µl	45 µl	10% APS	15 µl
TEMED	5µl	5µl	5µl	TEMED	3 µl

10. Isolation of mPIP

10.1. Production of recombinant mPIP in *E.coli*

10.1.1. Determining the signal peptide cleavage site

The location of the signal peptide cleavage site in the mPIP amino acid sequence was predicted using the SignalIP 3.0 online server (http://www.cbs.dtu.dk/services/SignalP, accessed September 2007).

10.1.2. Cloning and amplification of the mPIP cDNA

The cDNA corresponding to the secreted region of mPIP was amplified by PCR and the amplified product was cloned into the pET28a expression plasmid. The complete mPIP cDNA previously cloned into the same expression plasmid was used as a template for the PCR reaction. The 5' PCR amplification primer was 5'-GAGGCT**CC*ATG*G**GCCATCATCATCATCATCACGGCAGCGGCCAGGATGATGAAAA TGTCCGAAAG-3' which adds a *Nco*I restriction site (indicated in bold; also provides the Met initiation codon ATG indicated in italics) to the 5' end as well as a $(His)_6$ tag (underlined) to the N-terminal region of the resulting protein. The 3' PCR amplification primer was 5'-GTACC**GGATCC**TTAATTCATTCGCACAGTATTATA- 3' which added a *Bam*HI restriction site (indicated in bold) to the 3' end. Proofreading DNA polymerase, *Pfx* (Invitrogen, Burlington, ON), was used for the PCR reaction.

Both the amplified product and the expression plasmid pET28a were digested with the *NcoI* and *BamHI* restriction enzymes and then purified by agarose gel electrophoresis followed by extracting the DNA from the gel using the QIAquick® Gel Extraction Kit (QIAGEN Inc, Mississauga, ON). The DNA concentration of both plasmid

and PCR product (insert) was determined and the fragments were ligated together overnight at 14°C.

The resulting construct consisting of the pET28a plasmid with the mPIP cDNA inserted into the *NcoI* and *BamHI* sites was transformed into competent DH5α *E.coli* cells for amplification, as follows. Briefly, 50µl aliquots of frozen DH5α competent cells were thawed on ice for 30 minutes; the plasmid was added and incubated for 30 minutes on ice. The cells were then heat-shock treated for 45 seconds at 42°C and then placed on ice for another 2 minutes. One hundred µl of Difco™ LB (Luria Berthani) Broth (BD, Sparks, MD, USA) per transformation reaction was added to the cells which were then incubated for 1 h at 37°C with shaking.

The bacteria were plated on LB agar plates supplemented with kanamycin (30µg/ml) to select cells with incorporated plasmids and cultured overnight at 37°C. The plates were examined the next day and 18 single colonies were picked with sterile wooden toothpicks. Each colony was inoculated into 5ml LB broth supplemented with kanamycin (30µg/ml) and grown overnight at 37°C with shaking. The 18 bacterial cultures were designated as clones 1-18. Plasmids were isolated from the 18 bacterial clones using QIAprep® Spin Miniprep Kit (Cat# 27104, QIAGEN Sciences, Maryland, USA) and the plasmid DNA was analyzed by restriction enzyme digestion using *NcoI* and *BamHI,* followed by agarose gel electrophoresis.

10.1.3. Nucleotide sequence analysis

Three clones were randomly chosen from the ones that showed the expected restriction site pattern, the DNA concentration was determined and the appropriate amount of each clone (specifically clones 1, 10 and 18) was verified by sequencing (Robarts, London, Ontario). The results were compared with the nucleotide sequence of

the mPIP gene (gi:22135641) by using CLUSTAL W (1.83) multiple sequence alignment online tool (http://www.ebi.ac.uk/Tools/clustalw/, accessed September 2007).

In addition, the nucleotide sequence corresponding to the 131 amino-acids of the $(His)_6$-tagged mPIP protein was screened for the presence of *E. coli* underrepresented codons, known as rare codons.

10.1.4. Expression of recombinant mPIP in bacteria

The Rosetta 2(DE3) *E. coli* strain was chosen for protein expression because the cells carry a chloramphenicol-resistant plasmid with the tRNA genes that decode seven codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG) that are rarely used in *E. coli*.

Plasmid DNA isolated from clones 1 and 10 was used for transformation of aliquots of Rosetta 2 (DE3) competent cells (Cat# 71400-3, Novagen, EMD Biosciences Inc., USA), all procedures being performed according to the manufacturer's instructions. Transformed cells were cultured overnight at 37°C on LB agar supplemented with antibiotics (chloramphenicol 34µg/ml and kanamycin 30µg/ml) for selection of cells with incorporated plasmid.

One isolated bacterial colony was picked with a sterile wooden toothpick and used for inoculating 10ml LB broth supplemented with antibiotics (chloramphenicol 34µg/ml and kanamycin 30µg/ml) and cultured overnight (16h) at 37°C with shaking. The next day, the overnight bacterial culture was used to inoculate 90ml LB broth supplemented with the same antibiotics; the bacteria were cultured for approximately 2h at 37°C with shaking until they reached an OD_{600nm} of 0.5-0.6. The culture was divided into 5 tubes (10ml/tube) and protein expression was induced using different amounts of Isopropyl β-D-1-thiogalactopyranoside (IPTG), one tube was used as a control without IPTG. The IPTG treatment was performed while vigorously shaking the bacterial culture, at 37°C or room temperature, using 0.1mM or 1mM IPTG for each temperature.

Total cell protein fraction was extracted using 1 X SDS sample buffer (containing 4 X SDS sample buffer diluted 1:4 in ddH_2O, see the Western blot analysis section) from 1ml of each of the 5 bacterial cultures. The samples were separated by SDS-PAGE on a 15% separating gel, followed by Western blot analysis, following the protocol previously described.

10.1.5. Purification of recombinant mPIP from bacteria

Purification of $(His)_6$-tagged mPIP protein under native conditions was performed from one cell pellet resulting from 9ml overnight bacterial growth culture (protein expression induced with 0.1mM IPTG for 2.5h at room temperature) using the Ni-TED Protein Purification Spin Columns commercial kit with solutions (Cat# 98025, Active Motif, Carlsbad, CA, USA). All procedures were performed according to the manufacturer's instructions. Small aliquots of all the fractions collected during the procedure (specifically, the cleared lysates designated as "load", the flow through, the washes and the elutions) were saved and together with the insoluble portion of the cells were analyzed by SDS-PAGE on a 15% separating gel, followed by staining the gel with Coomasie blue or Western blot analysis, following the protocols previously described.

10.2. Purification of mPIP from mouse saliva by immunoaffinity chromatography

10.2.1. Preparing the protein A bead – antibody affinity column

Immunoaffinity purification of mPIP was performed using a column consisting of coupled mPIP Ab-2 with protein A agarose beads, (Immobilized Recomb® Protein A agarose beads, Pierce, Rockford, IL) using the protocol of Harlow and Lane (1988).

Briefly, 2ml anti-mPIP polyclonal antibody was mixed with 2ml of a 50% protein A agarose beads slurry and incubated for 1h at room temperature with gentle rocking. The beads were washed twice with 10ml 0.2M sodium borate (pH 9) and resuspended in 10ml 0.2M sodium borate (pH 9). The cross-linker DMP (dimethyl pimelimidate) (Pierce, Rockford, IL) was added to the beads to a final concentration of 20mM and incubated for 30min at room temperature with gentle rocking. The beads were then washed once in 10ml 0.2M ethanolamine (pH 8) and incubated for 2h at room temperature in 0.2M ethanolamine (pH 8), followed by centrifugation and resuspension in PBS to a final volume of 2ml. The efficiency of coupling was determined by boiling samples of beads taken before and after DMP cross-linking in 4 X SDS sample buffer, followed by SDS-PAGE electrophoresis on a 15% separating gel which was then stained with Coomasie blue. The coupled beads were stored at 4°C until further use.

10.2.2. Affinity chromatography

Protein A beads coupled with mPIP Ab-2 (0.5ml) were used to prepare an affinity chromatography column. The column was washed with 5ml 100mM Glycine (pH 2.5), and then equilibrated with PBS (2 x 5ml). Pooled mouse saliva, diluted 1:4 in PBS to reduce viscosity (14ml total), was then loaded and allowed to flow slowly thorough the column. The column was then washed with PBS (14ml) and the protein (antigen) bound to the column was eluted with 100mM Glycine (pH 2.5). Five 1ml elutions were collected separately, using 200µl 1M Tris (pH 8) per elution to neutralize the pH. The column was then washed with 10mL PBS and stored for further use at 4°C in PBS with 0.1% sodium azide as a preservative.

Fractions collected during the procedure (specifically the flow through, washes and elutions) were analyzed by SDS-PAGE on a 15% separating gel, followed by either

Coomasie staining of the gel or Western blot analysis, following the protocols previously described.

10.3. Recombinant mPIP protein from Abnova

Recombinant mouse PIP protein (Figure 6) produced in the wheat germ cell-free translation system was commercially obtained (Abnova Corporation, Taipei, Taiwan).

11. ELISA (enzyme-linked immunosorbent assay)

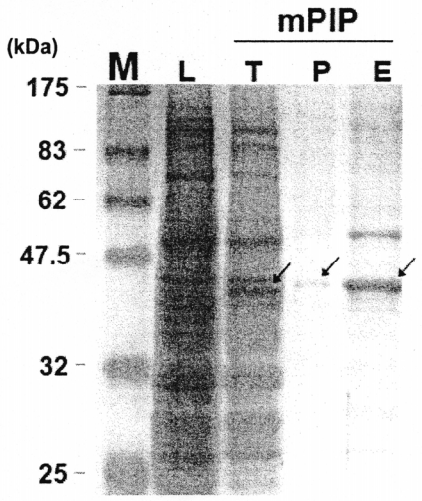
11.1. Development of indirect ELISA for salivary human PIP

Flat bottom 96-well immunoassay plates (Nalge Nunc International Rochester, NY) were coated with human saliva diluted 1:1000 in coating buffer (0.05M carbonate buffer, pH 9.6, 0.02% NaN_3). After overnight incubation at 4° C, the fluid was drained out and the plates were washed in PBS-Tween (phosphate-buffered saline, pH 7.4, 0.1% Tween 20). Blocking of non-specific binding was achieved by incubating 1 h in a blocking solution consisting of 0.1% BSA (bovine serum albumine) in PBS-Tween. After washing, rabbit polyclonal anti-PIP antibody at a dilution 1:1000 in blocking solution was added into the wells and incubated at room temperature for 2h with shaking. The plates were then washed again and subsequently incubated for 1h with HRP labeled swine anti-rabbit immunoglobulins (Dako, Denmark) diluted 1:1000 in blocking solution. After incubating and washing, OPD (o-Phenylenediamine Dihydrochloride) peroxidase substrate solution (SIGMA *FAST*™ OPD Tablets, Sigma-Aldrich, MO, USA) was added to the wells. The plates were incubated for 30 min at room temperature in the dark and then analyzed on a multi well plate spectrophotometer at 450 nm.

Figure 6. Specification sheet of recombinant mPIP protein produced *in vitro* in a wheat germ cell translation system.
This protein was produced by Abnova Corporation, Taipei, Taiwan.

Product Information			
Job Number	**MM931000543**	Gene Name	Mpip
Lot Number	UMN0041025JB01	Product Number	UMN0G438E
Storage Condition	-80℃	Tag	GST
Expression System	CFS* wheat germ cell free in vitro	Elute Buffer	Tris-HCl + Glutathione, pH=8.0
cDNA Source	Mouse	Molecular Weight	44.9 kDa (GST tag: 26 kDa)
Quantity	~10 ug	Notice	**Avoid repeated freezing and thawing**
Volume	182 ul	Concentration	0.055 ug/ul
Shipping type	(1) The protein was put at **-80**℃ for storage before shipping. (2) Tube x 1. (3) We put the tube in a piece of Styrofoam with ice pack (It is frozen in the -80℃ freezer at least 78 hours before shipping.), and than put it within the big Styrofoam box. Final, we put the big Styrofoam box into a carton.		

Final-QC Report



10ug/182ul (0.055ug/ul)
Total: ~10 ug
***** Mpip protein is recognized by anti-GST on western blot.**

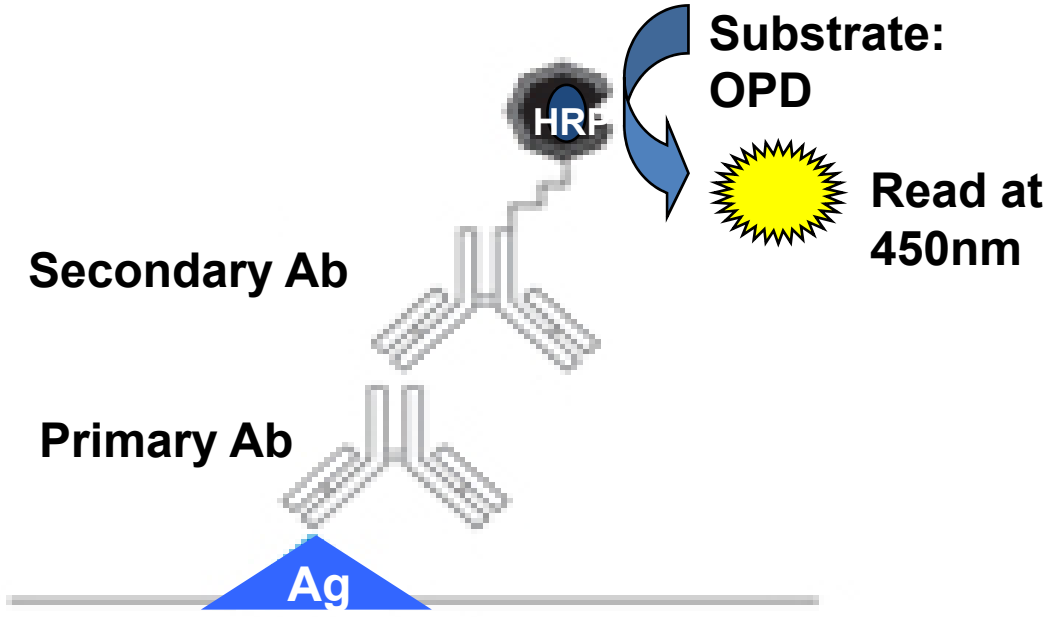
The standard curve was generated using purified native hPIP, isolated from aliquots of human saliva. The purified hPIP protein, the frozen samples of human saliva and the rabbit polyclonal anti-human PIP antibody were generously provided by Dr. Robert PC Shiu (Department of Physiology, University of Manitoba).

11.2. Development of indirect ELISA for salivary mouse PIP

Flat bottom 96-well immunoassay plates (Nalge Nunc International Rochester, NY) were coated with mouse saliva diluted 1:1000 in coating buffer (0.05M carbonate buffer, pH 9.6, 0.02% NaN_3). After overnight incubation at 4° C, the fluid was drained out and the plates were washed in PBS-Tween (phosphate-buffered saline, pH 7.4, 0.1% Tween 20). Blocking of non-specific binding was achieved by incubating 1h in a blocking solution consisting of 0.1% BSA (bovine serum albumine) in PBS-Tween. After washing, mPIP Ab-2 diluted 1:1000 in blocking solution was added into the wells and incubated at room temperature for 2h with shaking. The plates were then washed again and subsequently incubated for 1h with HRP labelled swine anti-rabbit immunoglobulins (Dako, Denmark) diluted 1:1000 in blocking solution. After incubating and washing, OPD peroxidase substrate solution (SIGMA *FAST*™ OPD Tablets, Sigma- Aldrich, MO, USA) was added to the wells. The plates were incubated in the dark for 30min at room temperature and analyzed on a multi well plate spectrophotometer at 450nm.

The standard curve was generated using 0.5-15ng/ml recombinant mPIP (Abnova Taiwan Corporation, Taipei, Taiwan). A diagram illustrating the experimental design of the indirect ELISA used for both human and mouse PIP is shown in Figure 7.

Figure 7. A schematic illustration of the indirect ELISA used for quantification of PIP in human and mouse saliva.
The plates were coated with antigen (Ag) represented by human/mouse saliva diluted in coating buffer or with known amounts of pure protein as standard. Plates were then incubated with primary antibody (anti-hPIP or mPIP Ab-2) followed by incubation with secondary antibody (anti-rabbit IgG, HRP-labelled). OPD peroxidase substrate solution was then added into the wells and after allowing the color reaction to develop for 30 minutes, the absorbance of each wells was analyzed on a spectrophotometer at 450nm.



11.3. Development of competitive ELISA for salivary mouse PIP

11.3.1. Determination of SMGP peptide optimal coating concentration

The optimal coating concentration of the SMGP peptide was determined by indirect ELISA, performed similarly to the method used for quantification of salivary human PIP.

Flat bottom 96-well immunoassay plates (Nalge Nunc International Rochester, NY) were coated with the SMGP peptide, at concentrations ranging between 0.1-1600 ng/100µl. The dilutions of mPIP Ab-2 and Swine anti-rabbit IgG HRP-conjugated secondary antibody (DAKO, Denmark) were fixed at 1:4000 and 1:1000 respectively. The mean absorbance measured at 450nm obtained from duplicates each standard was plotted against its concentration in ng/ml.

The concentration of SMGP corresponding to the mid-point of the linear part of the curve was considered to be the optimal coating concentration of peptide to be used in the competition ELISA.

11.3.2. Determination of the anti-mPIP antibody working dilution

The optimal primary antibody dilution to be used in the competitive assay was determined by indirect ELISA, performed similarly to the method used for quantification of salivary human PIP.

The wells were coated with the pre-determined optimal concentration of the SMGP peptide. After blocking 2h with blocking solution, the wells were incubated for 1h with serial dilutions of mPIP Ab-2 ranging from 1:64000 to 1:125, followed by incubation with swine anti-rabbit IgG HRP-conjugated secondary antibody (DAKO, Denmark) at a fixed 1:1000 dilution. The absorbance measured at 450nm was plotted against the dilution of the anti-mPIP antibody.

The antibody dilution corresponding to the mid-point of the linear part of the curve was considered as the optimal dilution to be used in the competition ELISA.

11.3.3. Competitive ELISA for quantification of salivary mPIP

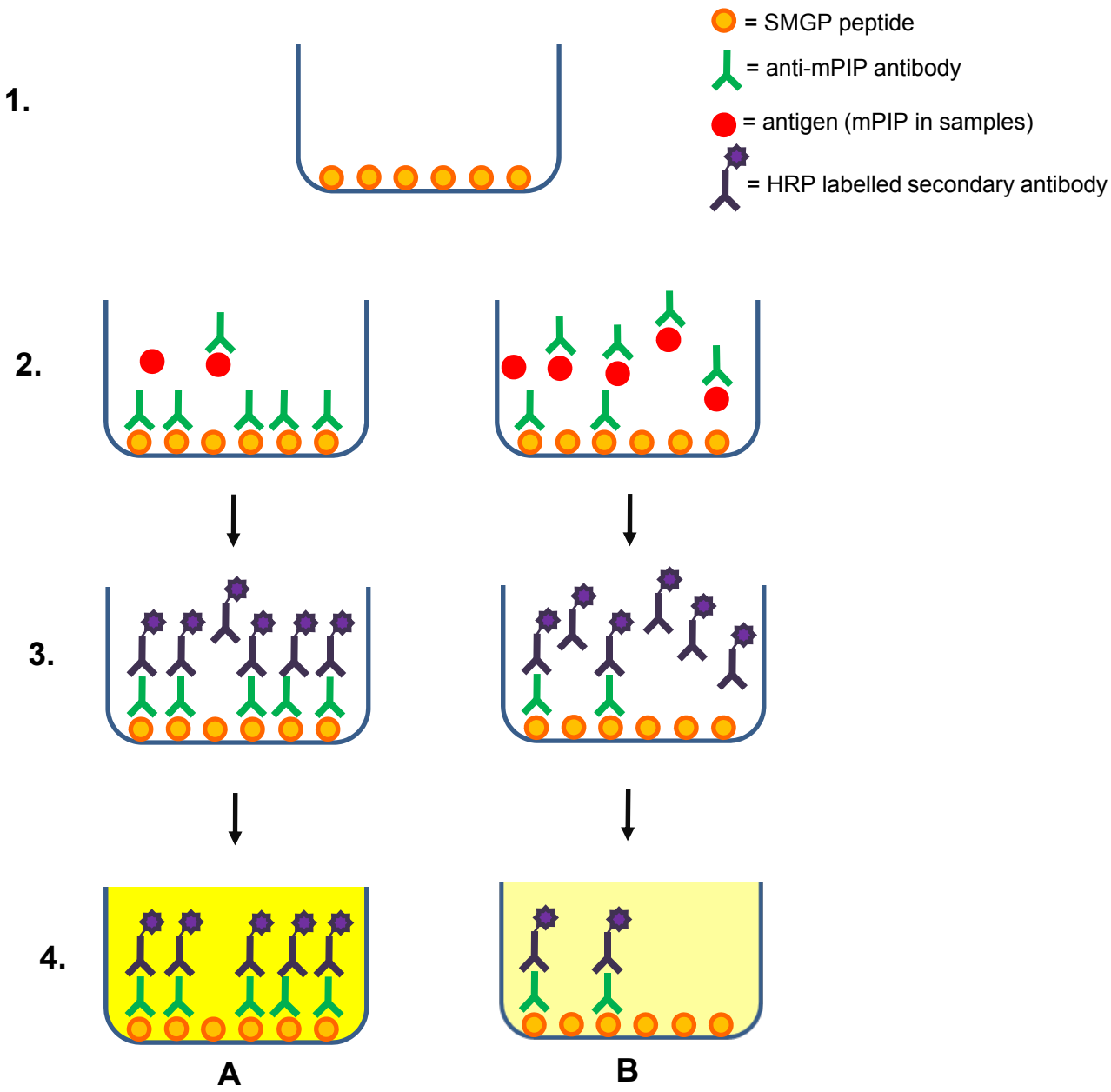
Flat bottom 96-well immunoassay plates (Nalge Nunc International Rochester, NY) were coated with 50ng of the SMGP peptide per well diluted in 100µl of the coating buffer (0.05M carbonate buffer, pH 9.6, 0.02% NaN_3). Following incubation at 4° C overnight, the fluid was drained out and the plates were washed in PBS-Tween (phosphate-buffered saline, pH 7.4, 0.1% Tween 20).

Blocking of non-specific binding was achieved by incubating for 1h in a blocking solution consisting of 1% BSA (bovine serum albumine) in PBS, followed by washing twice in PBS-Tween. The competition reactions between mPIP Ab-2 (final dilution 1:4000) and different dilutions of mouse saliva (or different amounts of recombinant mPIP as standards) were made in the wells by first adding the antibody, followed by the competitor, both diluted with blocking solution. The plates were incubated with the competition reactions for 1h. The plates were then washed again and subsequently incubated for 1h with HRP labeled swine anti-rabbit immunoglobulins (Dako, Denmark) diluted 1:1000 in blocking solution. All the antibody incubations, blocking and washings were performed on a plate shaker at room temperature.

Finally, OPD (o-Phenylendiamine Dihidrocloride) peroxidase substrate solution (SIGMA *FAST*™ OPD Tablets, Sigma-Aldrich, MO, USA) was added in the wells. The plates were incubated at room temperature in the dark for 30min and analyzed on a multi well plate spectrophotometer at 450nm**.** A diagram illustrating the experimental design of the competitive ELISA used mouse PIP is shown in Figure 8.

Figure 8. A schematic illustration of the competitive ELISA used for quantification of mPIP.

1. The wells were coated with equal amounts of SMGP peptide.
2. The primary antibody (mPIP Ab-2) was mixed with samples containing the antigen (mPIP protein) in the wells (sample A contains a lower amount of antigen than sample B).
3. Secondary antibody (HRP labelled) was then added into the wells.
4. OPD peroxidase substrate solution was added and the developed plate was then analyzed using a spectrophotometer . As expected, samples containing a lower amount of antigen (sample A) produced a more intense color reaction compared with samples containing more antigen (sample B).



12. Slot blot analysis

Quantitative slot blot analysis of mPIP protein in mouse saliva was performed using a Minifold® II Slot-Blot System (Scheliecher @ Schuell, Inc.,Keena, NH). Briefly, 0.25µl of frozen mouse saliva was diluted in PBS to a final volume of 100 µl and was denatured by boiling for 5 minutes. Different amounts of SMGP peptide, 0.625-20µg/slot, were used as standards and were treated the same way as the mouse saliva samples. The nitrocellulose membrane (0.45 µm, Bio-Rad Laboratories Inc., ON, Canada) was soaked in PBS for 5 min before being assembled into the apparatus. Each sample was analyzed in duplicate. The membrane was then removed from the system and treated as previously described in the "Western blotting" section. Briefly, blocking of non-specific binding was performed by incubation for 1h at room temperature in 5% nonfat dry milk in TBST. Then, the blot was incubated for 1 h with the mPIP Ab-2 diluted 1:4000 in blocking solution followed by washing (3 X 10 minutes in TBST) and then incubated for 1 h with Goat Anti-rabbit IgG HRP-conjugate (Bio-Rad) at 1:10000 in blocking solution and washed again (3 X 10 minutes in TBST). Finally, the blot was developed utilizing the Pico Chemiluminescence substrate (Pierce Biotechnology, Rockford, IL) and exposed to photographic film (Kodak Biomax MR, Baie d'Urfe, QC).

The resulting image was scanned and densitometry analysis was carried out on each peptide standard and mouse saliva sample. A standard curve was generated and the averaged values from the duplicate samples were plotted against the standard curve.

13. Morphopathological analysis of the mPIP knockout mice

Four 3-month-old male mPIP knockout mice together with wild-type controls were examined blindly for morphological and pathological changes by Dr Geoff Ward, (Samuel Lunderfeld Research Institute, Toronto, Ontario). Selected tissues were

dissected out, embedded in paraffin and histological analysis was performed. Both paraffin blocks and standard histological slides stained with H&E were received by our laboratory together with a detailed report.

14. Analysis of the mouse oral flora

14.1. Animals and sample collection

Sixteen adult mice (14 weeks-old; 4 wild-type and 4 mPIP knockout mice of each gender) were housed together in the same environment, with 4 mice/cage (2 wild-type and 2 mPIP knockout mice of the same gender). The mice were anesthetized by exposure to isoflurane. Sterile swabs (Calgiswab® type 1, Spectrum Laboratories, Dallas, TX) were used to swab the inside of each mouse's oral cavity, including under the tongue, without contamination from the lips. Each swab was placed into a sterile plastic tube containing 1 ml of Bacto™ Todd Hewitt Broth (Difco™, Becton Dickinson, MD, USA). The aluminum shaft of the swab was cut to allow capping of the tube. The tubes were mixed by vortexing to disintegrate the calcium alginate.

The animals were carefully monitored after the procedure and upon recovery were immediately returned to their cages. Male and female mice were studied as two distinct groups. Each sample collection procedure was performed on the same day on all the animals in a specific group.

14.2. Culturing the mouse oral bacteria

Serial dilutions of each suspension obtained from swabbing the mouse oral cavity were made in 1:10 and 1:100 ratios in Bacto™ Todd Hewitt Broth (Difco™, Becton Dickinson, MD, USA). Forty µl of each dilution, including the non-diluted suspension,

were plated on a blood-agar plate using a Spiral Plater Model C (Spiral System Instruments, Cincinnati, OH). The plates were incubated at 37ºC in a candle jar for 72h. The blood agar plates were made with blood agar base no. 2 (Oxoid, Hampshire, England), supplemented with 1% laked horse blood (Oxoid, Hampshire, England).

14.3. Quantitative analysis of the mouse oral bacteria

The most suitable dilutions were chosen for counting the colony forming units (CFUs). Two 1/8 diametrically opposite sectors were delineated on each plate, the CFUs observed on each of the sectors were counted and the average of the two values was calculated and multiplied by the dilution factor. For each animal, the number of CFUs/1ml suspension was determined.

Three successive sample collections, followed by counting of the CFUs were performed on each of the male and female group of animals. The males were analyzed at the 8, 9 and 11 months of age whereas the females were analyzed at 9, 11 and 13 months of age.

Statistical analysis was performed using GraphPad PRISM®, Version 3.02. A two-way repeated measure ANOVA analysis was performed on the results obtained from each group. In addition, the Student t test analysis was used to analyze the differences between the wild-type and mPIP knockout mice for each time point.

14.4. Qualitative analysis of the mouse oral bacteria

14.4.1. Sampling method, and isolation of pure strains

Qualitative analysis of the mouse oral bacteria was performed on the male and female mouse groups, aged 8 and 9, months respectively. Stratified sampling was used to select 16-18 single colonies from the same plates used for counting the CFUs.

Each bacterial colony was grown individually on blood agar for 24-48h in a candle jar. After 2 more consecutive rounds of sub-culturing from single colonies, the strains which grew were considered pure and were assigned codes, all of them starting with the letter 'M' followed by the mouse tag number and the strain number. Each strain was stored frozen at -80ºC in 1.5% BBL® Trypticase Soy Broth without Dextrose (Becton Dickinson Microbiology Systems, Cockeysville, MD) containing 30% glycerol.

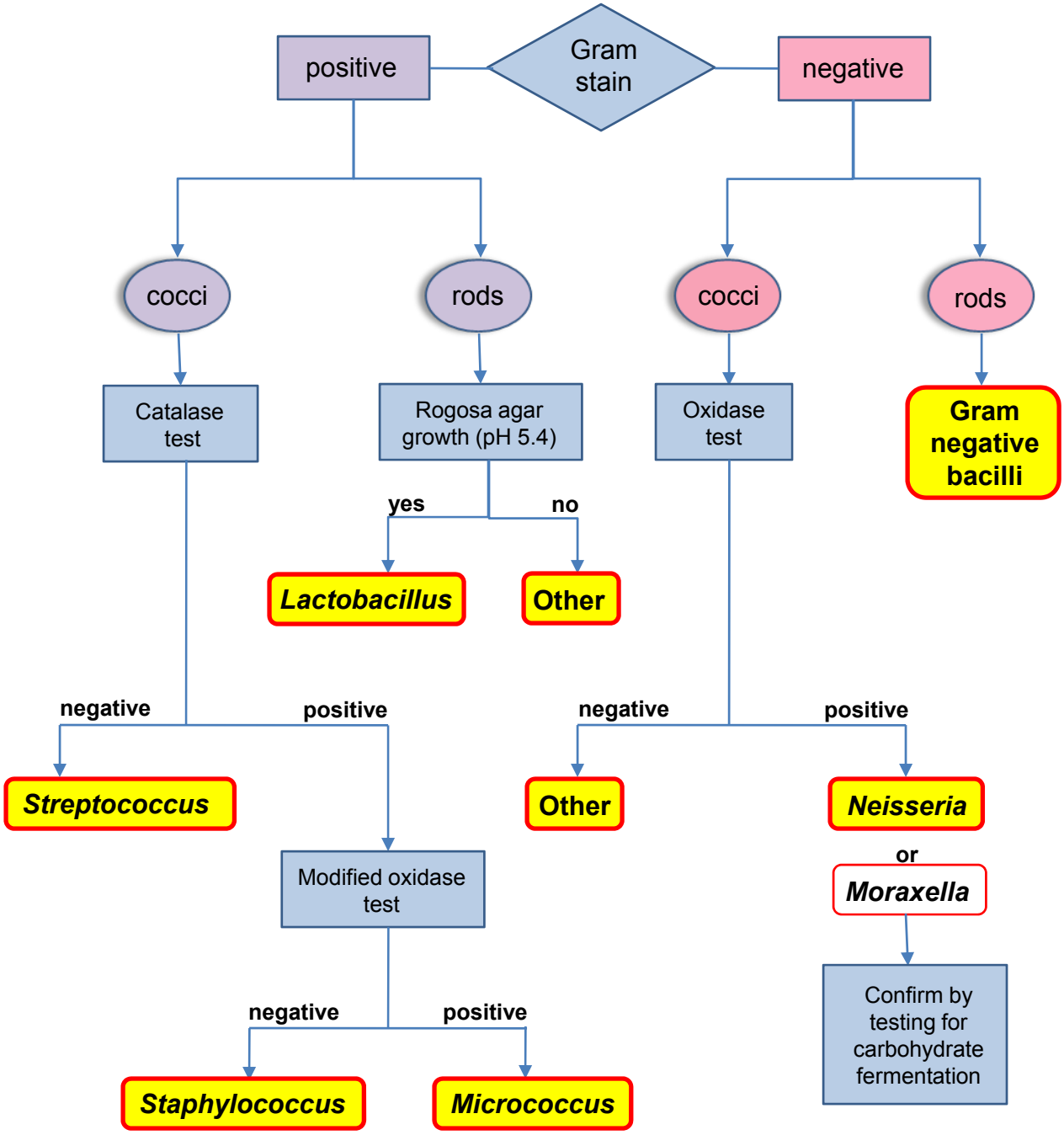
Standard microbiology techniques, such as Gram staining and biochemical tests were used to identify each strain of bacterial isolate. The algorithm used for bacteria identification is illustrated in Figure 9.

14.4.2. Identification of bacteria

14.4.2.a. Hemolysis

Bacteria were cultured on blood agar plate overnight in a candle jar. The presence of hemolysis was observed as discoloration of the blood agar surrounding the bacterial colonies. Alpha-hemolysis is a greenish discoloration, whereas beta-hemolysis is indicated by a zone of clearing of the blood agar.

Figure 9. The algorithm used for identification of mouse oral bacteria by genera.



14.4.2.b. Gram staining

A loop of bacterial cells cultured overnight on blood agar plate in a candle jar was placed on a microscope slide and heat fixed by passing the bottom of the slide through the tip of the burner flame several times (1s duration each time). The primary stain, crystal violet, was poured on the slide and incubated for approximately 30 seconds, followed by washing with water. Grams's iodine (Lugol) was then added to the slide and incubated for 1 minute. The slide was then rapidly washed with acetone for decolorization and rinsed with water. Counterstain, diluted carbol fuchsin (1:200 in distilled water), was applied and incubated for 30 seconds, followed by the final rinse with water. The excess water was removed and the slide was quickly dried by brief exposure to an open flame.

The slides were analyzed on a binocular microscope equipped with an 100X objective with an oil immersion lens. The cell shape and color were recorded. Gram-positive cells retain the primary stain and appear dark violet, whereas gram-negatives loose the primary stain, admit the counterstain and appear red-pink.

14.4.2.c. Catalase test

The catalase test was used to identify bacteria which produce catalase, an enzyme that converts hydrogen peroxide to water and oxygen. A standard loopful of bacteria was placed on a glass slide and a drop of hydrogen peroxide (30%) was added to the cells. The formation of bubbles due to oxygen production indicated a positive reaction.

This test was used to differentiate between Gram positive cocci, to differentiate the genus *Streptococcus* which is catalase negative, from *Staphylococcus* and *Micrococcus*, which are catalase-positive.

14.4.2.d. Identification of genus *Lactobacillus*

Lactobacilli are Gram positive rod-shaped bacteria, usually non-hemolytic. All bacterial strains with these characteristics were cultured on RogosaSL agar plates (Difco Laboratories, Detroit, MI, USA) for 72h in a candle jar at 37°C. These agar plates have a low pH (pH 5.4) and constitute a selective media for lactobacilli, which are acidophilic bacteria.

14.4.2.e. Modified oxidase test

The modified oxidase test is a method used for separating Gram positive, catalase positive cocci, distinguishing between staphylococci (except *Stapylococcus sciuri*) and micrococci based on their ability to produce cytochrome c oxidase (Faller and Schleifer, 1981).

A standard loop of bacteria was used to collect bacteria cultured on blood agar plate and smear it on Whatman No. 1 filter paper. One drop of a 6% solution of TMPD (tetramethylphenylendiamine) in DMSO (dimethyl sulfoxide) was added onto the bacterial smear. Bacterial smears that turned dark blue within 2 minutes were positive whereas smears negative for the test, remained colorless.

The bacterial genus *Micrococcus* yields a positive result, whereas *Staphylococcus* yields a negative result.

14.4.2.f. Oxidase test

The oxidase test determines whether gram-negative bacteria produce cytochrome c oxidase. This test is used for preliminary identification of *Neisseria* and *Moraxella* genera, which are both gram-negative and oxidase-positive cocci.

A standard loop of bacteria was used for collecting bacteria cultured on blood agar plate and smearing it on an Oxidase Test disk (Sigma-Aldrich, ON, Canada). Oxidase-

positive bacteria turns the disk deep purple blue within 2 minutes whereas the disk remains white with oxidase negative bacteria.

14.4.2.g. Identification of the genus *Neisseria*

Gram-negative, oxidase-positive cocci were tested for their ability to ferment different carbohydrates in order to differentiate between *Neisseria* and *Moraxella*. The organisms were cultured overnight at 37°C on agar plates supplemented with glucose, maltose, sucrose or lactose and containing a pH indicator. Organisms which ferment the specific carbohydrate in the medium will lower the pH due to the production of acids, changing the color of the agar from violet to yellow. The ability for each strain to ferment each of the 4 carbohydrates was determined and the pattern was compared with previously published data (Knapp, 1988).

15. Bacterial aggregation assay

Human strain SK120 (*St gordonii*) was selected for testing for mouse saliva induced aggregation because it was previously shown by us (Lee, 2000) to bind human PIP, and also because human saliva has been shown to influence aggregation and adherence of different strains of *S. gordonii* (Ligtenberg et al., 1992). The SK120 strain was initially isolated from the human oral cavity (Kilian et al., 1989) and was generously donated by Dr. George Bowden (Department of Oral Biology, University of Manitoba).

Strains M105/6 and M106/2 belonging to the genus *Streptococcus* were isolated from the mouse oral cavity and characterized during the initial qualitative analysis of mouse oral bacteria. These strains were randomly selected from the identified mouse streptococci based on their ability to grow well in Todd Hewitt broth, making them suitable for aggregation assays.

Bacterial cultures were grown overnight (16h) in 40ml Todd Hewitt broth and then harvested by centrifugation at 10,000 x g for 10 minutes. The pellets were washed twice in 40ml PBS and resuspended in PBS to an OD_{700nm} of 1 (± 0.02). The aggregation reactions were made by mixing bacterial suspension with mouse saliva to a final volume of 1ml. PBS was used as a control for bacterial auto-aggregation. The reactions were mixed into disposable cuvettes, the OD_{700nm} of each mixture was determined and the cuvettes were incubated at room temperature without further mixing. The OD_{700nm} of the mixtures was measured with a spectrophotometer at set time intervals (10-20 minutes) for a maximum of 2 hours. A decrease in the OD of the mixture, as well as the visualization of macroscopic aggregates floating or settled at the bottom of the cuvettes, was considered positive for saliva induced bacterial aggregation. The presence of aggregation in the cuvettes where PBS was used instead of saliva was considered bacterial auto-aggregation.

The degree of aggregation induced by saliva derived from wild-type mice was compared with that of mPIP homozygous knockout mouse saliva, which lacks mPIP. For each experiment, aggregation assays were set up in duplicates and the data obtained from each set of duplicates was averaged. Each bacterial strain was tested at least twice.

V. RESULTS

1. Mouse saliva collection

During a single procedure approximately 100-900µl mouse saliva was collected from each animal, with an average of about 300µl.

2. Generation of the anti-mPIP polyclonal antibody

The IgG concentration of the mPIP Ab-2 affinity pure antibody was 0.9mg/ml, as determined by the manufacturer (Alpha Diagnostics Intl., San Antonio, TX) using an ELISA technique.

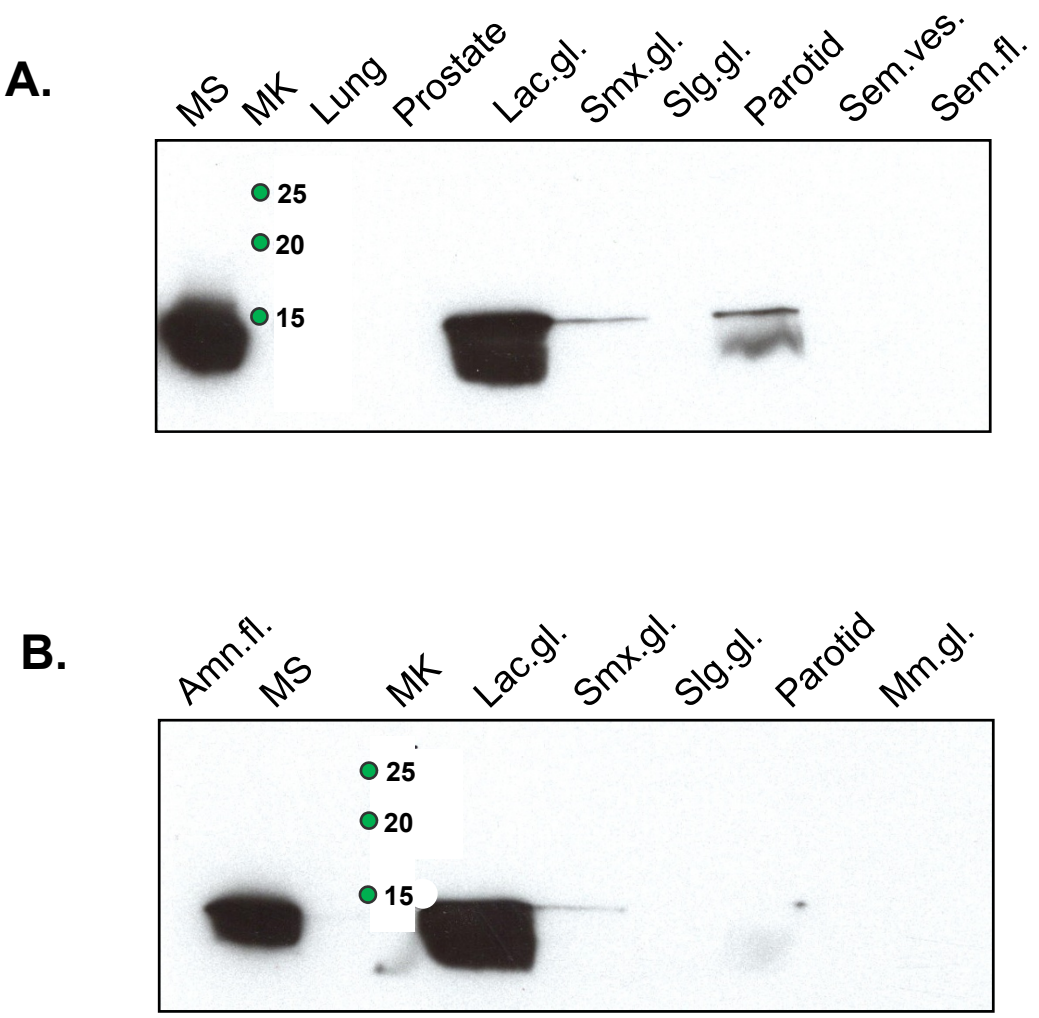
3. Expression of mPIP protein in different tissues and body fluids

Using Western blot analysis, an approximately 14kDa protein (the expected size of the mPIP protein) was detected by the mPIP Ab-2 in different mouse body fluids and protein extracted from different types of mouse tissues. This protein was present in mouse saliva and in protein extracts of both male and female lacrimal, submaxillary and parotid glands. It was absent from the sublingual gland, prostate, seminal vesicle, mammary gland and lung tissue as well as the seminal plasma and amniotic fluid (Figures 10.A and 10.B). The level of mPIP expression was higher in the lacrimal gland than in the submaxillary gland, as previously determined. In addition, we demonstrated for the first time that mPIP was also secreted into the mouse tears (see below, Figure 13.B).

Using immunohistochemistry, a high-intensity diffuse cytoplasmic staining pattern was observed in the serous acinar cells of both the submaxillary and lacrimal glands (Figure 11). No staining was detected in the mucous acinar cells of the submaxillary

Figure 10. Expression of the mPIP protein in male (A) and female (B) mouse tissues and body fluids.

Western blot analysis was performed using mPIP Ab-2. In both male and female, the highest level of mPIP expression was found in the lacrimal glands. The mPIP protein was also expressed in the submaxillary and parotid glands.

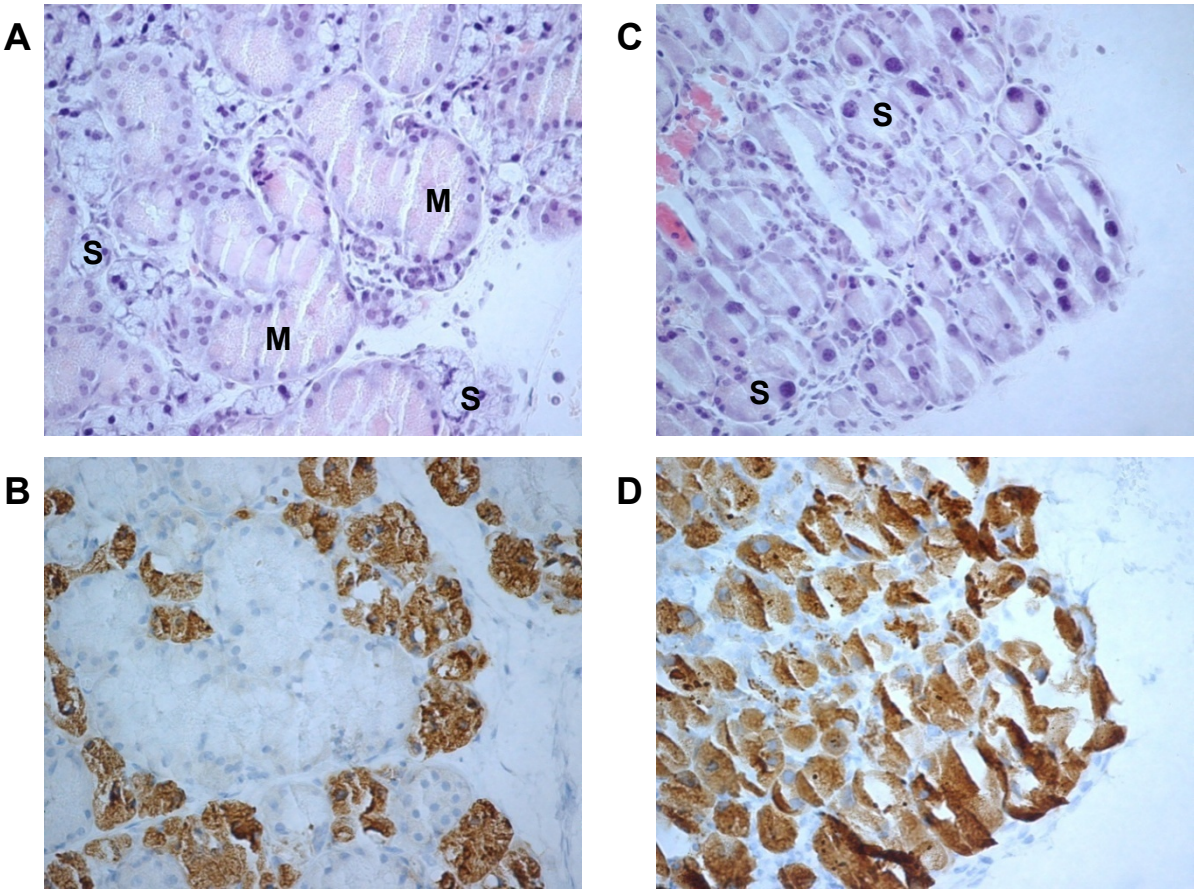


Total amount of protein loaded:
Tissue and seminal fluid: 60µg/lane
Mouse saliva and amn.fl.: 30µg/lane
(according to BCA protein assay)

MS = mouse saliva
MK = molecular weight marker (kD)
Lac.gl. = lacrimal gland
Smx.gl. = submaxillary gland
Slg.gl. = sublingual gland
Sem.ves. = seminal vesicle
Sem.fl. = seminal fluid
Mm.gl. = mammary gland
Amn.fl = amniotic fluid

Figure 11. Immunohistochemical analysis of mPIP in mouse submaxillary glands and lacrimal glands.

The level of mPIP protein expression is higher in the lacrimal gland than in the submaxillary gland. No staining was detected in the mucous acinar cells (M) of the submaxillary gland. Paraffin embedded tissue sections from submaxillary and lacrimal glands from wild-type CD1 mice were stained with the anti-mPIP polyclonal antibody.
A, C: Haematoxylin and Eosin (H&E) staining
B, D: Positive staining for mPIP in the serous acinar cells (S) of both submaxillary gland and lacrimal gland.



Mouse submaxillary gland (wild-type)

Mouse lacrimal gland (wild-type)

gland or in the epithelial ductal cells. The cells types were identified based on their histological characteristics determined by Haematoxylin and Eosin (H&E) staining (Figure 11).

4. Screening and verification of the mPIP knockout mouse model

4.1. Screening of ES cell clones

Screening of the ES cells transfected with the mutated mPIP gene construct was accomplished by Southern Blot hybridization using a 5' specific mPIP probe (Figure 12.A). The Southern Blot hybridization was performed in our laboratory by Ms. Anne Blanchard. Out of the 10 clones tested, 7 were found to be positive for the mPIP mutated allele. Three of the ES cells positive for targeted disruption of the mPIP gene were then chosen to be expanded into mouse blastocysts in order to produce chimeric mice. This procedure was performed at the University of Connecticut, CT, USA (see Material and Methods).

4.2. Genotyping of mPIP knockout mice

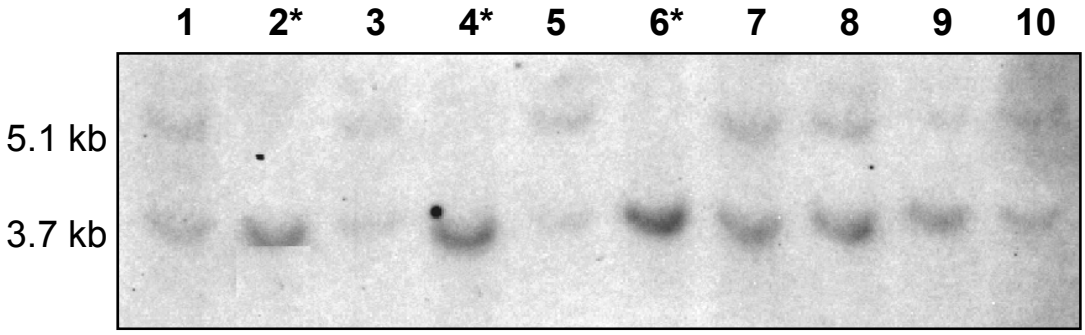
PCR analysis of genomic DNA resulted in a 289 bp band for the wild-type allele and a 395 bp band for the mutant allele of the mPIP gene. Amplification of wild-type (+/+) genomic DNA alone generated a PCR fragment of 395 bp, whereas a 289 bp fragment was generated by PCR from DNA obtained from the mPIP knockout homozygous mouse (-/-). Amplification of heterozygous (+/-) genomic DNA produced both bands (Figure 12.B). The PCR analysis was also performed by Anne Blanchard.

Figure 12. Screening and verification of the mPIP knockout mice.

A. Screening the mouse ES cells by Southern Blot analysis.

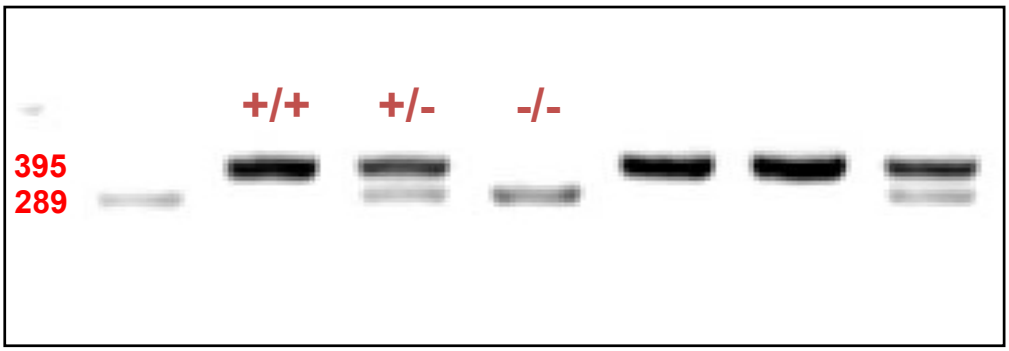
Genomic DNA derived from 10 (# 1-10) ES clones was digested with *HincII* and probed with the 5' external mSMGP probe. ES clones lacking the mutant gene were identified by the presence of a single 3.7 kb band. ES clones that contained the null mutation were identified by the presence of both 5.1 kb band and the 3.7 kb band.

* negative clones (wild type)



B. Verification of mPIP knockout mice by PCR analysis.

Amplification of wild-type (+/+) genomic DNA generated a PCR fragment of 395 bp, whereas a 289 bp fragment was generated by PCR from DNA obtained from an mPIP homozygous knockout mouse (-/-). Amplification of heterozygous (+/-) genomic DNA produced both bands.



4.3. Confirmation of the null mutation in the mPIP knockout mice

The absence of mPIP in saliva retrieved from knockout mice was confirmed by Western blot analysis (Figure 13.A). The mPIP Ab-2 recognized an approximately 14kDa (the expected size for mPIP) protein in saliva of wild-type and mPIP heterozygous mice, whereas this protein was absent in saliva of mPIP homozygous knockout mice. There was no reactivity with the pre-immune rabbit serum. The absence of mPIP in tears collected from the mPIP knockout mice was confirmed by Western blot analysis (Figure 13.B).

Expression of mPIP was not detected in the submaxillary gland tissue of knockout mice as assessed by immunostaining (Figure 14).

5. Production of pure mPIP protein

5.1. Generation of recombinant mPIP protein in *E.coli*

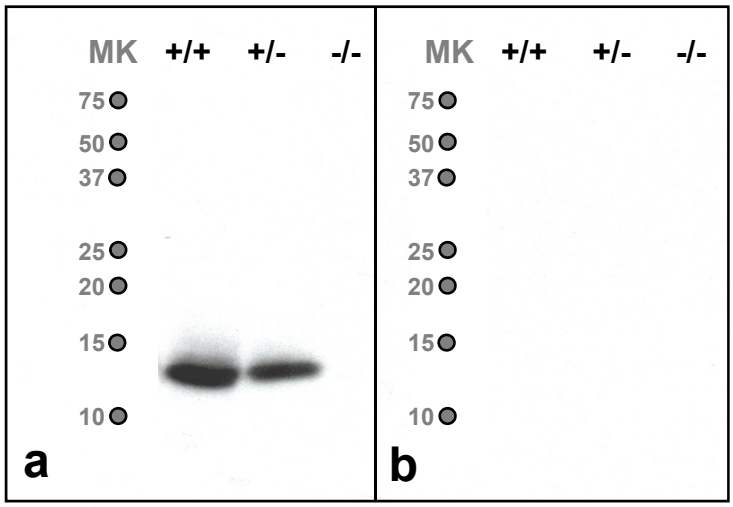
As mPIP is secreted from cells and must therefore pass through the secretory pathway, we used bioinformatics to examine its cDNA for the presence of a signal peptide and to predict where the amino terminus of the mature protein was located. A 0.906 probability of the signal peptide cleavage site location between amino acids 26 and 27 in the amino acid sequence of mPIP was determined using to the SignaIP 3.0 online server (Figure 15).

The cDNA corresponding to the mature secreted form of mPIP was successfully inserted in the pET28(a) plasmid and amplified in competent DH5α *E.coli* cells, by culturing the bacteria on plates containing Kanamycin for positive selection. Plasmid DNA was isolated from 18 bacterial clones grown from single colonies picked from these plates. The plasmid DNA was digested with *NcoI* and *BamHI,* followed by agarose gel

Figure 13. Demonstration of the absence of mPIP in saliva and tears of mPIP knockout mice (-/-).

Western blot analysis was performed using mPIP Ab-2. Saliva and tears collected from wild-type (+/+) and mPIP heterozygote (+/-) mice were used as positive controls. MK = molecular weight marker (kDa).

A. The mPIP protein was detected in wild-type and mPIP heterozygote mouse saliva and absent in mPIP knockout mouse saliva (a). Non-specific binding was ruled out by probing the same samples with pre-immune rabbit serum (b). Equal volumes (9µl) of mouse saliva were loaded on each lane.



B. The mPIP protein was present in saliva and tears from a wild-type mouse and absent from the mPIP knockout mouse saliva and tears. Equal volumes (6µl) of mouse body fluid was loaded on each lane.

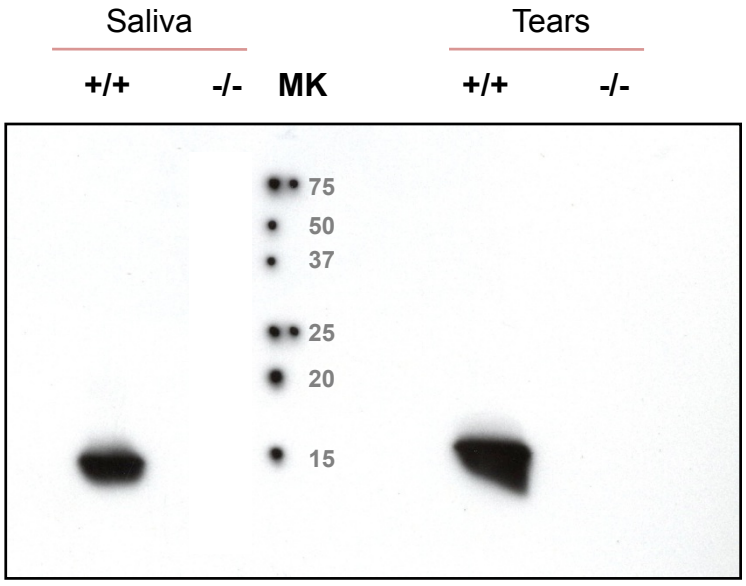


Figure 14. Demonstration of the absence of mPIP protein expression in the submaxillary glands of mPIP knockout mice.

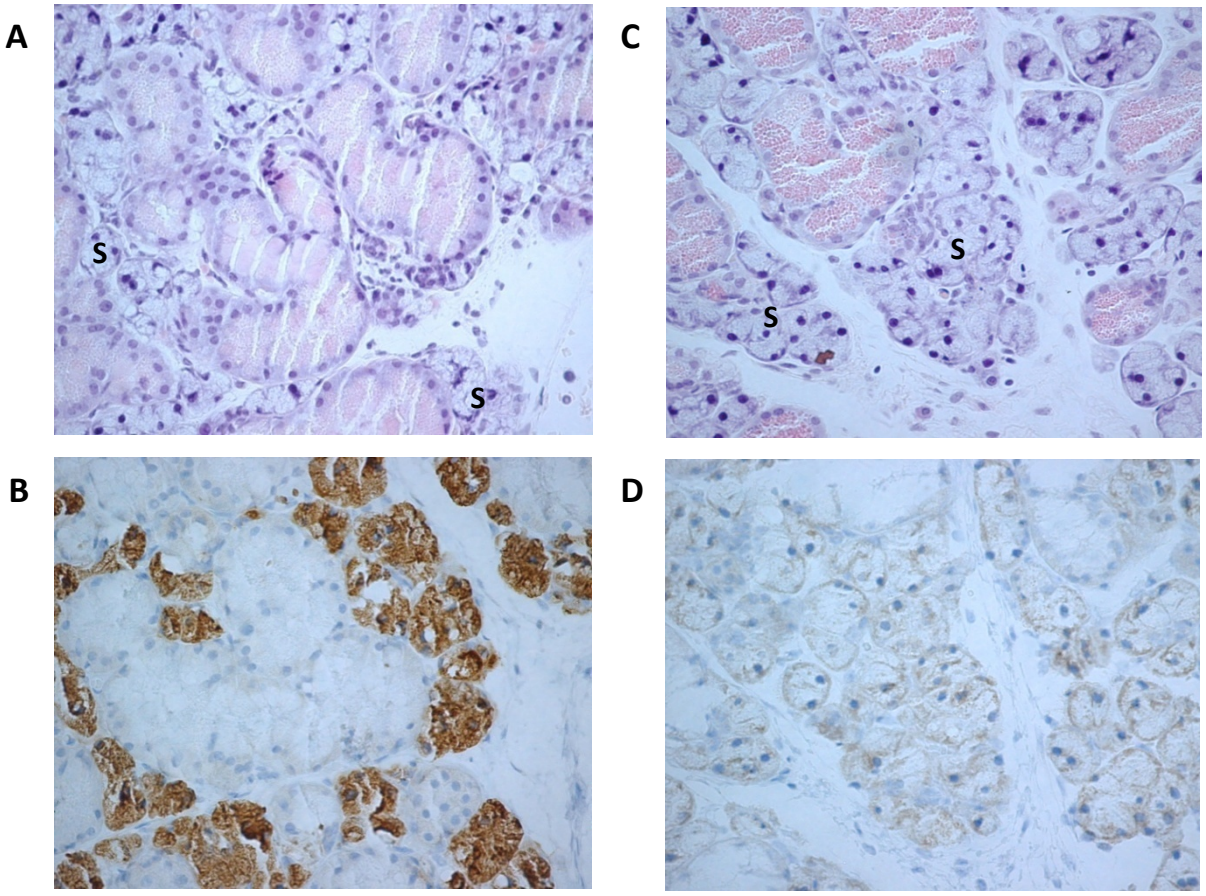
Mouse PIP was detected by immunohistochemistry in the serous acinar cells (S) of the wild-type submaxillary gland. No mPIP was detected in the serous acinar cells of mPIP knockout mice. Paraffin embedded tissue sections from the submaxillary glands of wild-type and mPIP homozygous knockout mice were stained with the anti-mPIP polyclonal antibody.

A: wild-type submaxillary gland stained with Haematoxylin and Eosin (H&E)

B: wild-type submaxillary gland stained with anti-mPIP antibody

C: submaxillary gland from mPIP knockout stained with H&E

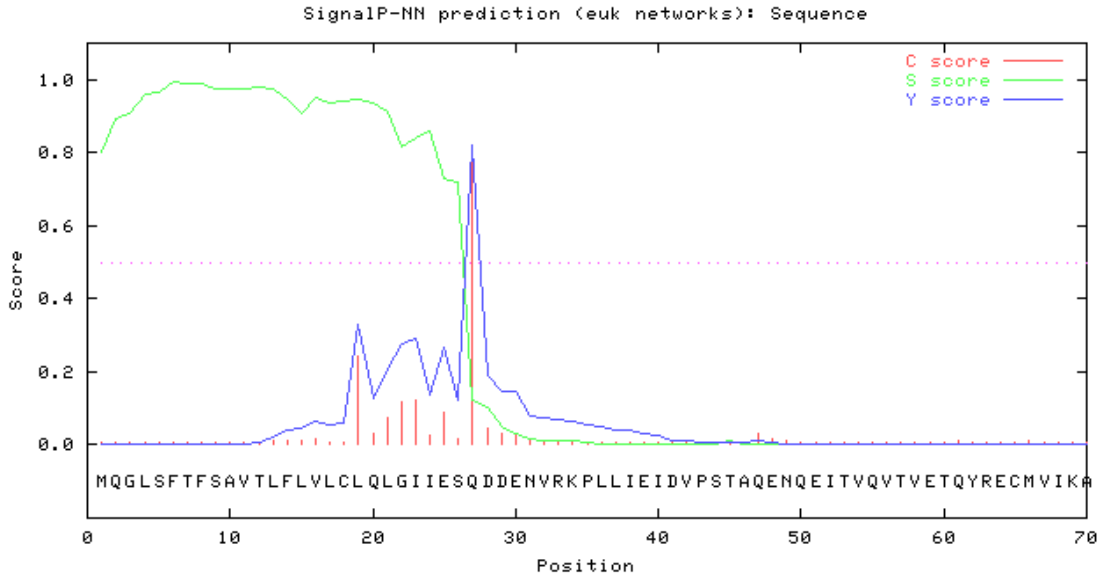
D: submaxillary gland from mPIP knockout stained with anti-mPIP antibody

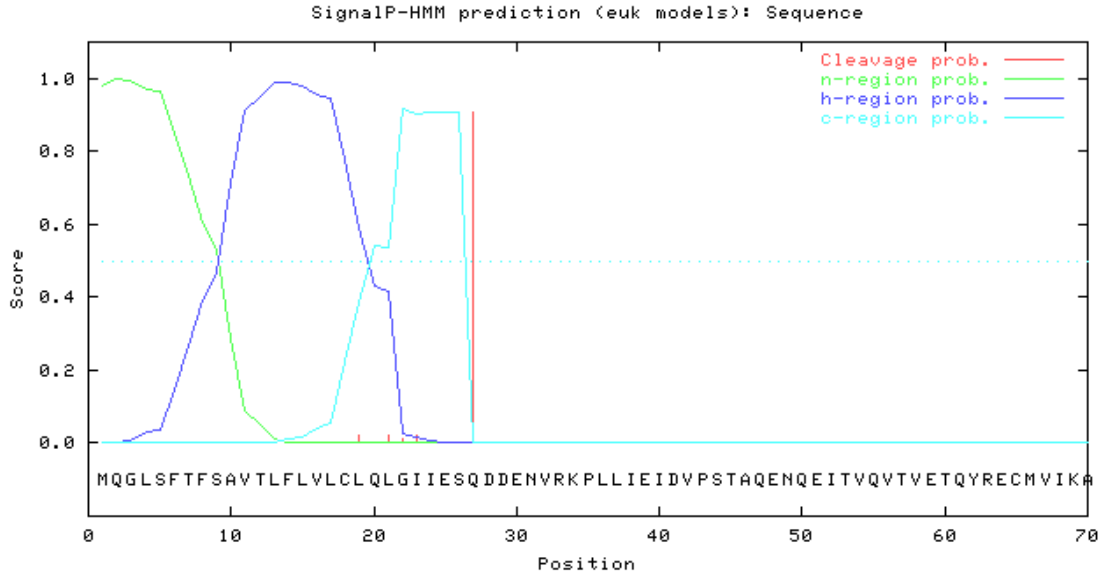


Mouse submaxillary gland (wild-type)

Mouse submaxillary gland (mPIP knockout)

Figure 15. Prediction of presence and location of signal peptide cleavage site in the amino acid sequence of mPIP (Signal IP 3.0 Server results) .





Prediction: Signal peptide
Signal peptide probability: 0.981
Max cleavage site probability: 0.906 between pos. 26 and 27

electrophoresis, leading to the identification of several positive clones. Three positive clones were randomly chosen for verification by sequencing, all of which showed the correct sequence of the mPIP gene inserted into the appropriate cloning sites of the pET28(a) vector. This was determined by aligning the results of sequencing with the nucleotide sequence of the mPIP gene (gi:22135641).

Seven “rare” *E.coli* codons out of a total of 131 cododns of the mPIP $(His)_6$-tagged protein were identified. The pET28(a) plasmid containing mPIP was successfully transformed into the Rosetta 2(DE3) *E. coli* strain and protein expression was induced with IPTG. Different concentrations (0.1mM and 1mM) of IPTG were used to induce protein expression at both room temperature and 37ºC. No significant differences in the level of protein expression were observed between the different conditions used (Figure 16.A).

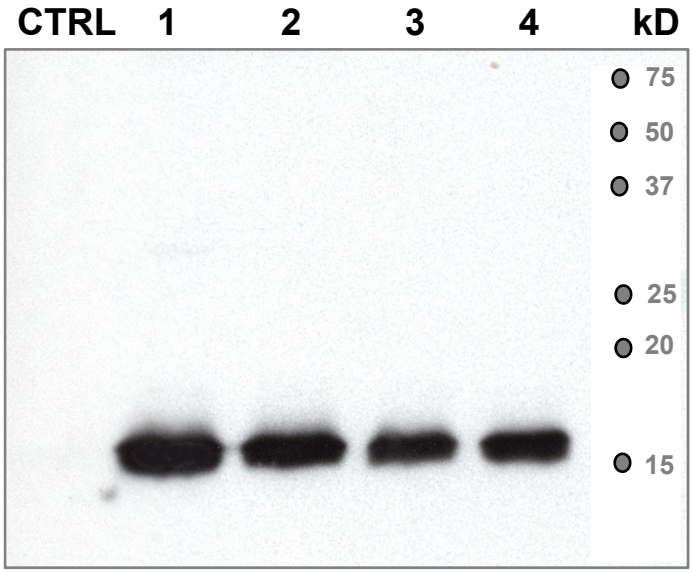
Although the $(His)_6$-tagged mPIP was successfully expressed by the Rosetta2(DE3) cells, the protein was mostly insoluble, in the form of inclusion bodies. Purification under native conditions of the low soluble amount of mPIP was attempted, relying on the high affinity of the $(His)_6$ tag for the Ni^{2+} column. The purified protein was not detectable by Coomasie staining of the separating gel used for electrophoresis of the fractions collected during the purification procedure. Low amounts of purified $(His)_6$-tagged mPIP were detected by Western blot analysis of the corresponding fractions (Figure 16.B). However, the protein yield was considered too little to pursue the method at a larger scale in order to produce sufficient protein to be used for *in vitro* assays.

5.2. Purification of mPIP from mouse saliva by affinity chromatography

Efficient coupling of the mPIP Ab-2 to the protein A agarose beads was determined by the presence of IgG heavy chains (MW = 50kDa) in the samples of beads taken before DMP cross-linking and their absence in the samples taken after cross-linking

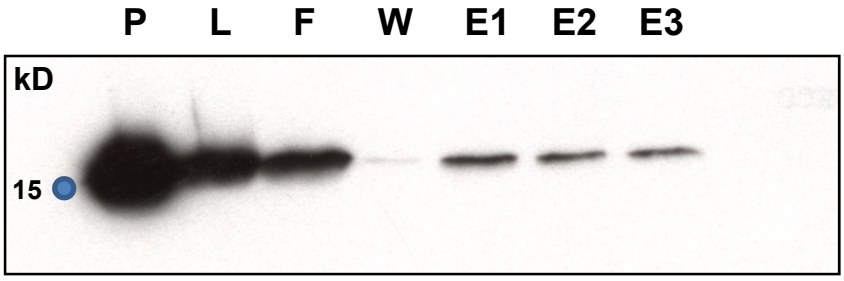
Figure 16. Induction of recombinant mPIP protein expression in Rosetta 2 (DE3) *E.coli* and purification of the recombinant protein from the bacterial cells.

A. Western blot analysis of total cell protein from Rosetta 2 cells transformed with mPIPs/ pET28(a). Protein expression was induced with different amounts of IPTG at room temperature (RT) or 37ºC; no significant differences in the level of protein expression were observed between the different conditions used.



CTRL = un-induced
1= 0.1mM IPTG / 37ºC
2 = 1 mM IPTG / 37ºC
3 = 0.1mM IPTG / RT
4 = 1mM IPTG / RT

B. Western blot analysis of the fractions obtained from purification of mPIPs/ pET28(a) / Rosetta 2 on a Ni-column. A high proportion of the recombinant mPIP protein was found in the insoluble pellet (P). Very low amounts of purified protein were identified in the elution fractions (E1-E3).



P = pellet (insoluble fraction)
L = load (clear lysate, soluble fraction)
F = flow through the column
W = washing
E1-E3 = elutions

(Figure 17.A). These were visualized by performing SDS-PAGE electrophoresis of the beads samples followed by staining with Coomasie blue.

After attempting purification of mPIP from mouse saliva by affinity chromatography on a column consisting of mPIP Ab-2 coupled with protein A agarose beads, no protein was detected in the elution fractions after SDS-PAGE electrophoresis and Coomasie staining of the separating gel. The fractions collected during the procedure were analyzed by Western blot analysis and mPIP was detected only in the flow through of the column (Figure 17.B).

6. Quantification of salivary PIP by ELISA

6.1. Quantification of salivary human PIP by indirect ELISA

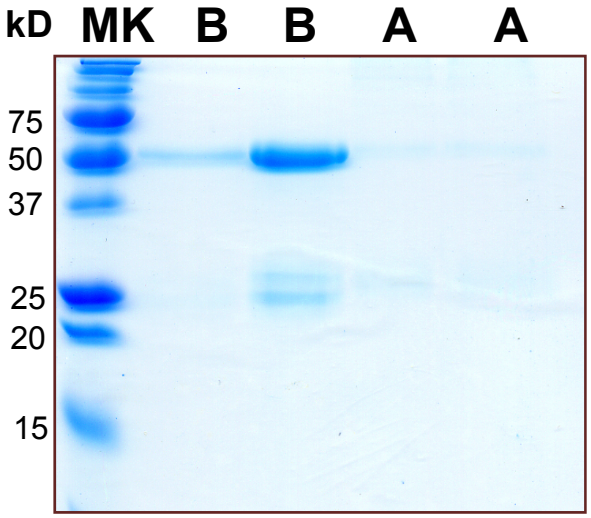
Frozen aliquots of unstimulated human saliva were used to determine the salivary concentrations of human PIP using an indirect ELISA assay developed in our laboratory. The level of PIP in human saliva was found to be 2-97 µg/ml (mean = 52.6 µg/ml, SD ± 30.8 µg/ml, n = 10), which corresponds to what is known in the literature (Haagensen, Jr. et al., 1979).

6.2. Quantification of salivary mouse PIP by indirect ELISA

Salivary mPIP levels in wild-type, heterozygote and homozygote mPIP knockout mice were determined using an indirect ELISA developed in our laboratory similarly to the assay used for quantification of hPIP in human saliva. The level of mPIP in saliva of animals expressing mPIP, wild-type and mPIP heterozygote knockout mice, was found to be 39.7-153.2 µg/ml (mean = 75.1 µg/ml, SD ± 45.8 µg/ml, n = 6). Using this method, the absence of mPIP in the saliva of knockout mice was confirmed, determined as OD

Figure 17. Purification of mPIP from mouse saliva by immunoaffinity chromatography.

A. SDS-PAGE and Coomasie staining of protein A agarose beads coupled with anti-mPIP antibody. The IgG heavy chains (50kD) were present in the samples taken before cross-linking and absent after cross-linking.



MK = molecular weight marker
B = Before cross-linking
A = After cross-linking

B. Western blot analysis of the fractions collected during the attempt to purify mPIP from mouse saliva. The mPIP protein was only detected in the flow through the column.

F = flow through the column
W = washing
E1-E5 = elutions

readings similar to the blank controls. These results were obtained by coating the plates with mouse saliva diluted 1:1000, similar to the technique used for quantification of human PIP. However, during the process of verifying the results by assaying different mouse saliva dilutions, several difficulties were encountered, such as the OD readings did not correspond with the level of dilution of the sample and the maximum OD produced by the mouse saliva samples was below 0.18, irrespective of the dilution factor (Figure 18). Therefore, the newly developed indirect ELISA assay failed to accurately quantify mPIP in mouse saliva.

6.3. Quantification of salivary mouse PIP by competitive ELISA

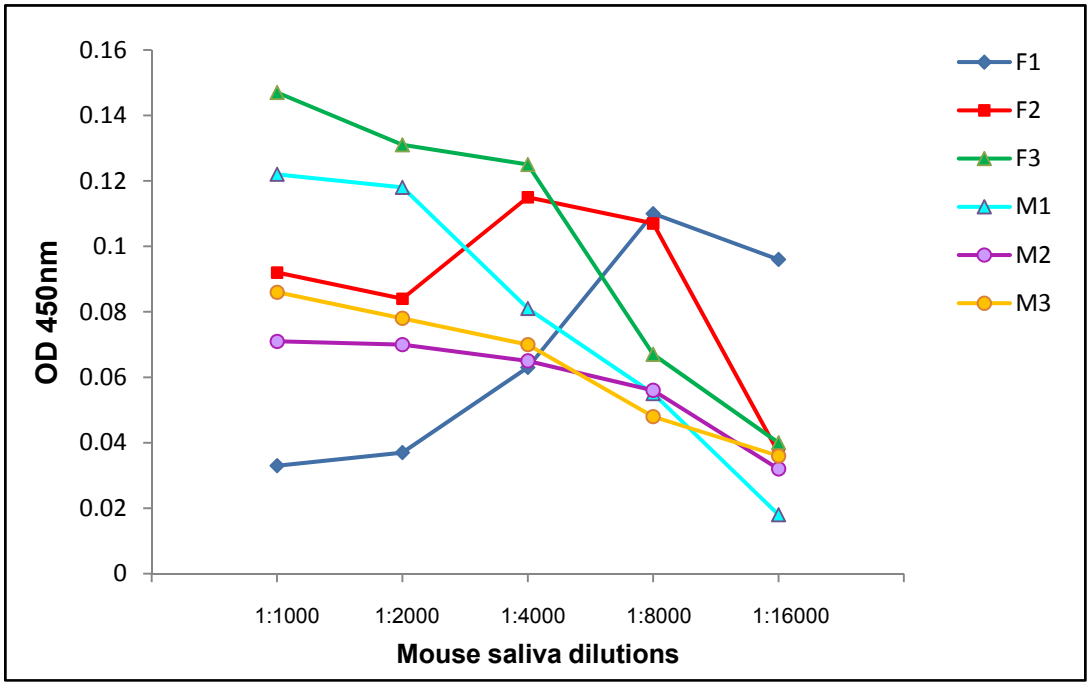
6.3.1. Determination of the optimal coating concentration of SMGP peptide and mPIP Ab-2 working dilution

A standard curve was obtained by performing indirect an ELISA assay, coating the plate with different dilutions of SMGP peptide in the range 0.1-1600ng/100µl. The linear portion of this standard curve (Figure 19.A) ranged between 25-200ng peptide per well (100µl) and was used to determine the optimal coating concentration of SMGP peptide. At 60ng (100µl), the absorbance at 450nm was approximately 50% of the maximum absorbance observed and therefore this was estimated as the optimal coating concentration of SMGP peptide. In order to facilitate the dilution process, 50ng/100µl/well of SMGP was chosen as the coating concentration to be used in the indirect ELISA assays.

The optimal working dilution for mPIP Ab-2 was estimated at 1:6000, using the linear part of the antibody titration curve (Figure 19.B). However, the working dilution of 1:4000 (which was located on the linear part of the standard curve, not far from the estimated optimal working dilution) was chosen for consistency with the dilutions of the same

Figure 18. Quantification of mPIP in mouse saliva by indirect ELISA.
Twelve wild-type mouse saliva samples (6 females F1-F6 and 6 males M1-M6) were used to coat ELISA plates. Indirect ELISA was performed as described in the Materials and Methods section, A and B represent 2 sets of samples tested on different plates. The OD did not vary according to the serial dilution of the samples. Quantification of mPIP levels by this strategy was not possible in our hands.

A.



B.

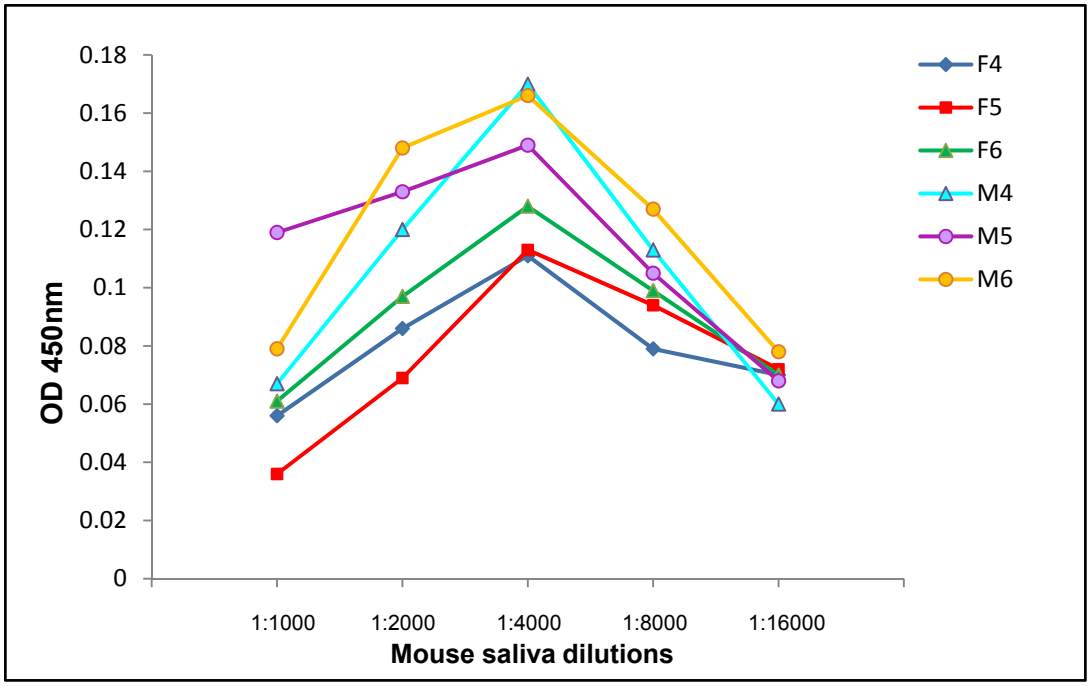
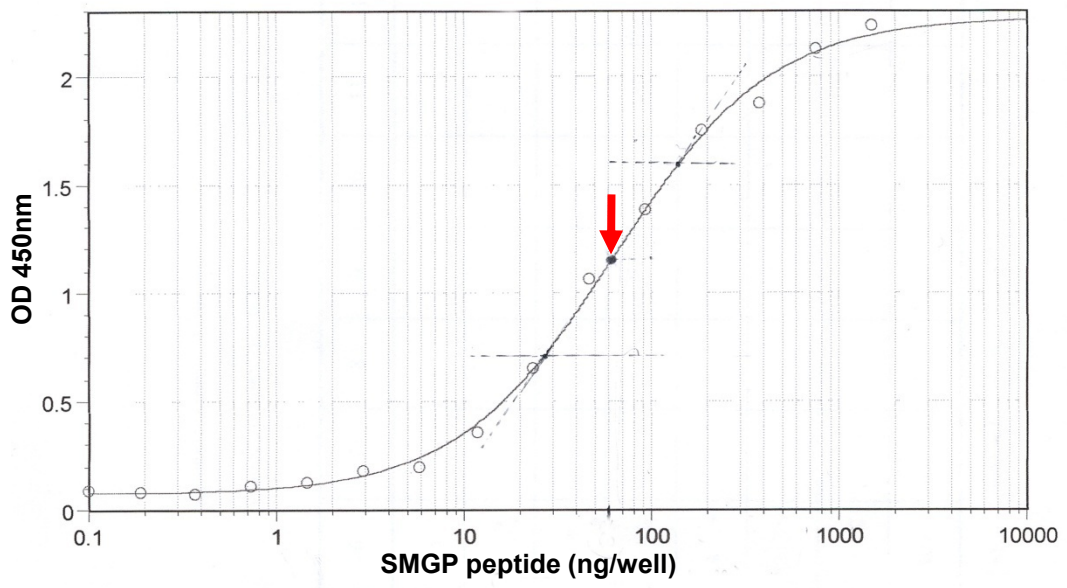


Figure 19. Optimization steps of competitive ELISA for quantification of mPIP protein.

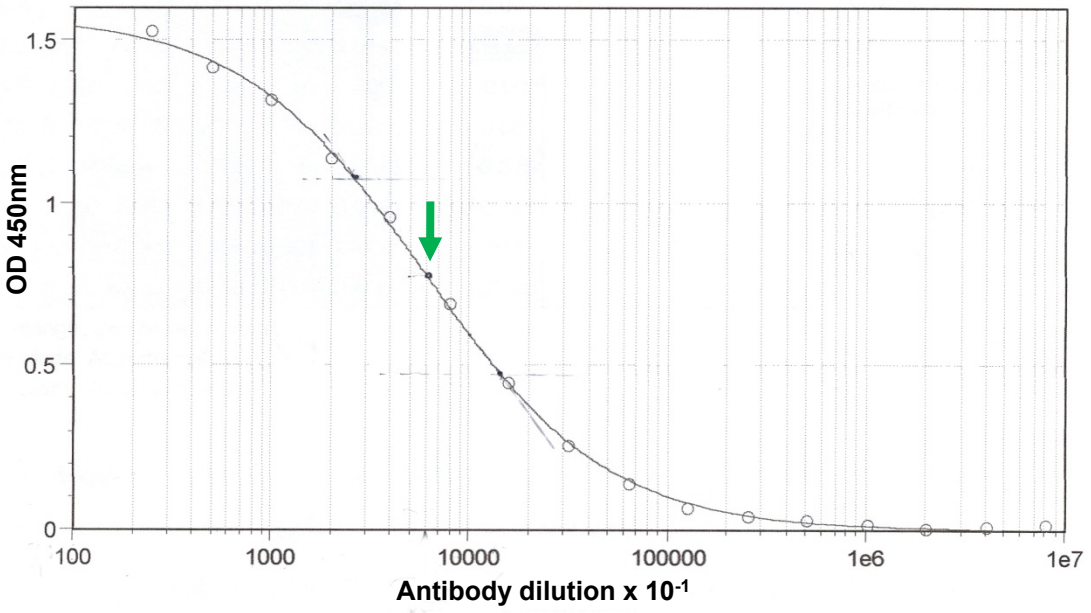
A. Determination of optimal SMGP peptide coating concentration.

A standard curve was generated by performing indirect ELISA by coating the plate with SMGP peptide, 0.1-1600ng/100µl/well. The linear portion of the standard curve was determined and the concentration of SMGP corresponding to the middle of this linear portion was considered as being the optimal SMGP coating concentration (60ng/100 µl/well, indicated by the red arrow).



B. Determination of the optimal mPIP Ab-2 working dilution.

The optimal working dilution for the Anti-mPIP antibody was estimated as corresponding to the middle of the linear part of the antibody titration curve. (1:6000, indicated by the green arrow).



antibody used in our studies for other techniques such as Western and slot blot analysis and immunohistochemistry.

6.3.2. Competitive ELISA for quantification of salivary mPIP

A standard curve was successfully generated by mixing different amounts of recombinant mPIP with mPIP Ab-2 diluted 1:4000 (Figure 20.A). A reaction equivalent to the presence of a high concentration of mPIP was generated by some of the mouse saliva samples collected from mPIP knockout mice (Figure 20.B, see sample KO3) although these samples had already been proven to lack mPIP by Western blotting. Therefore, the newly developed competitive ELISA was not reliable for quantitation of mPIP in mouse saliva.

7. Quantification of mPIP in mouse saliva by blotting techniques

7.1. Relative quantification of mPIP in mouse saliva by Western blot

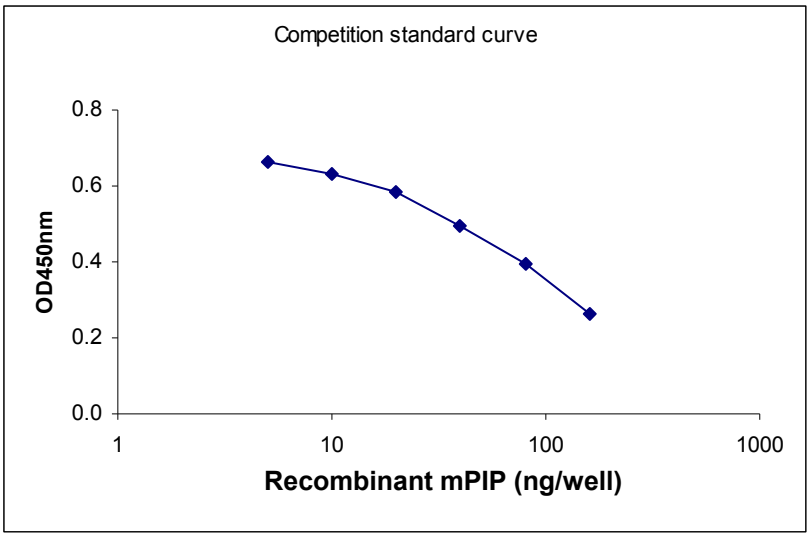
After ELISA techniques were shown to be unable to accurately quantify mPIP in mouse saliva, blotting techniques were considered in order to accomplish this objective. To determine whether there are differences between the levels of mPIP in saliva of male and female individuals, equal volumes of saliva collected from 4 male and 4 female adult mice were analyzed by Western blot analysis using mPIP Ab-2. The salivary mPIP levels were similar in both male and female mice (Figure 21).

7.2. Slot blot analysis

Slot blot analysis was chosen for quantification of mPIP in mouse saliva because the SMGP peptide was available in known amounts in order to generate a standard curve

Figure 20. Competitive ELISA for quantification of mPIP in mouse saliva.

A. Different amounts (5-160ng/100µl/well) of recombinant mPIP protein were mixed with mPIp Ab-2 generating a standard curve for the competition reactions.



B. Saliva collected from 7 mice (4 wild-types WT1-WT4 and 3 mPIP knockouts KO1-KO3) was used for analysis. Each sample was diluted as shown. A strong decrease in absorbance was recorded in samples WT1, WT3 and KO3 while sample KO2 did not produce any positive competition reaction.

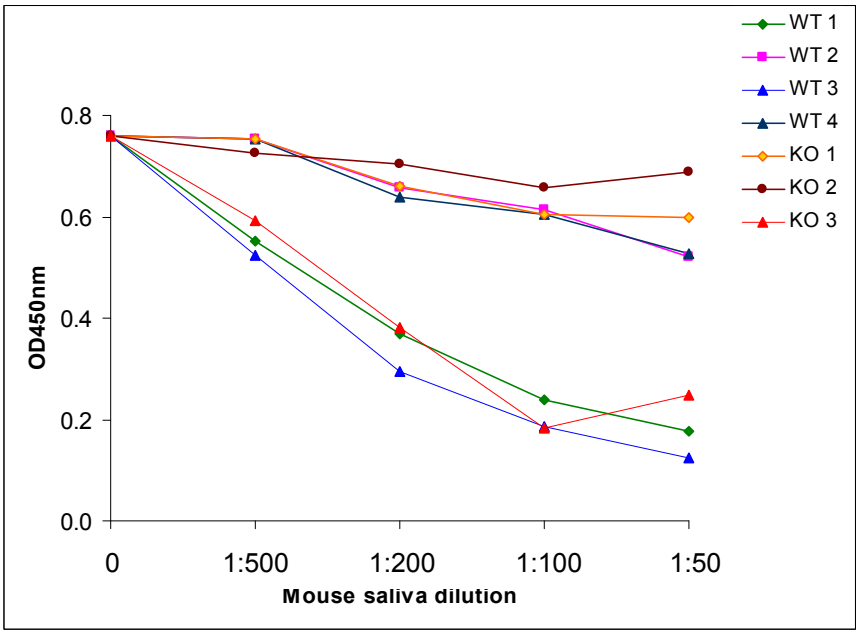
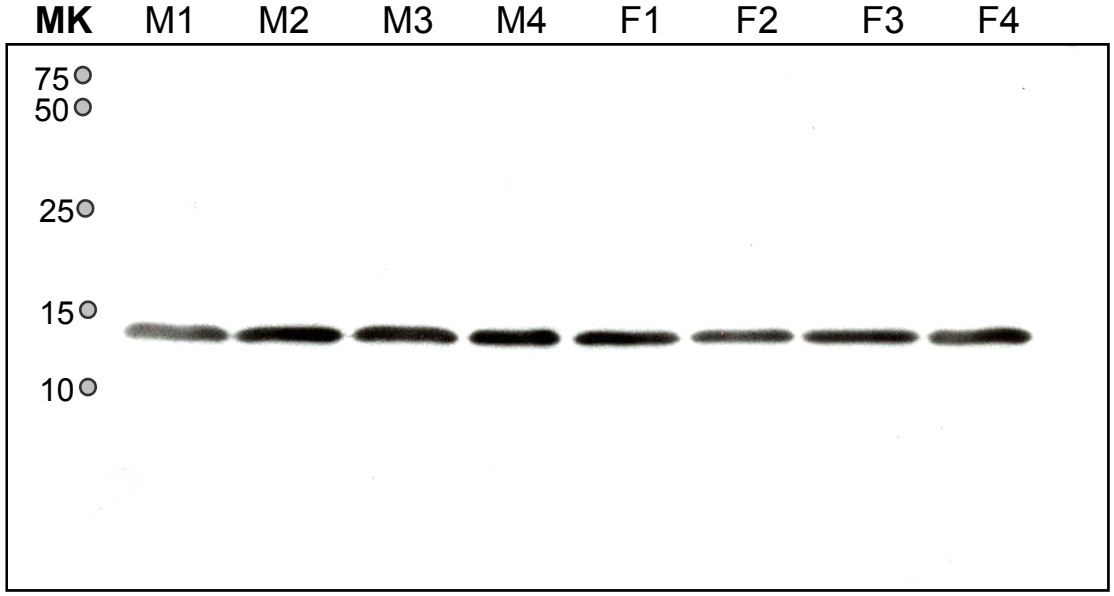


Figure 21. Relative quantification of mPIP in saliva from male and female mice by Western blot analysis.

Western blot analysis was performed using mPIP Ab-2. Equal volumes of mouse saliva collected from 4 male (M1-M4) and 4 female (F1-F4) wild-type mice were analyzed on each lane. MK = molecular weight marker (kDa). No difference in mPIP levels was observed between the male and female groups.



and the molecular weight of the peptide (approximately 2.5kDa) did not permit regular Western blot analysis.

Slot blot analysis was performed on mouse saliva samples collected from 12 wild-type mice (6 males and 6 females) and 2 mPIP knockout mice (Figure 22). A standard curve of different amounts of SMGP peptide was generated. Wild-type mouse saliva samples were found to contain a quantity of mPIP equivalent to 54.1 µg of the SMGP peptide per µl (range = 11.7-79.5µg/ml, SD± 18.64 µg/ml, n = 12). No mPIP protein was detected in the mPIP knockout mouse saliva samples.

8. Morphopathological analysis of the mPIP knockout mice

Both male and female mPIP knockout mice were viable and fertile and they did not show any obvious phenotypic differences when compared with wild-type mice.

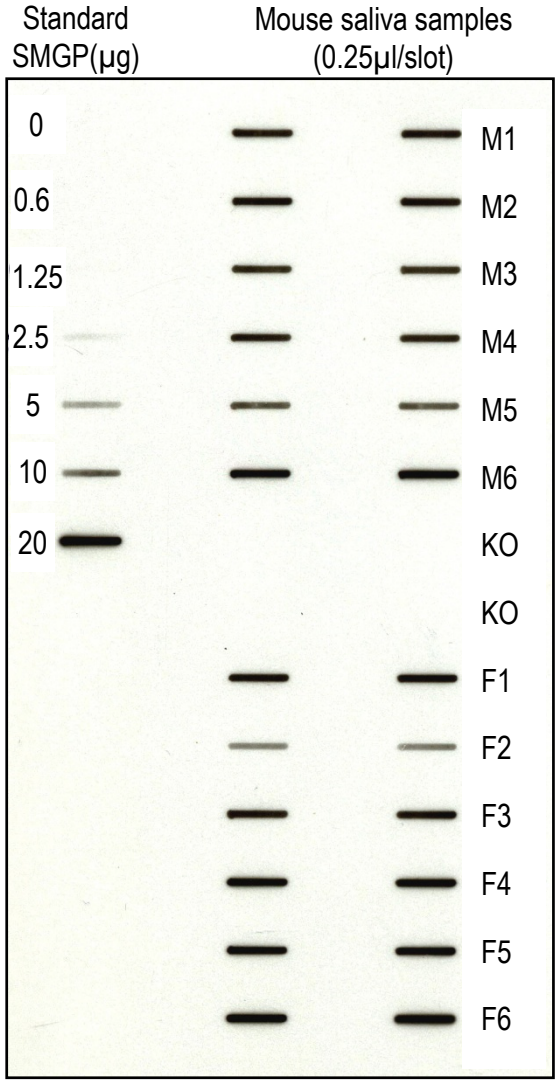
Histological analysis of several tissues (see Materials and Methods) revealed aggregation of lymphocytes in the prostates of knockout mice; also, enlarged lymph nodes with multiple germinal centres with high apoptotic and mitotic rates were observed in the submaxillary gland (Figure 23).

9. Analysis of the mouse oral flora

9.1. Quantitative analysis of the mouse oral bacteria

Sixteen adult mice (4 wild-type and 4 mPIP knockout mice of each male and female gender) were included in the study. Different dilutions (non-diluted, 1:10 and 1:100) of the suspension obtained from swabbing the oral cavities of the mice were cultured on blood agar plates in a candle jar. The candle jar was chosen as the optimal environment for culturing the mouse oral flora because it was previously determined that similar

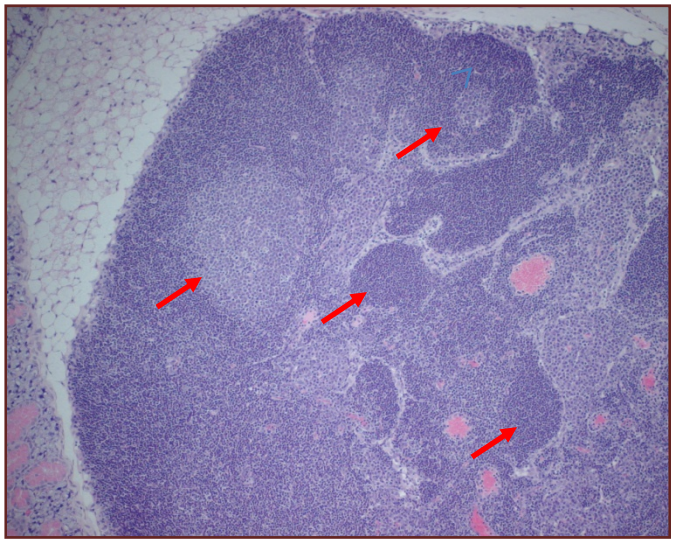
Figure 22. Quantification of mPIP in mouse saliva by slot blot analysis.
All samples were analyzed in duplicate, 0.25µl of mouse saliva diluted in PBS was loaded in each slot. No mPIP was detected in the mPIP knockout mouse saliva. Wild-type mouse saliva contain a quantity of mPIP equivalent with 54.1 µg SMGP peptide per µl (range = 11.7-79.5µg/ml, SD± 18.64 µg/ml, n = 12).



F1-F6 = wild-type female mice
M1-M6 = wild-type male mice
KO = mPIP knockout mice

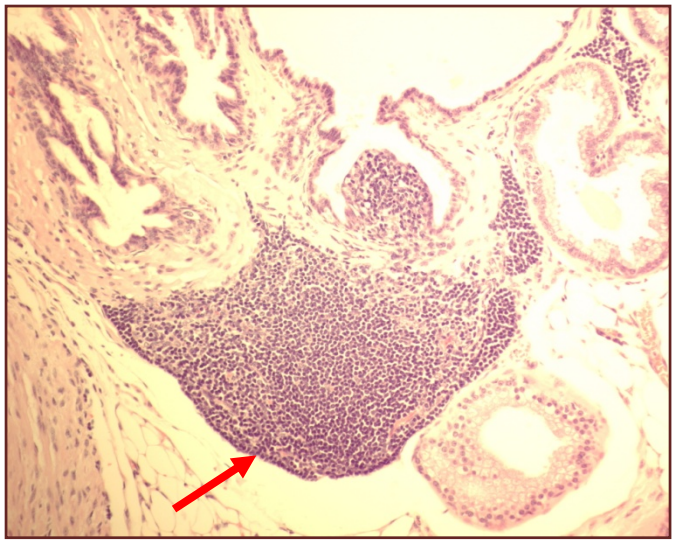
Figure 23. Histological analysis of mPIP knockout mouse tissues.
Selected tissues were dissected from 3 month-old male mPIP knockout mice and wild-type controls, embedded in paraffin and then analyzed by histology (procedures performed by Dr Geoff Ward, Samuel Lunderfeld Research Institute, Toronto, Ontario).

A. Submaxillary gland (magnification100X).



Enlarged lymph node containing multiple germinal centers (indicated by red arrows) with high mitotic and apoptotic rates.

B. Prostate gland (magnification 200X).



Aggregate of lymphocytes (indicated by the red arrow).

colonies result from incubating mouse oral suspension in either an anaerobic chamber or a candle jar (Lee et al., 2002) and the latter is more cost effective and easier to use. The colony forming units were counted for the most suitable dilutions and the numbers multiplied by the dilution factor. Male and female mice were analyzed as two separate groups.

For the female mice, a two-way repeated measure ANOVA analysis did not reveal any significant differences between the wild-type and knockout mouse group or between experiments. In addition, student t test analysis of each of the three time points did not reveal any differences in the total amount in the oral bacteria in the female wild-type and knockout mice (Figure 24.A).

For the male mice, the two-way repeated measure ANOVA analysis revealed significantly more oral bacteria residing in the oral cavity of wild-type mice compared with mPIP knockouts ($p<0.001$). However a significant difference was also identified between the three time points. In addition, student t test analysis of each of those time points revealed a significant difference in the amount of oral bacteria only in the first two of the three experiments (Figure 24.B).

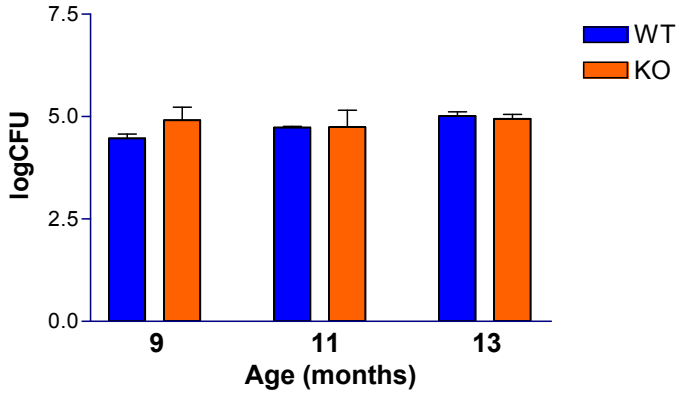
9.2. Qualitative analysis of the mouse oral bacteria

A total of 228 pure bacterial strains were isolated from the mouse oral cavity, 110 strains from the female mice (55 each from the wild-type and mPIP knockout group) and 118 strains from the male mice (59 each from the wild-type and mPIP knockout group).

Gram staining was used as the first step in identification of all strains (Figure 25). Using standard microbiology techniques following the algorithm illustrated in Figure 7, we identified bacteria belonging to several different genera, as summarized in Table 3.

Figure 24. Quantitative analysis of PIP knockout mouse oral flora.
Male and female mice were analyzed as two separate groups (8 mice of each gender; 4 wild-type and 4 mPIP knockout mice in each group). Comparisons between the mPIP knockouts and wild-type mice were performed for each group.

A. Female mice. No significant differences were found between the wild-type (WT) and mPIP knockout (KO) mice.



B. Male mice. Wild-type mice (WT) presented a more numerous oral resident bacteria compared to mPIP knockout (KO) mice; the difference was significant at ages of 8 and 9 months.

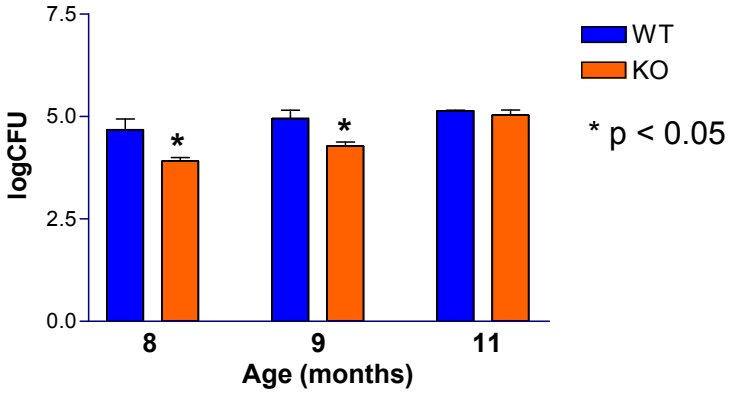
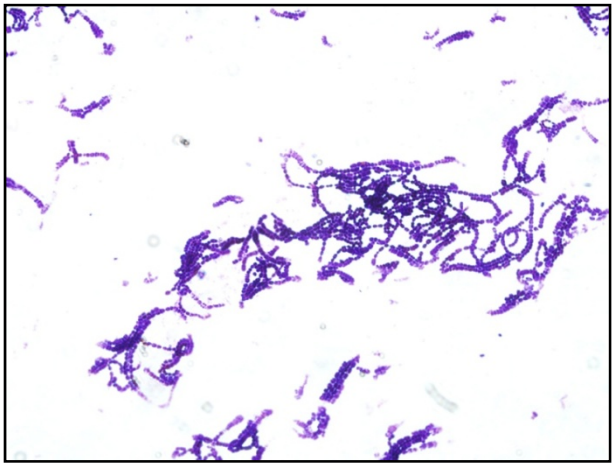


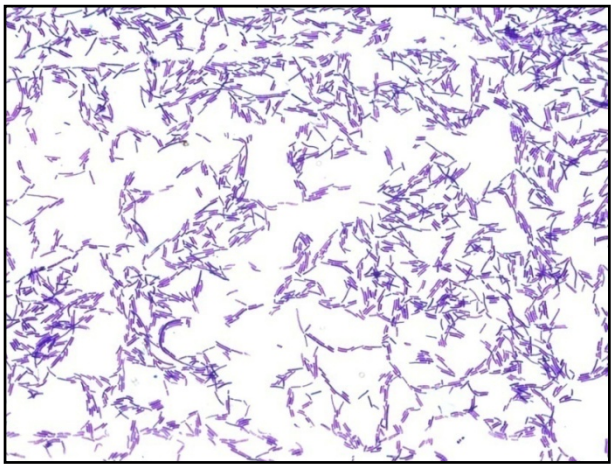
Figure 25. Gram staining of the most common mouse oral bacterial strains identified (magnification 1250X).

A.



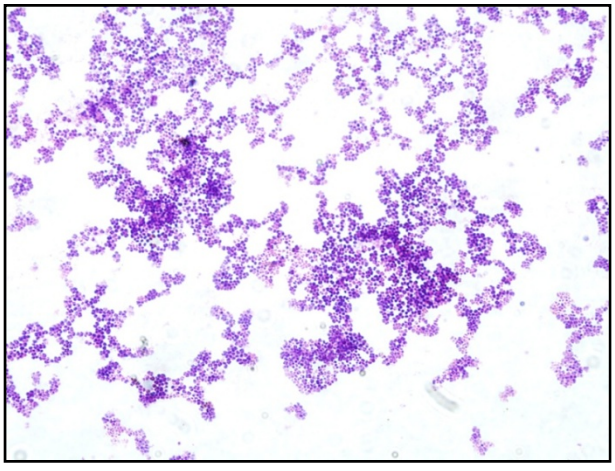
Streptococcus

B.



Lactobacillus

C.



Staphylococcus

For both male and female groups, *Streptococcus* was the dominant genus. In both sexes, streptococci represented a higher proportion of the oral bacteria of the mPIP knockout when compared to the wild-type mice. In addition, the results show that certain genera identified in the wild-type mice were absent from the oral cavity of the mPIP knockouts (Table 3), such as *Neisseria* isolated from the wild-type but not from the knockout males and Gram negative rods isolated from the wild-type but not from the knockout females.

10. Mouse saliva induced bacterial aggregation

Human oral bacterial strain SK120 (*S. gordonii*) and mouse oral streptococci M105/6 and M106/2 were tested for mouse saliva induced aggregation. Bacterial aggregation was recorded as a decrease of the optical density of the mixture of bacterial suspension and mouse saliva (or PBS for control), expressed as percentage from the optical density of that specific mixture at the starting point of the assay. For each saliva-bacterial suspension mixture, the proportion of the optical density determined at each time point from the initial OD value was plotted against time on a XY graph, the starting point representing time 0 and 100% optical density (Figure 26.A-C).

Both wild-type and mPIP knockout mouse saliva were able to induce different levels of aggregation of all three bacterial strains tested (Figure 26.A-C). The level of aggregation induced by the wild-type mouse saliva was higher than that induced by mPIP knockout mouse saliva. This finding was consistent with all the strains tested. No autoaggregation was detected in the controls where PBS was used instead of saliva.

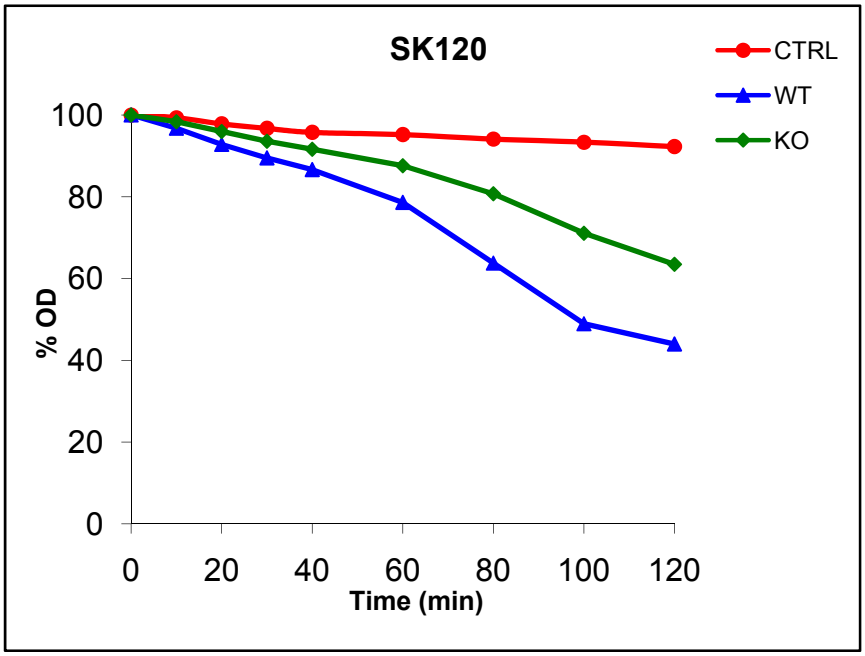
Table 3. Qualitative analysis of the oral flora isolated from the wild-type (WT) and mPIP knockout (KO) oral cavity.

Male and female mice were analyzed as two separate groups. The numbers show the proportions represented by each genus from the total number of stains isolated from that group. For both groups, *Streptococcus* was the dominant genus. Regardless of the mice gender, streptococci represented a higher proportion of the oral bacteria of the mPIP knockout when compared to the wild-type mice.

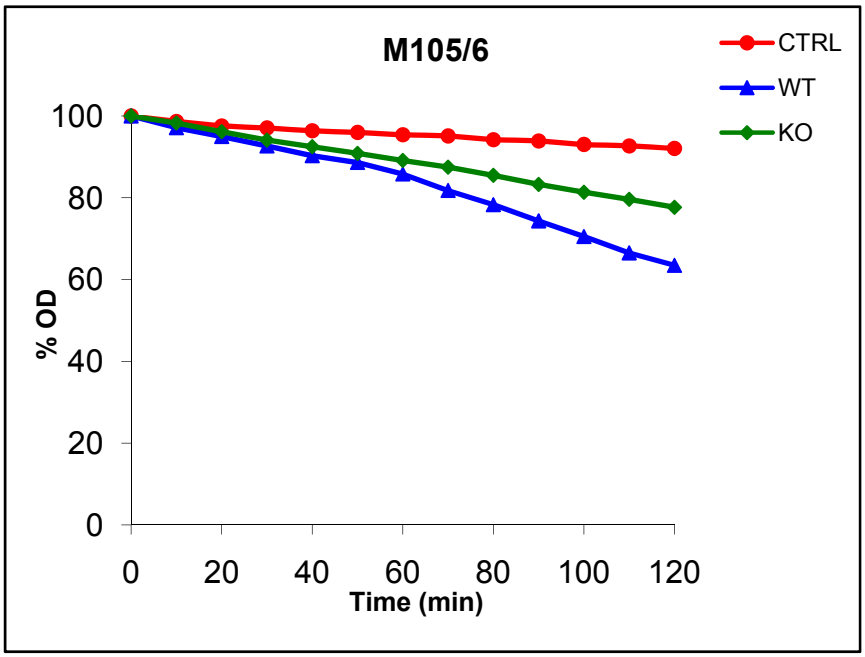
Genus of bacteria	Females		Males	
	WT (%)	**KO (%)**	**WT (%)**	**KO (%)**
Streptococcus	70.9	80.0	66.1	86.4
Staphylococcus	3.6	5.5	11.9	10.2
Lactobacillus	16.4	10.9	1.7	3.4
Micrococcus	0	1.8	3.4	0
Gram neg. rods	7.3	0	0	0
Neisseria	0	0	13.6	0
Others	1.8	1.8	3.4	0
Total	100	100	100	100

Figure 26. Bacterial aggregation assays.
Both wild-type (WT) and mPIP knockout mouse (KO) saliva induced aggregation of the human *S. gordonii* strain SK120 (A) and of mouse oral streptococci M105/6 (B) and M106/2 (C). For all 3 strains, WT saliva induced a higher degree of aggregation than KO. No autoaggregation was observed in the PBS controls (CTRL). Each bacterial strain was tested at least twice.

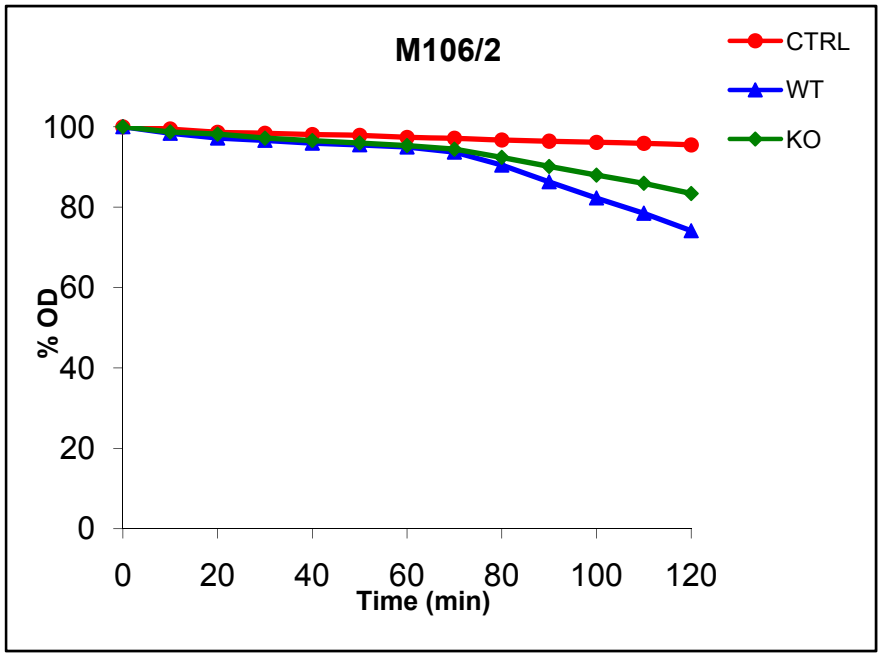
A.



B.



C.



VI. DISCUSSION

1. Gene and protein expression of mPIP

Gene expression of mPIP was detected by RT-PCR analysis in submaxillary, sublingual, parotid and lacrimal glands of mice ranging from 5 days to adulthood (Lee et al., 2003). The observation that mPIP was expressed in these tissues was confirmed by Osawa et al (2004), who demonstrated expression of mPIP gene in adult mouse lacrimal glands as well as all three major salivary glands, by RT-PCR analysis. *In situ* hybridization studies showed that localization of mPIP expression in the mouse submaxillary gland is restricted to the acinar cells (Lee, 2000), as has been previously shown for the rat homologue (Mirels et al., 1998; Myal et al., 1994). In addition, our laboratory also found mPIP to be expressed in the mouse prostate during early postnatal development, with the expression being turned off by 10 weeks of age (Lee et al., 2003).

Through Western blot analysis, the mPIP protein was detected in adult mouse lacrimal gland tissue (Lee, 2000) and secreted mPIP protein was identified in mouse saliva (Lee et al., 2002).

In the present study, a commercially made anti-mPIP polyclonal antibody (Alpha Diagnostics Intl. Inc., San Antonio, TX), designated as mPIP Ab-2, generated in rabbit against a 20 amino-acid peptide encoding the C-terminus region of the mPIP protein was used to identify the mPIP protein by Western blot analysis. The specificity of the antibody was demonstrated by probing a blot containing known positive mouse saliva controls with pre-immune rabbit serum (Figure 13.A). Both the sensitivity and specificity of the mPIP Ab-2 were higher than that of an anti-mPIP antibody previously used in our laboratory.

A single protein of approximately 14kDa molecular weight was detected in both male and female mouse submaxillary, lacrimal and parotid gland tissue lysates (Figure 10)

with the mPIP Ab-2. Lacrimal glands showed the highest level of mPIP protein expression of all the tissues analyzed. The presence of secreted mPIP in mouse saliva was established by Western blotting using this new antibody (Figure 13.A) and used as a positive control for Western blot analysis of mouse tissues (Figure 10). For the first time we demonstrated that mPIP is also secreted into mouse tears (Figure 13.B).

Immunohistochemical analysis of adult mouse submaxillary and lacrimal glands using the mPIP Ab-2 showed positive cytoplasmic staining of serous acinar cells of both tissues (Figure 11). No staining was observed in the mucous acinar cells of the submaxillary gland. Therefore, the higher level of expression of mPIP identified by Western blot analysis in the lacrimal gland when compared with submaxillary gland may be explained by the fact that that the lacrimal gland contains mainly serous acini whereas the mouse submaxillary gland is a mixed gland and that mPIP is not expressed in the mucous acinar cells.

Differences between human and mouse PIP genes have been previously identified. Human PIP occurs as a unique functional gene localized on the human chromosome 7q32-36 (Myal et al., 1989), whereas mouse PIP belongs to a cluster composed of five active genes (PIP, SVA and SVAL1-3) out of which SVA is expressed in the seminal vesicles and colon and SVAL-2 in the lactating mammary gland, in addition to the lacrimal and salivary gland expression (Osawa et al., 2004). Therefore, it has been suggested that different members of the mPIP gene cluster have evolved in order to perform different functions in various secretory body fluids, such as saliva, milk, and seminal plasma (Osawa et al., 2004).

Our results further delineate differences in the pattern of expression at the protein level between mouse and human PIP. Although human PIP is present in a variety of physiological fluids such as saliva, seminal plasma, amniotic fluid and breast milk (Autiero et al., 1991; Autiero et al., 1995; Haagensen, Jr. et al., 1980; Murphy et al.,

1987b; Myal and Shiu, 2000), we did not observe mouse PIP protein in mouse body fluids other than saliva and tears (Figures 10 and 13). This finding is consistent with the pattern of mPIP gene expression (Myal et al., 1994; Osawa et al., 2004), suggesting once again that aside from mouse salivary and lacrimal glands the levels of mPIP must be lower or non-existent in these other bodily secretions.

2. Isolation of mPIP protein

2.1. Production of recombinant mPIP protein in bacteria

To conduct various studies that could further our insight about the PIP protein, an adequate supply of isolated mPIP protein was required.

The production and isolation of recombinant mPIP protein was initially attempted using *E. coli*, the most common system used for expression of eukaryote proteins. The Rosetta 2(DE3) *E. coli* strain was chosen as a host because it carries tRNA genes for decoding mammalian codons which are rarely used in *E. coli*. Seven such codons were identified in the 131 amino-acids of the $(His)_6$-tagged mPIP protein. Subsequently, the expression of $(His)_6$-tagged mPIP protein using this system was successfully induced (Figure 16.A) by this strategy. However, most of this protein was insoluble, in the form of inclusion bodies (Figure 16.B). Thus, only low amounts of soluble $(His)_6$-tagged mPIP could be purified from the bacterial cells as determined by Western blot analysis (Figure 16.B). It was therefore necessary to redirect our efforts towards other strategies that would result in a soluble usable protein.

Attempts to express recombinant human PIP in bacteria resulting in the production of a misfolded insoluble protein have been documented by others (Caputo et al., 1999), suggesting that our result is not unexpected. Formation of insoluble protein aggregates

known as inclusion bodies is the most common problem which occurs when *E. coli* systems are used for production of recombinant proteins (Baneyx, 1999; Cutler, 2004).

Attempts to denature the inclusion bodies in order to solubilize the protein, as this process usually results in a loss of biological activity of the products, were not pursued. Retaining protein function is important, particularly to address interactions of mPIP with bacteria. However, denatured purified mPIP protein could have been useful for quantitation purposes.

2.2. Purification of mPIP from mouse saliva by immunoaffinity chromatography

Immunoaffinity chromatography was another strategy attempted to purify mPIP from mouse saliva as mouse PIP is abundant in mouse saliva and the new mPIP Ab-2 was highly specific and sensitive in detecting mPIP (Figures 10 and 13). Immunoaffinity chromatography is considered one of the most powerful techniques for isolation of proteins (Harlow and Lane, 1988).

Although the mPIP Ab-2 polyclonal antibody was successfully linked to a solid matrix consisting of protein A agarose beads (Figure 17.A), problems arose during the protein purification process. It appeared that the mPIP Ab-2, though very specific for Western blot and immunohistochemical analyses, did not recognize the native mPIP in mouse saliva. The lack of the affinity of the anti-mPIP antibody to the native protein could be due to the fact that the antibody was raised against a 20 amino-acid peptide representing the C-terminal end of the mPIP and its accessibility in the native protein is not known. Therefore, the epitope recognized by our antibody might be inaccessible for the IgG molecules coupled to the column.

To our knowledge, this is the first attempt to purify mPIP from a native source of protein. However, several research groups experienced purification difficulties of human PIP from body fluids such as human seminal plasma and breast gross cystic disease

fluid, demonstrating that it is a difficult task, attributable to the tendency of the PIP protein to aggregate and form multimeres or complexes with other protein species (Autiero et al., 1991; Haagensen, Jr. et al., 1979).

An anti-mPIP antibody which could be raised against the entire native mPIP protein may be a valuable alternative of purification of mPIP using immunoaffinity chromatography. However, because of the sequence homology between mPIP and hPIP, there may be a similar tendency to form protein aggregates and mPIP may be equally difficult to purify in its native form required for functional studies.

3. Quantification of mPIP in mouse saliva

3.1. Quantification of mPIP in mouse saliva by ELISA

To date, there is no available method to determine the levels of mPIP protein secreted in mouse body fluids such as saliva and tears. The levels of hPIP in human saliva were previously determined to be 10-70 μg/ml by radioimmunoassay (Haagensen, Jr. et al., 1979). Currently, the use of RIA has been generally replaced by ELISA (Enzyme-Linked ImmunoSorbent Assay), with the antigen-antibody complexes being measured by colorimetric signals instead of radioactivity (Lequin, 2005). Since the values of hPIP levels in saliva were available for comparison, I developed an indirect ELISA for quantification of human PIP as a point of reference. The level of hPIP in saliva was determined to be 2-97 μg/ml, similar to previously published values for hPIP (Haagensen, Jr. et al., 1979), validating this strategy as a method for accurately quantifying PIP in saliva.

Development of an indirect ELISA for quantification of mPIP provided values of salivary mouse PIP (39.7-153.2 μg/ml) comparable to the human levels. In addition, this

method was very successful for validating the absence of mPIP in saliva of mPIP knockout mice when compared with wild-type controls. Unexpectedly however, was the finding that when used to compare the levels of mPIP in different dilutions of mouse saliva, the OD readings obtained by indirect ELISA did not vary according to the level of dilution of the sample (Figure 18). In addition, the maximum OD values produced by the mouse saliva samples were below 0.18, regardless of the dilution used (Figure 18).

It is possible that the difference observed between the two assays was due to the lack of an anti-mPIP antibody that would recognize the native protein (like the anti-hPIP antibody). In the assay for quantification of mPIP, epitopes recognized by our antibody on the antigen used for coating the plates (Figure 7), might be inaccessible for the IgG molecules in the folded protein. Therefore, an antibody raised against the full length protein may increase the chance of the antigen linked to the surface of the plate being recognized by the antibody. In addition, the human samples used in the assay consisted of frozen, unstimulated whole saliva, whereas the mouse samples were obtained by stimulating salivary secretion with pilocarpine, a parasympathetic agonist, whose effects on the composition and properties of saliva are not known.

Competitive ELISA (Figure 8) was selected as an alternative method in an attempt to overcome the possible problems mentioned above generated by using saliva to coat the plates. When this assay was used, consistent positive values for mPIP were found in saliva of mPIP knockout mice which do to contain the protein (Figure 20.B, sample KO3). This was very puzzling. These false positive values suggest the presence of some non-specific interference of the anti-mPIP antibody with another salivary component, resulting in an inhibitory effect on the assay. Altogether, we were unable to overcome the problem and to accurately quantify mPIP in mouse saliva using either indirect or competitive ELISA techniques.

3.2. Quantification of mPIP in mouse saliva by blotting techniques

Slot blot analysis was a viable alternative to ELISA for quantitation of the levels of mPIP in mouse saliva, as the protein samples are usually denatured. Denaturation would destroy any existing protein complexes and unfold native proteins, thus facilitating the access of the antibody to the antigen. Using slot blot analysis, a standard curve of different amounts of SMGP peptide (0.625-20 μg) was successfully generated and the absence of mPIP in saliva of mPIP knockout mice was again verified, thus validating the reliability of this assay (Figure 22). The average mouse saliva sample was found to contain the equivalent of 54.1μg/μl SMGP peptide. Relative quantification of mPIP in male and female individuals was performed by Western blot analysis. Saliva samples were retrieved from 4 mice from each gender. Similar mPIP levels were detected in saliva of male and female mice (Figure 21).

Based on the assumptions that the mPIP Ab-2 possessed the same level of affinity for the SMGP peptide and the denatured salivary mPIP protein, the ability of salivary mPIP to bind to the nitrocellulose membrane was similar to that of the peptide, and the amounts of peptide used to determine the standard curve were accurate, these results are equivalent to 305.4 μg mPIP per μl of mouse saliva. While stimulated mouse saliva samples usually contain below 10 μg/μl of total protein (as determined by BCA protein assay; for example see Figure 10 where 7.5 μl mouse saliva corresponding to 30 μg total protein was loaded on a lane for Western blot analysis), the results of the slot blot analysis were inaccurate. These could be tested by measuring the concentrations of the standards used and collecting the fractions of the samples flowing through the membrane, followed by determining the presence of mPIP protein in those fractions. However, it was difficult to identify any differences between the levels of affinity of the antibody for the SMGP peptide and the secreted mPIP protein in mouse saliva.

Therefore, at the present time, attempts to accurately quantify mPIP using either blotting or ELISA techniques have been unsuccessful.

4. Analysis of the mPIP knockout mice

4.1. Morphopathological analysis of the mPIP knockout mice

The null mutation in the mPIP knockout mice was confirmed by demonstrating the absence of mPIP in knockout mouse saliva by numerous approaches: Western blot analysis (Figure 13), indirect ELISA and slot blot analysis (Figure 22). As well, the absence of mPIP in serous acinar cells of the submaxillary gland was determined by immunohistochemistry (Figure 14).

The mPIP knockout mice did not show any obvious phenotypic differences when compared with the wild-type mice. The submaxillary and lacrimal glands appeared normal, suggesting that mPIP is not essential for their development. However, histological analysis revealed increased lymphocytic proliferation in both submaxillary and prostate glands of knockout mice (Figure 21), suggesting a possible role in immune host defense. Interestingly, human PIP was shown to be involved in activity of lymphocytes by inhibiting $CD4^+$ T lymphocyte apoptosis, suggesting a functional relevance of this protein in defense against infections or tumors (Gaubin et al., 1999).

4.2. The influence of the mPIP null mutation on mouse oral flora

Analysis of the mPIP knockout mouse oral flora compared with wild-type controls was carried out to determine whether the previously shown interaction of mPIP with oral bacteria (Lee et al., 2002) has any effect on quantity or composition of mouse oral flora.

4.2.1. Quantitative differences

Quantitative differences between mouse oral flora of 8 and 9 months old male mPIP knockout mice were identified and significantly more oral bacteria resided in the oral cavity of wild-type mice compared with mPIP knockouts (Figure 24.B). Such differences were no longer observed when the same animals were analyzed at 11 months of age. In females, no significant differences were identified between the amounts of oral resident bacteria of mice analyzed at three different ages (Figure 24.A).

The fact that the differences in the amount of oral bacteria of the male mice were not found as being significant when the animals reached 11 months of age, despite living together in the same environment, may be explained by previous results showing that mouse oral flora varies with age (Wolff et al., 1985). In addition, environment has been shown to mask the effects of the genetic background of animals (Gadbois et al., 1993).

A sexual dimorphism has been identified in the amounts of mouse resident oral flora (Figure 24), suggesting a possible hormonal influence to the effect of presence or absence of mPIP in mouse saliva. This is not an isolated finding, as differences exist between the activity of the mouse male and female salivary glands (Pinkstaff, 1998; Tucker, 2007) and the oral microbiota of mice has been found to be influenced by hormonal changes such as those induced by pregnancy and lactation (Coulombe and Lavoie, 1995).

4.2.1. Qualitative differences

The oral flora composition of mPIP knockout mice revealed differences when compared with the flora of wild-type mice in both male and female individuals. Irrespective of gender, *Streptococcus* represented a higher proportion of the oral bacteria of mPIP knockout mice (Table 3). In addition, the absence of certain genera from the oral cavity of these mice was observed. Isolation the bacterial strains, which

were then characterized, was performed using a stratified sampling method consisting of sub-culturing proportional numbers of samples from each type of colony of mouse oral bacteria. Therefore, it is possible that some bacterial genera underrepresented in the oral cavity of mPIP knockout mice have been deemed as absolutely absent, when in reality it was not the case. Underrepresentation of certain genera in the normal resident flora has an important functional significance because antibodies specific the indigenous flora may cross-react with external pathogens, playing a role in host immunity (Liljemark and Bloomquist, 1996). For example, antibodies to *Neisseria meningitidis*, a common causative agent of meningitis, have been found in human individuals colonized with the normal resident species of *Neisseria* (Liljemark and Bloomquist, 1996). Resident *Neisseria* have been isolated from the in the oral cavity of wild-type male mice but not from the corresponding mPIP knockout mice, representing a lack of a host protection factor in the mPIP knockout mice. Therefore, it is possible that mPIP might play a role in non-innate host defense by modulating the resident oral flora.

It is known that oral flora benefits the host in different ways, protecting the organism against disease (Liljemark and Bloomquist, 1996). While our laboratory animals are housed in a very clean constant environment, it is possible that a phenotypic difference between the mPIP knockout mice and wild-types may become evident when the animals immune systems are challenged by external pathogens, such as induced infection with virulent pathogens.

5. Bacterial aggregation assays – *in vitro* analysis of interaction of mPIP with oral bacteria

Previous work from our lab (Lee et al., 2002) has demonstrated that mPIP binds to human and mouse oral bacteria with the highest affinity for streptococci. However, the

consequences of this interaction were not known. Possible reasons for such binding of mPIP to bacteria may be that it facilitates bacterial aggregation, adhesion to oral tissues, or lysis, as a mechanism of innate host defense. Aggregation is thought to promote the clearance of bacteria from the oral cavity (Mandel, 1979). Also, the effect of different components of saliva on the aggregation of oral streptococci has previously been explored (Ligtenberg et al., 1990a; Ligtenberg et al., 1990b; Yamaguchi, 2004).

To address the possibility that mPIP plays a role in aggregation of oral bacteria, we examined whether saliva from wild-type mice induced aggregation of oral bacteria compared to saliva from mPIP knockout mice. Mouse saliva from mPIP knockout mice induced a lower level of aggregation of all three oral bacterial strains tested when compared with wild-type mouse saliva (Figure 26.A-C). Therefore, mPIP contributes to saliva-induced bacterial aggregation demonstrating its role in host defense by modulating the resident oral flora.

VII. SUMMARY

1. Expression of the mPIP protein in lacrimal, submaxillary, and parotid glands of both male and female adult mice was confirmed by Western blot analysis using a newly generated anti-mPIP specific antibody (mPIP Ab-2) that worked well in our hands. The presence of secreted mPIP in mouse saliva was also verified and the presence of secreted mPIP in mouse tears was demonstrated for the first time.

2. Using the polyclonal antibody mPIP Ab-2, the localization of mPIP was confirmed by immunohistochemical analysis as a diffuse cytoplasmic staining in the serous acinar cells of the mouse lacrimal and submaxillary gland.

3. Attempts to purify mPIP protein from either native or recombinant sources were unsuccessful.

4. Quantification of mPIP in mouse saliva by either ELISA or slot blotting techniques was not successful. However, quantification human PIP in saliva by indirect ELISA was successful, suggesting that there may be other mitigating factors in either mouse saliva or mPIP Ab-2 that complicated this approach.

5. Null mutation in the mPIP knockout mice was confirmed by PCR, Western blot analysis, immunohistochemistry, slot blot analysis and indirect ELISA.

6. Morphopathological analysis of the mPIP knockout mice revealed increased lymphocytic proliferation in both submaxillary and prostate glands.

7. Both quantitative and composition differences in the oral flora of mPIP knockout mice in comparison with wild-type controls were identified.

8. The mPIP protein was shown to contribute to aggregation of oral bacteria.

VIII. FUTURE DIRECTIONS

1. Further elucidation of the functions of mPIP through *in vitro* studies requires the development of an accurate method for physiological determination of mPIP in body fluids and identification of a reliable source of purified mPIP protein. An anti-mPIP antibody raised against the entire native mPIP protein may represent a valuable tool in both purification and quantitation of mPIP.

2. The lymphocytic proliferation in submaxillary and prostate glands of mPIP knockout mice needs to be further investigated by further analysis of animals of different genders.

3. While a possible role of mPIP in host defense has been proposed and considering that laboratory mice are living in an almost sterile environment lacking contact with external pathogens, the immune systems of mPIP knockout mice need to be challenged by external factors, such as induced infection with virulent pathogens, in order to delineate a possible phenotypic difference between the mPIP knockout mice and wild-type controls**.**

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