Developmental and Functional Analysis of the Mouse Submaxillary Gland Protein (mSMGP)

By

Beverley Lee

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

Master of Science

Department of Pathology
Faculty of Medicine
University of Manitoba
Winnipeg, Manitoba

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ABSTRACT

The mouse submaxillary gland protein (mSMGP) gene is highly expressed in the submandibular and lacrimal glands of the adult mouse. The mSMGP was first isolated as an abundant mRNA species in the mouse submandibular gland by Windass et al. in 1984. Analysis of the SMGP between several species has shown that the SMGP sequences are conserved, suggesting that it may have an important role in submandibular gland biology (Myal & Shiu, 2000). The human homologue of the mSMGP, prolactin-inducible protein (hPIP) has been found in saliva, tears, sweat, seminal plasma, and submucosal glands of the lung. More recently, binding of hPIP to certain bacterial strains found in the oral cavity, ear canal and human skin has been reported (Schenkels et al., 1993 &1997). Therefore, it has been suggested that the hPIP may be involved in non-immune host defense. Since the hPIP and the mSMGP share a 51% homology on the amino acid level, this current study hypothesized that the role of the mSMGP may be involved in non-immune host defense of the mouse oral cavity. As a first step in elucidating the role of the mSMGP in the submandibular gland, gene expression in the developing mouse was examined. Using reverse transcriptase polymerase chain reaction (RT-PCR), Southern and Northern blot analyses, mSMGP gene expression in the prenatal submandibular gland was detected as early as embryonic day 14 (E14), the approximate time at which submandibular gland development begins. By *in situ* hybridization studies of the embryonic gland, mSMGP gene expression was localized to the acinar cells, the major secretory cells of the submandibular gland. The early and continued expression of the mSMGP gene in the embryo suggests that the mSMGP may be integral during submandibular gland development in the mouse.

Sequence analysis of the mSMGP in our laboratory has revealed the presence of a signal peptide, suggesting that the mSMGP is a secreted protein (Myal et al., 1994). Antibodies to the mSMGP have been generated and through Western blot analysis, we have indeed detected the mSMGP in mouse saliva. To further investigate the role of this secreted protein in the mouse oral cavity, binding interactions with bacteria were carried out. Several bacteria obtained from the mouse oral cavity were characterized and found to belong in the genus *Streptococcus*, *Aerococcus*, *Vibrio, Aeromonas, Sphingomonas, Pseudomonas* or *Staphylococcus.* These bacteria were further incubated with ^{35}S-labeled *in vitro* translated mSMGP. The mSMGP was found to bind specifically to certain strains with some selectivity, showing the highest affinity to the *Streptococcus* species.

Since the function of the mSMGP is still undetermined, this present study suggests an important role for this novel protein during embryonic development and provides insight into a possible involvement of the protein in host defense. The discovery of the biological role of the mSMGP in the submandibular gland will further increase our understanding of this complex organ.

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ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometer
$(NH_4)_2SO_4$	Ammonium sulphate
°C	Degree Celsius
10X	Ten times
1X	One time
2X	Two times
^{32}P	Phosphorus-Isotope 32
^{35}S	Sulphate-Isotope 35
4X	Four times
5X	Five times
bp	Base pairs
BSA	Bovine Serum Albumin
CaCl	Calcium Chloride
cDNA	Complimentary Deoxyribonucleic Acid
CO_2	Carbon Dioxide
cpm	Counts per minute
dCTP	Deoxycytosine Triphosphate
ddH_2O	Deionized distilled water
DEPC H_2O	Diethylpyrocarbonate Water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetate
ER-α	Estrogen Receptor alpha
g	Gravity
GRB	Gel Running Buffer
H_2	Hydrogen
HCL	Hydrochloric Acid
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulforic Acid
hPIP	Human Prolactin-Inducible Protein
K_2HPO_4	Potassium phosphate
KCL	Potassium Chloride
kD	Kilodaltons
kg	Kilogram
KH_2PO_4	Potassium phosphate monobasic
LB	Luria-Bertani
M	Molar

MAP	Multiple antigen peptide
mg	Milligram
mGAPDH	Mouse glyceraldehyde-3-phosphate dehydrogenase
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium sulphate
ml	Millilitre
mM	Millimolar
mm	millimeter
MMLV	Moloney Murine Leukemia Virus
MOPS	3N-morpholino-propanesulfonic acid
mSMGP	Mouse Submaxillary Gland Protein
N_2	Nitrogen
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NaH_2PO_4	Sodium phosphate
NaOAc	Sodium acetate
NaOAc	Sodium Acetate
NaOH	Sodium Hydroxide
ng	Nanogram
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PTC	Programmable Thermal Controller
RNA	Ribonucleic Acid
rPIP	Rat Prolactin-Inducible Protein
RPM	Revolutions Per Minute
RT	Reverse Transcriptase
RTF	Reduced Transport Fluid
SDS	Sodium dodecyl sulfate
SMG	Submandibular Gland
SSC	Standard Saline Citrate
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA
TBST	Tris Buffered Saline, Tween
Tris-Cl	Tris Chloride
UTP	Uridine triphospate
V	Volts
v/v	Volume per volume
w/v	Weight per volume

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1. INTRODUCTION

1.1. The Submandibular Gland

1.1.1. Structure of the Mouse Submandibular Gland

The submandibular gland, the largest of the three major salivary glands in the mouse, is a complex organ that plays a vital role in the maintenance of the health of the organism. The submandibular gland does not only contribute to the production of saliva; it also provides a variety of components needed for many different biological processes. It is the source of many essential proteins necessary for such functions as cell growth and differentiation, homeostasis, intracellular regulation, and digestion (Barka, 1980).

The histological structure of the rodent submandibular gland was first described in 1926 (Tupa, 1926). The gland is a compound, "tubuloacinar" structure connected to the oral cavity by a complicated duct system involving four morphologically distinct segments. Secretory tubules, capped by acini, make up the first part of the branching duct system (Pinkstaff, 1980) and are found at the end of a second segment called the intercalated duct. This intercalated duct is connected to the granular convoluted tubule, which then leads into a striated excretory duct and into the mouth (Figure 1).

The submandibular gland comprises of two main cell types, the acinar and ductal. The acinar cells are the major secretory endpieces that generate all the saliva fluid and approximately 85% of exocrine proteins in the gland (Sabbadini & Berczi, 1995). There are two major types of acinar cells, serous and mucous, each defined by the physical consistency of the secretions produced (Denny et al., 1997). Mucous acinar cells secrete viscous material largely made up of mucins, large glycoproteins that comprise of about 70% of carbohydrates (Denny et al., 1997; Navia, 1977).

Conversely, serous acinar cells produce watery secretions that are made up of a general variety of proteins and lack mucins. In the submandibular gland, the acini are of mixed type, consisting of both serous and mucous acinar cells, although serous cells predominate. This distinguishes the gland from the parotid gland, in which only serous acinar cells are found. As in the acini of the submandibular gland, those of the sublingual gland are also of mixed type. However, the predominant secretory cells in the latter are mucous acinar cells (Eroschenko, 1996).

The submandibular glands are usually larger in the male compared to that of the females (Atkinson et al., 1959; Raynaud, 1964; Chai et al., 1993). However, sexual dimorphism of the submandibular gland is apparent not only in respect to the size, but also on the histological and biochemical level. The ductal segments between the acinar cells and striated excretory ducts vary with the endocrine state depending on the sex (Junqueira et al., 1949). In adult mice, granular convoluted tubules are found to be larger and more numerous in the males than the females (Lacassagne, 1940; Fekete, 1941; Gresik, 1980). They occupy about 50% of the gland's volume in the male, whereas the proportion in the female is only 15-20% (Barka, 1980). The average volume of acinar cells was found to be much greater in females than in males (Chai et al., 1993). This difference is thought to be an indirect effect due to compression of the acinar cells by the enlarging granular convoluted tubule cells in the male (Chai et al., 1993).

1.1.2. Development of the Mouse Submandibular Gland

Development begins in the embryo at approximately day 14 of gestation (Kaufman & Bard, 1999). More recently, it has been reported to begin as early as day 12 (Macauley et al., 1997; Jaskoll & Melnick, 1999). Development starts as an *Initial Bud stage* where thickening of the oral epithelium and a downgrowth of this

undifferentiated cell mass into the surrounding mesenchyme occurs. This initial bud elongates into a cord-like structure, which will ultimately give rise to the excretory duct. At the distal end of this structure, a solid "terminal bulb" is found (Cutler & Chaudhry, 1974). As growth continues into the mesenchyme, this cell mass interacts with the newly formed basement membrane situated at the mesenchymal-epithelial interface. This mesenchymal invasion stimulates inductive interactions between the bud and the surrounding mesenchyme, producing small cleavages in the terminal bulbs. As a result, these terminal bulbs begin to bifurcate, an indication of the onset of branching (Cutler & Chaudhry, 1974; Kaufman & Bard, 1999). Several branches then extend from the main stalk and each terminates in one or two cellular "end buds", resulting in a pseudo-lobular appearance. This stage is known as the *Pseudoglandular stage* where the branching epithelium is surrounded by closely packed mesenchyme (Jaskoll & Melnick, 1999). Development continues into the *Canalicular stage*. In this stage, the lumens begin to form within the branched stalks, then elongate and form lobules, called "terminal tubules". Some of these cells from the terminal tubules begin to contain accumulations of irregularly shaped secretory granules in the apical region, and are referred to as Type I proacinar cells (Cutler & Chaudhry, 1974). Loosely packed mesenchyme surrounds the terminal tubules and lumena begin to form in the terminal end buds only at the end of this stage. Finally, in the following stage called the *Terminal Bud Stage,* distinct lumena can be seen in the end buds and these lumena eventually become continuous with the ductal lumena (Jaskoll & Melnick, 1999). At this point, the Type I proacinar cells have evolved into a second granule cell type (Type II proacinar cells) and can be observed just prior to birth. These cells in turn develop into Type III proacinar cells, which appear in increasing numbers in the first postnatal week (Cutler & Chaudhry, 1974). As

development continues, maturation of these proacinar cells progresses into one more stage (Type IV) before reaching the mature adult form (Type V). These stages, from Type I to V are morphologically distinguishable as different granule types and differentiation continues into three weeks after birth, when both Type IV proacinar cells and mature acinar cells are present. While the acini develop from the end buds, the terminal tubules gradually lose their granules and these segments become converted into the final arrangement of the intercalated duct system (Gresik, 1980). The number of Type V acinar cells increases as the animal reaches maturity until they are the only type of cells seen in the gland (Cutler & Chaudhry, 1974). While acinar cells are already apparent during prenatal development, granular convoluted tubules are not present in the duct system until at least the beginning of postnatal day 20 (Gresik, 1980). As well, sexual dimorphism is not apparent until later in adult life (at approximately day 60), when sexual maturity has been reached (Harvey, 1952; Gresik, 1980).

1.1.3. Protein and Gene Expression in the Mouse Submandibular Gland

Development of the submandibular gland involves a complex and well-orchestrated epithelial-mesenchymal interaction and expression of extracellular matrix molecules that control morphogenesis and cytodifferentiation of the epithelium. Some of these matrix molecules include collagen, fibronectin, laminin B_2 and elastin (Macauley et al., 1997). Gene and protein expression of these extracellular-matrix proteins were detectable as early as embryonic day 16 (Macauley et al., 1997). Several other proteins, such as Muc-1 mucin and amylase, have also been reported to be expressed early in embryonic development. Muc-1 was found to be expressed as early as embryonic day 15 (Braga et al., 1992), and amylase, a digestive enzyme, at embryonic day 18 (Poulsen et al., 1986). Interestingly, the

expression of these genes correlated with important developmental stages of the submandibular gland involved with epithelial differentiation and initial branching (Macauley et al., 1997). Mucins in general, are thought to play a protective and/or lubricative role in secretory epithelial tissues (Braga et al., 1992). In humans, mucins have been shown to interact with host cells such as gingival fibroblasts, to modulate wound healing, to have an effect on mineralization, to form complexes with other salivary molecules, such as the antimicrobials lysozyme and secretory immunoglobulin A (sIgA) and to modulate the oral colonization of a variety of microorganisms (Scannapieco & Levine, 1993).

Mouse submandibular glands are also known to synthesize many other important proteins including the nerve growth factors and the epidermal growth factors. Nerve growth factor (NGF) plays an important role in the development and functional maintenance of sympathetic and some sensory neurons and is found in high concentrations in this gland (Barka, 1980). Epidermal growth factor (EGF) has many functions including that of a mitotic stimulant for a variety of cell types and an accelerator of tooth eruption, eyelid opening and skin thickening in young mice (Kaplan & Brewar, 1983). This growth factor is found within the granular convoluted tubule cells of submandibular glands and is first detectable around postnatal day 20 (Barka, 1980). Other major proteins synthesized in the mouse submandibular glands include kallikrein, renin and a variety of digestive enzymes such as amylase and proteases (Barka, 1980). Many of the submandibular gland products are secreted via ducts into the saliva. However, this gland can also act as an endocrine organ as it synthesizes several hormones such as glucagons and erythropoietin (Barka, 1980). The spatial and temporal gene expression of many submandibular gland proteins have been studied extensively (Poulsen et al., 1986; Braga et al., 1992; Gresik et al., 1997;

Macauley et al., 1997; Denny et al., 1999; Jaskoll & Melnick, 1999). Some of these proteins are hormonally regulated and others are not (Barka, 1980). Some are synthesized and secreted by the acinar cells while others originate from the granular convoluted tubule cells (Barka, 1980). In addition, the functions of several of these proteins are not yet known. These facts demonstrate the complexity involved in defining the function of this gland. Therefore, establishing gene and protein expression in the submandibular gland during development is crucial, as this information will provide important clues into how this organ ultimately forms and functions.

1.2. The Mouse Submaxillary Gland Protein (mSMGP)

1.2.1. Cloning and Sequencing of the mSMGP gene

One of the genes that are highly expressed in the submandibular glands is the mouse submaxillary gland protein (mSMGP). This gene was first isolated as an abundant mRNA species in the mouse submandibular gland by Windass et al. (1984). This novel mRNA species is approximately 700 bases long by Northern blot analysis while sequencing revealed the cDNA clone to be of 576 base pairs (bp) (Windass et al., 1984, Myal et al., 1994). A 14 kD protein was obtained by *in vitro* translation of this mRNA species (Windass et al., 1984). This protein was identified as one of the major products of the submandibular glands and its gene expression did not demonstrate sexual dimorphism (Windass et al., 1984). SMGP gene expression was also tissue specific. In the rat and mouse, it was found to be expressed in the adult lacrimal and submandibular glands (Myal et al., 1994). Hormonal studies carried out in the rat demonstrated that SMGP gene expression was inhibited by androgens in the lacrimal glands, but not in the submandibular glands, while prolactin and estrogen had

no effect on SMGP gene expression in either glands (Myal et al, 1994). This lack of hormonal regulation further confirmed the initial observations that gene expression of the SMGP in the submandibular gland was similar in both sexes (Windass et al., 1984). Identification of the cells expressing the SMGP gene within the adult submandibular glands of the rat has also been studied and it has been confirmed to be limited to the acinar cells (Myal et al., 1994).

Southern blot analysis of the SMGP gene in several species including the human, monkey, dog, cow, rabbit, chicken and yeast, has shown that the SMGP gene sequences are conserved, suggesting that it may have an important role in submandibular gland biology (Myal & Shiu, 2000).

1.2.2. Characteristics of the mSMGP

Based on sequence analysis, Windass and colleagues (1984) reported that the putative polypeptide lacked a signal sequence, suggesting that the mSMGP was an intracellular submandibular gland protein. However, following the isolation and sequencing of the mSMGP cDNA, Myal et al. (1994) identified a small sequencing error in the previously reported cDNA. The new sequence revealed that the putative polypeptide contained a 25 amino acid long signal peptide. The overall length of the mSMGP was 146 amino acids and had a molecular weight of 17 kD. Upon cleavage of this signal peptide, a secreted peptide of 14 kD is produced. No glycosylation sites have been identified for this protein. Interestingly, salivary peroxidase, an enzyme known to protect the oral mucosa by inhibiting bacterial growth and detoxifying hydrogen peroxide, exhibits similar expression and distribution patterns as SMGP (Mirels et al., 1998).

1.2.3. The Human Prolactin-Inducible Protein and its Homologues

At the amino acid level, a 51% homology exists between the mSMGP and a human protein, the prolactin-inducible protein (hPIP; Myal et al., 1994). This hPIP is believed to be the human homologue of the mSMGP. The human PIP was initially identified as a novel protein secreted by a human breast cancer cell line, T-47D, in response to prolactin, hence the name prolactin-inducible protein (Shiu & Iwasiow, 1985). Androgen, estrogen and glucocorticoids have also been found to regulate expression of this gene (Murphy et al., 1987a, 1987b, Myal & Shiu, 2000). Independently, another group isolated this protein from gross cystic disease fluid in benign tumors of the breast and thus identified the protein as Gross Cystic Disease Fluid Protein 15 (GCDFP-15; Haagensen & Mazoujian, 1986). Although the hPIP gene is expressed in more than 90% of human breast cancer biopsies, its expression is not found in the normal mammary gland (Myal et al., 1998). Human PIP is synthesized in the apocrine glands of the axilla, perineum, Molls glands of the eyelids and the ceruminous glands of the ear (Mazoujian et al., 1983; Haagensen et al., 1986; Myal & Shiu, 2000) as well as in several exocrine tissues, such as the lacrimal, salivary and sweat glands (Haagensen et al., 1990). In addition, hPIP gene expression has been identified in low levels in healthy human ovaries (Clark et al., 1999). A variety of physiological fluids have also been found to contain hPIP, including saliva, tears, sweat, amniotic fluid, breast milk, blood, and fluid from the seminal vesicle and bronchial submucosal glands (Murphy et al., 1987a). More recently, other groups have isolated proteins identical to the hPIP. These include the extra parotid glycoprotein (EP-GP; Rathman et al., 1989; Schenkels et al., 1993), the seminal actin-binding protein (SABP; Akiyama & Kimura, 1990; Schaller et al., 1991) and the human seminal fluid gp 17 (Autiero et al., 1991, 1995). The function of the

hPIP/GCDFP-15/EP-GP/SABP/gp17 is not known. However, several possible functions have been suggested. First, since this protein has been found in various secretions such as saliva, sweat, tears and seminal plasma (Murphy et al., 1987a), and was recently found to bind to several strains of bacteria, the hPIP was suggested to play a role in non-immune host defense (Schenkels et al., 1993). Secondly, the hPIP has been proposed to provide protection from HIV infection, as it was able to obstruct the interaction between the HIV envelope gp120 with CD4 (Autiero et al., 1991, 1995 & 1997). The hPIP may also play a role in fertilization because it was found associated with fertilizing spermatozoa in seminal fluid (Schaller et al., 1991). In addition, inhibition of CD4-mediated T-cell programmed cell death has been observed after the binding of the hPIP to CD4. This, along with the fact that this protein has been found to stimulate the growth of several human breast cancer cell lines (Gaubin et al., 1999), suggests a potential role of the hPIP in cell survival as an apoptosis inhibitor and mitogen (Autiero et al., 1995; Cassoni et al., 1995).

1.2.4. The Rat Prolactin-Inducible Protein

Recently, a rat homologue to the mSMGP, rat Prolactin-Inducible Protein (rPIP) has been cloned. At the amino acid level, the rPIP has 56% overall identity and 80% similarity to the mSMGP (Mirels et al., 1998; Figure 2). rPIP gene and protein expressions were analyzed in neonatal and adult submandibular, sublingual and parotid glands (Mirels et al., 1998). The levels of gene expression appeared to be similar between postnatal days 0 to 7, increasing from days 7 to 14 and finally reaching a constant level throughout adulthood. The secreted rPIP was found to be 17 kD in size, with N-glycosylated forms of 20-22 kDs. Protein expression of the rPIP was localized to the developing and mature acinar cells of the submandibular and parotid glands (Mirels et al., 1998). In addition, low levels of this protein were

detected in the acinar cells of the sublingual gland (Mirels et al., 1998). Whether expression of this protein is hormonally regulated in these tissues is not known.

1.3. Saliva

1.3.1. The Composition of Saliva

In humans and animals, salivary glands are composed of highly differentiated epithelial cells that can secrete fluid made up of water, electrolytes and proteins (Sabbadini & Berczi, 1995). Secretions from the salivary glands, along with the gingival crevicular fluid (fluid originating from the crevice between the teeth and gingiva (gums)), contribute to the complex mixture of the oral fluid known as 'whole saliva'. This term is commonly used to describe the fluid normally present in the mouth, whereas the term 'duct saliva' refers to the fluid flowing from the individual glands (Edgar, 1992). The secretions from the submandibular glands in particular, contribute many biologically active proteins to saliva. In the mouse, these proteins have been classified into several functional categories, including those involved in growth and differentiation, homeostasis, intracellular regulation and digestion (Barka, 1980). Furthermore, several of these proteins (i.e. mucins) have been implicated in anti-microbial activity in the oral cavity, playing a role in host defense.

1.3.2. The Functions of Saliva

Before foreign organisms invade the host, they must first pass the external barriers. Like the skin and mucous membranes of the eyes, ear and lungs, the oral cavity acts as the first line of defense for the host (Campbell, 1993). The constituents of the secretions (tears, sweat and saliva) from these structures, plays major roles in combating these foreign invaders.

The functions of saliva extend beyond the obvious role of keeping the oral tissues hydrated and lubricated. Saliva acts as a permeability barrier by coating the oral tissues, flushes the oral cavity and modulates the composition of the oral flora (Levine et al., 1987). Moreover, saliva moistens ingested food and lubricates the bolus to facilitate its swallowing and passage through the esophagus (Tabak, 1995; Eroschenko, 1996). Because the mouth is the port of entry to the gastrointestinal system, regulation of its colonization by bacteria is important to maintain the health of not only the oral cavity itself, but of the entire organism. Saliva controls oral colonization by immune and non-immune host defenses (Marsh & Martin, 1992; Xu & Oppenheim, 1993) (Table 1).

In the immune system, the cells involved in specific antigen recognition are the T- and B-lymphocytes (Kuby, 1992). Both T- and B-cell precursors originate from a common multipotential hematopoietic stem cell and differentiation into T- or B-lymphocytes occur mainly in the embryonic thymus or liver, respectively (Gallagher & Miller, 1988). Development of the thymus begins between E10 and E11 of embryonic development (Douagi et al., 2000). Although T cell precursors are already detected in the thymic rudiment by E11, the first mature T-cells are not detected until E18 (Ema et al., 1997). Liver development begins approximately at E8.5 (Kaufman & Bard, 1999) and this organ becomes the main site where hematopoietic precursors proliferate and differentiate (Ema et al., 1998). However, these precursors do not populate the liver until E10 to E11 (Cumano et al., 1993). Moreover, at E12, B-cell precursors in the embryonic liver remain uncommitted, where at least half of them are able to give rise to both B-cells and macrophages (Cumano et al., 1993). Fundamental components of oral immune defense are the salivary immunoglobulins (predominantly sIgA), which aggregate specific bacteria

and prevent their adhesion to oral hard and soft tissues (Edgar 1992). The first detectable sIg-bearing B-lymphocytes have been reported to occur at E17 (Delassus et al., 1998).

The non-immune defense system, on the other hand, encompass a wide array of components that combat bacteria in a variety of ways. For example, salivary lysozyme has the potential to hydrolyze the peptidoglycan component that provides rigidity to bacterial cell walls. Also, the salivary peroxidase enzyme system (sialoperoxidase) produces hypothiocyanite at neutral pH or hypothiocyanous acid at low pH in the presence of hydrogen peroxide (H_2O_2), which inhibit glycolysis in bacteria (Marsh & Martin, 1992). Rodent peroxidase activity was first detected in the submandibular gland at E17 (Yamashina & Barka, 1973; Osugi, 1977) and one day after birth in the parotid gland (Redman & Field, 1993). Several proteins such as mucous glycoproteins (MG2) interact with specific bacteria and promote their attachment to oral surfaces, leading to subsequent proliferation of these organisms or through aggregation, promote the oral clearance of others (Gibbons & van Houte, 1975; Tabak et al., 1982; Tabak, 1995). Many other proteins, such as statherins, histatins, histidine-rich polypeptides and proline-rich proteins have also been shown to have specific binding capabilities and are found to be major secretions of human parotid and submandibular glands (MacKay et al., 1984; Lamkin & Oppenheim, 1993; Xu & Oppenheim, 1993; Nagata et al., 1997; Kataoka et al., 1999).

Although the functions of the major salivary proteins, such as sIgA and mucins, are well characterized, the unique roles of many other salivary polypeptides within the oral cavity have yet to be defined.

1.4. Bacteria in the Oral Cavity

1.4.1. The Oral Environment

In humans and animals, the environment of the oral cavity fluctuates under certain conditions, producing a variety of niches that support different organisms. The main components of the oral cavity are the teeth and their supporting structures, the saliva and the flora colonizing the oral tissues (Navia, 1977). Colonization by the oral flora occurs on two distinct tissue surface types within the mouth: the hard tissues (teeth) and the mucosal surfaces (lips, cheek, palate, tongue and gums).

1.4.2. Factors Influencing the Oral Microbial Community

The mouth provides an ideal environment for the growth and survival of bacteria (Marsh & Martin, 1992). There are many factors, such as host species, age, individual habits, the external environment and diet, that influence the oral microbial community at any given time.

The oral flora of several animal species used most commonly as models for dental disease has been examined. Compared to primates, cats and dogs, the oral flora of the mouse is relatively simple (Jordan, 1971; Syed et al., 1982; Trudel et al., 1986; Mikx et al., 1990; Gadbois et al., 1993; Madianos et al., 1994; Renvert et al., 1996; Hudspeth et al., 1999). The flora in the laboratory mouse oral cavity is limited to approximately 18 species (Trudel et al., 1986), whereas hundreds of different species can be found in the oral cavity of primates, cats, dogs or humans. The oral flora of primates is closest to that of humans (Beighton, 1985; Beighton & Hayday, 1986; McMahon et al., 1990).

Although it is established that the composition of the human oral flora becomes more complex as an individual matures (Marsh & Martin, 1992), relatively little is known about changes in the oral flora of other animals as they develop.

However, microbial changes between a periodontitis-susceptible mouse strain (STR/N) and a non-susceptible strain (Swiss-Webster) were compared (Wolff et al., 1985). With advancing age, there was a progressive increase in bone loss in the STR/N mice, which correlated with a significant increase in the proportion of *Streptococcus* spp., *Neisseria* spp., *Actinomyces* spp, and *Haemophilus* species. In contrast, the Swiss-Webster mice did not have any significant bone loss and only demonstrated a significant increase in *Actinomyces* spp. with advancing age. Therefore, not only does the oral flora vary between different mouse strains at any age, the composition of bacteria within the same animal can also fluctuate as it becomes older.

Many habits of mice provide bacteria mechanisms for entry into the oral cavity that may also be found in other animals but not in humans. Mice often groom themselves and their young by licking (Keele, 1977; Trudel et al., 1986). Additionally, members of the *Enterobacteriaceae* are often isolated from the mouse oral flora because mice, like several other animals, are coprophagic (Trudel et al., 1986). These social habits are likely to affect the composition of the oral flora in these animals, as it is well known that social conditions and attitudes among humans influence dental disease and to some extent the composition of the oral flora (Horton & Sumnicht, 1967; Fejerskov & Thylstrup, 1994; Koch et al., 1994; Locker et al., 1997; Moynihan, 2000).

The external environment provided for the experimental animal can also have an effect on the oral microbial community. Microorganisms from their habitat can easily find their way into the animals' mouths. An observation by Keele (1977) revealed that rats as young as 12 days begin exploring their immediate environment by nibbling the cage and chewing the bedding. In mice, Marcotte and coworkers

(1995) discovered variations in the oral flora within the same strain but from different breeders. An unidentified streptococcus was the predominant species in BALB/c mice from Jackson Laboratories, yet this strain was absent from BALB/c mice from Charles River Laboratories (Marcotte et al., 1995). Thus, although mice may be genetically the same and housed under tightly controlled conditions, variations in their oral microflora can still occur when the external environment (breeding facility) is not identical.

The host's diet can also influence the composition of the oral flora by a) its composition, which will select those bacteria that are best suited to utilize the components as substrates, b) the patterns of intake, which determines the frequency of nutrient availability to the oral flora, and c) the food textures, which can influence retention of food within the mouth (Johansson & Birkhed, 1994). For example, dietary sucrose can be broken down into acids and also be converted to glucans and fructans. Glucans contribute to the matrix of dental plaque by strengthening the attachment of bacteria to the tooth surface, while fructans serve as extracellular nutrient storage compounds. Furthermore, the low pH generated by fermentation may also disrupt the survival of the indigenous microflora, as many of these oral microorganisms require a pH around neutrality for growth (Marsh & Martin, 1992). In one study, the effects of varying the concentrations of sucrose, starch, proteins, lipids, vitamins and minerals in the diet of BALB/c mice were examined (Blais and Lavoie, 1990). It was found that the protein content had the most pronounced effect on the indigenous oral bacterial population. *Lactobacillus murinis* (*L. murinis*) demonstrated the fastest growth rate with a high protein diet, whereas the proportion of this species lowered as the protein level decreased.

In addition, the host's diet and nutrition can influence the composition of saliva. Deficiencies in vitamin D, zinc, protein, and caloric intake resulted in reduced salivary protein, atrophy of the salivary glands, or hypocalcemia and hyperparathyroidism (Johnson, 1993). These changes in saliva influence the oral flora by reducing the levels of molecules such as sIgA and mucin, which help to control the microbial community.

1.4.3. The Significance of the Oral Resident Flora

In general, the oral flora, as with the resident flora at other sites in the body, is beneficial to the host. It prevents colonization of the host by exogenous potentially pathogenic organisms by either competing for receptors or ligands for adhesion, competing for essential endogenous nutrients and cofactors, creating micro-environments that discourage the growth of exogenous species or producing inhibitory substances (Marsh & Martin, 1992). For example, some streptococci produce inhibitory levels of hydrogen peroxide, which is active against Gram-negative rods such as *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*), *Capnocytophaga sputigena* (*C. sputigena*), *Fusobacterium nucleatum* (*F. nucleatum*), *Wolinella recta* (*W. recta*), and *Bacteroides* species, all of which are implicated in periodontal disease (Theilade, 1990).

Interactions between the different species present in oral bacterial communities can influence the composition of dental plaque to help maintain a healthy balance (homeostasis) between the oral flora and the host (Marsh & Martin, 1992). Salivary proteins and glycoproteins can be degraded by certain bacterial species to produce carbon and nitrogen, which is then used for nutrition (Beighton et al., 1986; de Jong and van der Hoeven, 1987; Marsh, 1989). Nutrient competition *in vitro* for individual amino acids, peptides and carbohydrates has been shown to

noticeably influence the final proportions of species in mixed continuous culture studies (McKee et al., 1985; Rogers et al., 1987; Marsh, 1989) and similar effects can be shown *in vivo* (Bowden & Li, 1997). Physico-chemical interactions between bacterial species also contribute to formation of a dental plaque community that can be beneficial to the host. For example, colonization by *Veillonella* sp., a genus that utilizes lactic acid and increases plaque pH, is thought to help reduce the cariogenicity of dental plaque (Mikx et al., 1972; Marsh, 1989; Theilade, 1990). This bacterial species cannot adhere directly to the tooth surface, but can coaggregate with other species, such as *Streptococcus salivarius* (*S. salivarius*), for retention and colonization in the oral cavity.

Alternatively, several oral bacteria found in small numbers in a healthy individual can be selected for under certain environments and cause dental disease. These organisms are opportunistic pathogens and only express their pathogenicity under suitable environmental conditions. A classic example is *Streptococcus mutans* (*S. mutans*), an organism strongly implicated as a causative organism of dental caries (Hamada & Slade, 1980; Bleiweis & Oyston, 1993). Sucrose is essential for *S. mutans* accumulation on teeth and initiation of carious lesions on smooth enamel surfaces (Bleiweis & Oyston, 1993). Normally, *Streptococcus sanguis* (*S. sanguis*) and *Streptococcus mitior* (*S. mitior*) are found in higher proportions in dental plaque compared to the concentrations of *S. mutans*. In the absence of sugar, *S. sanguis* and *S. mitior* can inhibit *S. mutans* by producing hydrogen peroxide. However, this "protective" effect is compromised with frequent sugar consumption, leading to accumulation of acids and a lowered pH, favoring the growth of *S. mutans*. Consequently, the cariogenicity of dental plaque is increased as these aciduric bacteria

continue to produce acid to higher final concentrations, which can demineralize the tooth (Theilade, 1990).

Several other species such as *Bacteroides gingivalis* (*B. gingivalis*), *A. actinomycetemcomitans* and *Treponema* sp., have been implicated in periodontal diseases. Growth of these species is favored by a reduction in the quality of the oral hygiene of the host (Theilade, 1990). Furthermore, the pathogenic potential of the resident oral microflora can extend beyond the mouth. Acute or chronic infections commonly occur by mixtures of resident oral bacteria. These infections may spread systemically via the blood or lymphatic systems. Bacteria from dental plaque can be found in the bloodstream when wounds within the mouth expose vessels to infection. Although these organisms are normally removed by host defense mechanisms, a compromised immune system may allow abscesses to develop in the brain, liver or lung. Additionally, individuals with heart disease, which compromises the valves and endocardium, can commonly acquire endocarditis caused by oral bacteria, mainly oral streptococci (Theilade, 1990).

1.4.4. Resident Flora in the Mouse Oral Cavity

Several words have been used to describe the resident oral flora, including normal, indigenous, autochthonous and commensal. These all refer to the mixtures of microorganisms that have the ability to inhabit the oral surfaces and thus are naturally found within the oral cavity. Conversely, the term transient describes the microorganisms that do not remain in the oral cavity for long periods, as they are unable to establish themselves (Bowden et al., 1979). Thus, transient bacteria can be found in the oral cavity at certain times but are removed either mechanically (i.e. swallowing) or physiologically degraded by host defense mechanisms.

The bacteria in the mouth of humans and animals are normally present in biofilms on the soft (mucosal) and hard (teeth) surfaces. The biofilms on the teeth are dental plaque, which is made up of micro-colonies of bacteria within a matrix of carbohydrate and protein polymers (Keele, 1977; Bowden et al., 1979; Costerton et al., 1999; Sutherland, 1999). Several studies have been completed on the accumulation and composition of dental plaque in experimental animals (Wolff et al., 1985; McMahon et al, 1990). Generally, dental plaque accumulation follows a similar pattern of adhesion, coaggregation and growth by selected bacteria in both humans and animals (Bowden & Li, 1997). Although, only a limited amount of literature is available on the development and composition of dental plaque in normal mice (Wolff et al., 1985), Trudel and colleagues (1986) made a thorough examination of the indigenous oral flora of BALB/c mice. They identified 671 isolates from different sites of the oral cavity, saliva, tongue, teeth and mucosa, and found only 18 different species. This observation demonstrated that the mouse oral flora, at least in a laboratory setting, is relatively simple compared to the several hundred present in humans (Theilade, 1990). The predominant species, *L. murinis* and *Staphylococcus aureus* (*S. aureus*), contributed 38% and 37% respectively, of the total cultivable flora. The other species identified are shown in Table 2 (Trudel et al., 1986). In addition, the composition of the resident oral flora in six genetically different mouse strains, BALB/c, C3H/He, C57BL/6, C57BL/10, DBA/2 and CD1 were examined on receipt and after housing for 1 week by Gadbois et al. (1993). Table 3, listing the flora isolated from the CD1 mouse strain, partially summarizes the results of this study. Although the oral flora of the mouse is relatively simple with less variety than in humans, there can be significant differences in predominant bacterial species found among strains of different mice housed under different conditions. Therefore, it is

important to establish the composition of the oral flora in the animals used for each study before the interactions between these bacteria and the host's salivary proteins are examined further.

1.4.5. Resident Flora in the Human Oral Cavity

The human body is comprised of over 10^{14} cells, of which only around 10% are actually mammalian (Marsh & Martin, 1992). The remaining 90% consists of bacterial cells, protozoa, yeasts and fungi, which comprise the resident or indigenous microbial flora of the human body. One of the most complex of the bacterial communities associated with humans is the oral bacterial community (Marsh & Martin, 1992). Different species of these organisms are found to colonize specific habitats within the mouth. For example, *S. salivarius* prefers to colonize on the dorsal surface of the tongue, whereas *S. sanguis* and *S. mutans* have a preference on the teeth (Gibbons & van Houte, 1975). *Streptococcus* spp. make up the majority of the bacteria found in most oral habitats and these are the most extensively studied oral bacteria.

1.4.6. Interactions Between Salivary Molecules and Oral Bacteria

Interactions between the surfaces of oral bacteria and molecules in saliva are significant both for the survival of specific bacteria and the removal of others from the oral community (Scannapieco, 1994). The interaction between the salivary deposits (pellicle) on the tooth surface and lectins and other molecules in saliva are fundamental for the adhesion of bacteria to surfaces (Ofek & Doyle, 1994), which in turn, is crucial for their survival. Coaggregation, as well, is significant in plaque accumulation (Kolenbrander et al., 1992, 1999). In contrast, other molecules such as mucins, antibodies and antimicrobial peptides (Amerongen et al., 1995; Helmerhorst

et al., 1999; Boman, 2000) can kill bacteria and prevent their adherence and subsequent colonization of the mouth.

2. Rationale and Hypothesis

Although the mSMGP gene is abundantly expressed in the adult mouse submandibular gland (Windass et al., 1984; Myal et al, 1994), its expression in the developing mouse has not been addressed. The expression of the mSMGP gene during embryonic development may suggest an important function for this protein in submandibular gland biology, as the role of the mSMGP is not yet known. A human homologue of the mSMGP, the human prolactin-inducible protein (hPIP)/gross cystic disease fluid protein 15 (GCDFP-15), has been identified in several physiological fluids including saliva, tears and sweat (Murphy et al., 1987a). Recently, the hPIP/GCDFP-15 has been shown to bind to bacteria that reside in the human oral cavity, skin and ear canal (Schenkels et al, 1993, 1994, 1997). These studies suggest to us that the mSMGP may be involved in some non-immune host defense response. In addition, previous studies in our laboratory have shown that the mSMGP gene sequences are conserved in several species (Myal & Shiu, 2000). Therefore, the function of the protein may also be conserved.

The current study focuses on the hypothesis that the mSMGP plays a role in non-immune host defense. To address this hypothesis, the following questions are proposed:

1) *Is the mSMGP gene expressed in the mouse submandibular gland during embryonic development?*

   Expression of the mSMGP gene in the embryo may suggest that this protein plays an important role during development, possibly in the protection of the developing mouse via non-immune defenses.

2) *Is the mSMGP secreted into mouse saliva?*

Previous studies have identified a signal peptide sequence in the putative mSMGP polypeptide suggesting that this protein is secreted. Identification of the mSMGP in saliva will suggest that the action of this protein takes place extracellulary, in regions having contact with saliva, such as the oral cavity.

3) *Does the mSMGP selectively bind to certain oral bacteria?*

Previous studies on the human homologue, hPIP, have shown a binding interaction of this protein with certain bacteria (Schenkels et al., 1993, 1997). If the mSMGP can similarly bind to microorganisms such as bacteria, the hypothesis that the mSMGP plays a role in non-immune host defense may be further supported.

4) *Will submandibular gland development and the health of the individual be altered in the absence of the mSMGP gene?*

The function of the mSMGP can further be elucidated by observing the effects, if any, that occur when the protein is absent and no longer providing a role in the biology of the submandibular gland and the overall organism.

The first three questions proposed for our hypothesis will be addressed in this study.

3. Research Objectives

The objective of this study is two-fold:

1) To examine mSMGP gene expression during embryonic development

Specific aims:

a) *To determine tissue specificity of mSMGP gene expression in the developing mouse submandibular gland.* Whole embryos will be sectioned and analyzed for mSMGP gene expression.

b) *To determine the temporal and spatial mSMGP gene expression in the embryonic submandibular glands.* E14-E21 embryos will be collected. mSMGP gene expression will be analyzed by Northern and Southern blot analysis, RT-PCR and *in situ* hybridization.

2) To investigate the function of the mSMGP by examining the binding of the protein to bacteria.

Specific aims:

a) *To establish whether the mSMGP is present in mouse saliva.* The mSMGP gene is highly expressed in the mouse submandibular gland and the protein sequence contains a signal peptide. In addition, the human homologue has been detected in human saliva. Therefore, the mSMGP may similarly be found in mouse saliva. Studies will be carried out using Western blot analysis.

b) *To determine if the mSMGP binds to oral bacterial strains.* The hPIP has been shown to selectively bind to bacteria (Schenkels et al., 1993, 1994, 1997). We will investigate whether the mSMGP can bind to oral bacteria. The binding of the mSMGP to oral bacteria will lend support to our hypothesis that this protein may play a role in non-immune host defense.

4. Significance of the Study

The demonstration of mSMGP gene expression in the developing submandibular gland will suggest that the mSMGP may play an important role during mouse embryonic development. If the mSMGP is involved in non-immune host defense, its presence during embryonic development may be to provide protection for the embryo. Since cells of the immune system have not fully matured until approximately E17 and E18 (Ema et al., 1997, Delassus et al., 1998) the mSMGP may help to provide defense against foreign organisms while the immune system continues to develop.

Establishment of specific interactions between the mSMGP and certain oral bacterial strains may provide insight into a possible function of this novel protein. For example, the result of such interaction may impede the movement of organisms or facilitate their removal. Therefore, the potential function of the mSMGP may be in non-immune host defense. Altogether, these studies will contribute to further increasing the knowledge about the maintenance and regulation of the oral microbial community among the murine species. In addition, the results may have important implications for the treatment of dental caries and periodontal diseases in humans.

5. MATERIALS & METHODS

5.1. Animal Housing and Breeding

Four to six week CD1 mice were obtained from Charles River Laboratories (St. Constant, PQ, Canada) and housed at the facilities of the Central Animal Care Services department, Faculty of Medicine, University of Manitoba (Winnipeg, Manitoba, Canada). The mice were housed in plastic cages with wood chip bedding and fed Agway PROLAB rodent chow (RMH 3200; Agway Inc., Syracuse, NY, USA) and tap water *ad libitum*. Female mice were mated overnight with male mice. The next morning, the females were checked for vaginal plugs. If vaginal plugs were present, this day was counted as day 1 of gestation. Pregnant females were sacrificed at day 14, day 15, day 16, day 17, day 18, day 19, day 20, day 21 of gestation (E14-E21) and day 1 (D1) of birth and the fetuses were collected for mSMGP gene expression studies (See Section 5.2. Tissue Collection).

5.2. Tissue Collection

Adult female (4-8.5 wk, 5 mo) and embryonic mice (E14-E21, D1) were sacrificed and tissues were dissected and immediately frozen in 2 ml polypropylene microcentrifuge tubes (Fisher Scientific, Whitby, ON, Canada), placed on dry ice or fixed in 4% paraformaldehyde in Fisherbrand clear glass screw threaded 5.5 ml vials (Fisher Scientific). Frozen tissues were stored at –70°C until RNA isolation. Paraformaldehyde treated tissues were fixed overnight at 4°C, rinsed with and stored in 70% ethanol at room temperature to be later used for *in situ* hybridization studies (See Section 5.10. *In Situ* Hybridization). Since the embryos were very tiny with many organs still underdeveloped, the bodies were simply sectioned into two or three parts for gene

expression analysis. E14 to E18 embryos were sectioned into an upper (U) and lower (L_1) section. For the larger embryos E19 to E21, and D1, the bodies were sectioned into an upper (U), middle (M) and lower (L_2) section (Figure 3). The upper section consisted of everything from below the forelimbs and higher, while the middle section included the abdominal region and the lower sections included the pelvic region, tail and hindlimbs. In the E14, E15 and E16 embryos, the SMGs were not separated from the upper section because these glands were too small to be isolated. The SMGs from all other ages were either removed from the upper body section or left intact.

5.3. Isolation of Total RNA

Total RNA was extracted from frozen tissues using TRIzol Reagent (GibcoBRL, Burlington, ON, Canada) according to the protocol outlined by the manufacturer. Briefly, 1 ml of TRIzol Reagent was added per 50-100 mg tissue sample and homogenized using a Brinkmann Polytron Homogenizer (Brinkmann Instruments, Westbury, NY, USA) for 10 to 15 seconds at maximum speed or until complete homogenation. Homogenized samples were incubated at room temperature for a minimum of 5 minutes and 0.2 ml chloroform was added for every 1 ml of TRIzol Reagent. Tubes were capped securely, shaken vigorously for 15 seconds and left on the bench for 2 to 3 minutes. Smaller volumes (less than 2 ml) were centrifuged in an IEC Micro-MB centrifuge (International Equipment Company; IEC, Needham Heights, MA, USA) using the 9-84 rotor at maximum speed for 15 minutes at 4°C. Centrifugation of sample volumes greater than 2 ml was performed in a Beckman J2-21 Centrifuge (Beckman Instruments, Palo Alto, CA, USA) with the JA-20 rotor at 4°C and 12,000 g

for 15 minutes. Following centrifugation, the colorless aqueous upper phase, which contains the RNA, was carefully transferred to a new tube and the white interphase and red phenol-chloroform lower phase was discarded. RNA was precipitated from the aqueous phase using 0.5 ml isopropyl alcohol for every 1 ml TRIzol Reagent used. Following the addition of isopropyl alcohol, tubes were capped and inverted 3 to 4 times and allowed to stand at room temperature for 10 minutes. Centrifugation was repeated under the same conditions as above, but only for 10 minutes. The RNA pellet was washed with cold 75% ethanol; to every 1 ml of TRIzol reagent, 1ml of ethanol was added, mixed for 10 to 15 seconds with a vortex (Scientific Industries, Springfield, MA) and centrifuged for 5 minutes. The ethanol was discarded, briefly centrifuged again and remaining ethanol was removed with a pipette. RNA pellets were allowed to dry at room temperature and redissolved in diethylpyrocarbonate water (DEPC H_2O). The concentration of the RNA was determined by spectrophotometric absorbance readings at 260 nm with a Spectronic 1001 Plus Spectrophotometer (Milton Roy, Rochester, NY, USA). The following equation was applied to determine the RNA concentration:

Equation 1:

$$\frac{\text{(Optical Density)(40)(Dilution Factor)}}{1000} = \text{RNA } \mu g/\mu l$$

Purity of the RNA isolated was indicated by readings at 260 nm and 280 nm. A 260/280 ratio between 1.7 and 2.0 reflects a sample with high RNA purity. The total RNA isolated was stored at –70°C for Northern blot analysis and reverse transcription-polymerase chain reaction studies.

5.4. Mini-Gel Electrophoresis

To determine the integrity of the extracted RNA, 1 μg of total RNA was separated in 1.3% (w/v) agarose gel containing 1X Gel Running Buffer (GRB; 1XGRB= 0.04 M Mops, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, pH 8.0). To each 1 μg sample of RNA, 10 μl of a master mix solution (64.5 % Formamide, 2.8 M Formaldehyde, 1X GRB) and 2.5 μg of ethidium bromide were added. Samples were denatured at 65°C for 10 minutes, immediately placed on ice and briefly spun down. Prepared samples were then electrophoresed in the RunOne Electrophoresis System (EmbiTec, San Diego, CA, USA) at 100 V for 30-45 minutes to assess the integrity of the RNA. RNA was visualized using long wave ultraviolet light and photographed with a CCD camera and the Microcomputer Imaging Device (MCID) M4 software version 2.0 (Imaging Research Inc., Brock University, St. Catherines, ON, Canada).

5.5. Northern Blot Analysis

Total RNA (10 μg) was prepared in denaturing solution as outlined by Maniatis et al. (1982) and incubated at 65°C for 15 minutes prior to electrophoresis. Total RNA was separated in 1.5% (w/v) agarose gel containing 0.04 M Mops, pH 7.0, 10 mM sodium acetate, 1 mM EDTA, pH 8.0, 2.2 M formaldehyde and 5 μg of ethidium bromide. Initially, samples were electrophoresed at 100 V for 10 to 15 minutes and the voltage was reduced to 20 V to continue overnight. The RNA was transferred onto 0.45 μm nitrocellulose membrane (Micron Separations, Westborough, MA, USA) using 20X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate) as the transfer buffer. The transfer was allowed to proceed for at least 24 hours with 2 or 3 changes of paper towel.

Nitrocellulose membranes were baked after transfer for 2 hours at 80°C in a dry oven (Lab-Line Instruments, Melrose Park, IL, USA).

5.6. Hybridizations and Autoradiography

Baked membrane filters were placed in glass hybridization tubes (Robbins Scientific, Sunnyvale, CA, USA) containing 10 ml of pre-hybridization solution containing 50% (v/v) formamide, 5X SSPE (1X SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 1mM EDTA, pH 7.7), 5X Denhardt's Solution (1X Denhardt's = 0.02% (w/v) each of Ficoll 400, Polyvinylpyrrolidone and BSA), 0.1% sodium dodecyl sulfate (SDS) and 250 μg/ml denatured salmon sperm DNA. The tubes were placed in a hybridization incubator (Robbins Scientific) and allowed to rotate for a minimum of 1 ½ hours at 42°C. Labeling of the cDNA probe with ^{32}P was performed with the T7 QuickPrime Kit (Amersham Pharmacia, Baie d'Urfé, PQ, Canada) using ^{32}P-labeled dCTP isotope and the probe was purified with NICK columns (Amersham Pharmacia) containing Sephadex G-50 DNA grade. The amount of cDNA labeled for each reaction was 30 ng. The labeled probe was denatured by boiling for 5 minutes just prior to hybridization, quickly cooled and added to a fresh 10 ml of hybridization solution. The solution used during prehybridization was discarded and replaced with the prepared hybridization solution containing the labeled probe. Filters were hybridized at 42°C in the rotating hybridization incubator overnight for approximately 20 hours.

After hybridization, filters were washed twice with 2X SSC/0.1% SDS at room temperature for 10 minutes on a shaker followed by pre-warmed 0.1X SSC/0.1% SDS at 65°C on a shaker for 5 minutes. Radioactivity was monitored with a radioactivity survey

meter (Model 3, Ludlum Measurements Inc., Sweetwater, TX, USA) and the filters were washed with 0.1X SSC/0.1% SDS at 65°C one or two more times if necessary. Filters were then placed on Whatman® chromatography paper (3mm; Whatman International, Maldston, England) to soak up excess liquid evenly and placed into a heat-sealable pouch (Fisher Scientific). For autoradiography, Kodak X-OMAT AR film (InterScience, Markham, ON, Canada) and an intensifying screen were used for varying exposure times at -70°C.

5.7. Dot Blot Analysis

mSMGP gene expression was analyzed using the mouse RNA Master Blot™ (Clontech, Palo Alto, CA, USA), a positively charged nylon membrane containing poly A^+ RNA from selected mouse tissues. A prehybridization solution was prepared by warming 15 ml of the provided ExpressHyb solution at 50-60°C and mixed with 1.5 mg of denatured (boiled for 5 minutes and quickly chilled on ice) salmon sperm DNA. The Master blot™ was then placed into a heat-sealable pouch filled with 10 ml of the prepared prehybridization solution. The sealed bag was put into a Tupperware container with the bottom immersed in a water bath and incubated at 65-70°C for 1 hour in continuous agitation. During this time, a cDNA probe was prepared with 30 ng of mSMGP cDNA, labeled and purified in the same manner as described in Section 5.6. "Hybridizations and Autoradiography". One hundred and five µl of this labeled probe was then mixed with 30 µg of C_0t-1 DNA (Boehringer Mannheim, Indianapolis, IN, USA), 150 µg of sheared salmon sperm DNA and 50 µl of 20X SSC to a total volume of 200 µl. All 200 µl of this mixture containing the labeled probe was boiled for 5 minutes followed immediately by

incubation at 68°C for 30 minutes. The labeled probe was added to the remaining 5 ml of fresh prehybridization solution and this mixture was used as the hybridization solution for incubation with the RNA Master Blot™. Following the removal of the prehybridization solution, fresh hybridization solution was added and the pouch was resealed. The bag was then placed into the Tupperware container for an overnight incubation in a 65°C waterbath with continuous agitation. The next day, the RNA Master Blot™ was washed with 200 ml wash solution 1 (2X SSC, 1% SDS) at 65°C for 20 minutes and monitored for radioactivity with the survey meter before a second wash with solution 1 was carried out. A final wash was performed with prewarmed wash solution 2 (0.1X SSC, 0.5% SDS) at 65°C for 20 minutes, after which the excess fluid was removed from the blot with Whatman® chromatography paper and the blot was quickly sealed for autoradiography. Kodak X-OMAT AR film (InterScience) and an intensifying screen was used and placed in -70°C for varying exposure times.

5.8. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The reverse transcription reaction consisted of 5X RT buffer (GibcoBRL; 1X RT buffer = 50 mM Tris-HCL, 75 mM KCl, 3 mM $MgCl_2$), 200 nM of each dNTPs (Amersham Pharmacia), 250 ng Oligo dT RT primer, 1 μg BSA (New England Biolabs; NEB, Mississuaga, ON, Canada), 0.01 M DTT (GibcoBRL), 19 units of RNA guard (Amersham Pharmacia) and 200 units of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (GibcoBRL). One μg of RNA was used for each RT reaction in a final volume of 10 μl. As a negative control, the above mixture was prepared without RNA in a separate tube and treated under the same conditions. To rule out genomic

DNA contamination, parallel tubes of some RNA samples were set up minus the RT enzyme (RT-). Reverse transcription was performed at 37°C for 2 hours using a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA, USA). One tenth of each RT reaction was used for PCR with 10X PCR buffer (Amersham Pharmacia; 1X PCR buffer = 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl), 10 mM dNTPs, 2.5 units of *Taq* DNA Polymerase (Amersham Pharmacia) and the appropriate primers (Table 4) to a final volume of 50 µl. As a negative control for the PCR reaction, the above reaction mixture was prepared without the cDNA template and treated under the same conditions. In addition, a plasmid containing the mSMGP cDNA was amplified under the same conditions as a positive control. The mouse housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) was used as an internal control gene, as it is ubiquitously and constitutively expressed in most cells. Conditions for PCR are outlined in Table 5 for each primer used.

5.9. Southern Blot Analysis

RT-PCR products were electrophoresed on a 1% (w/v) agarose gel in 1X TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). The gel was then soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 hour at room temperature and constant agitation, followed by 1 hour in neutralization solution (1 M Tris-Cl, pH 8.0, 1.5 M NaCl) under the same conditions. After neutralizing the gel, transfer, hybridization and autoradiography methods were carried out as described in Section 5.5. "Northern Blot Analysis".

5.10. *In Situ* Hybridization

In situ hybridization was performed as previously described by Al-Haddad et al. (1999). Sense and antisense RNA probes were generated from a linearized plasmid consisting of the mSMGP cDNA insert (~600 bp) in a PVZI vector (3.6 kb) (Figure 4). Using the restriction enzymes Sal I (5 µl) and EcoRI (5 µl), 20 µg of the DNA were linearized in 20 µl of the corresponding 10X restriction enzyme buffer in a final volume of 200 µl. The digestion was performed at 37°C for 1½ hours. The DNA was then purified using a phenol/chloroform solution. The purified sample was electrophoresed in a 0.7% agarose gel for ½ an hour using TAE buffer (1X TAE = 0.04 M Tris-acetate, 0.001 M EDTA). The bands were excised from the gel and further purified with the GFX PCR DNA & Gel Band Purification Kit (Amersham Pharmacia). The purified DNA was concentrated by adding 400 µl of 100% ethanol and 20 µl of sodium acetate (NaOAc) followed by incubation at -20°C for 1 hour. The samples were then centrifuged for 15 minutes at 4°C at 12,5000 RPM and the recovered pellet was washed with 200 µl of 75% ethanol. All of the ethanol was removed and the pellet was left to dry at room temperature for about 15 minutes. Each sample was dissolved in ddH_2O and stored at -70°C after the concentration was determined by spectrophotometric absorbance at 260 nm with a Milton Roy Spectronic 1001 Plus Spectrophotometer (Fisher Scientific).

This linearized mSMGP cDNA was used to generate UTP^{S35} sense and antisense probes with the Riboprobe Gemini II Core System kit (Promega, Madison, WI, USA) according to the manufacturer's protocol and purified using a Quick spin G50 sephadex column (Boehringer Mannheim). The RNA probes were then stored at -70°C. To

calculate the amount of RNA probe to use per ml of hybridization solution, the following formula was applied following ^{35}S scintillation counts of each sample:

Equation 2:

$$\frac{1 \times 10^6 \text{ (the desired count per minute; cpm)}}{\text{Initial cpm}} = \mu\text{l antisense/sense needed per ml of hybridization solution}$$

Sense and antisense probes with a concentration of 1×10^6 cpm/μl were applied to 5 μm paraffin sections and pretreated with triethanolamine/acetic anhydride and proteinase K. Coverslips were then placed over each section, sealed with rubber cement and incubated overnight at 42°C in a humid chamber. The following day, coverslips were gently removed and sections were incubated with posthybridization solution (20 mM Tris, 1mM EDTA, 0.3 M NaCl, 10 mM DTT and 50% deionized formamide) for 10 minutes at 55°C and buffered RNase A (0.5M NaCl, 10 mM Tris and 1mM EDTA and 20 μg/ml of prewarmed RNase A (20 μg/ml; Boehringer Mannheim)) for 30 minutes at 37°C. Weakly bound nonspecific label was washed off using standard saline citrate buffer in the following descending dilutions: 2X SSC twice each for 5 minutes, 1X SSC once for 15 minutes and 0.1X SSC three times each for 15 minutes. Slides were dehydrated with graded concentrations of ethanol beginning with 50% ethanol to 70%, 95% and twice with 100% containing 300 mM ammonium acetate and finally dried overnight under the fumehood. For autoradiographic detection, slides were dipped in Kodak NTB-2 (InterScience) emulsion at 40°C, dried in a humid chamber for 1 hour and stored in light tight slide boxes at 4°C for 4 weeks. Slides were counterstained with Lee's methylene blue and basic fuchsin.

5.11. Preparation of Antibodies

5.11.1. The Anti-mSMGP Antibody

Polyclonal antibodies to the mSMGP were commercially prepared by Research Genetics (Huntsville, AL, USA) using the multiple antigen peptide (MAP) technique to synthesize the desired mSMGP immunogenic peptide. Eight copies of the 18 amino acid long peptide QENQEITVQVTVETQTRE, beginning at the 47th amino acid of the mSMGP protein sequence, (Figure 2) were synthesized on a MAP carrier core. This MAP-peptide was emulsified by mixing with complete Freund's adjuvant for the initial injection and incomplete Freund's adjuvent for subsequent injections. Freund's adjuvent was injected into three subcutaneous dorsal sites of New Zealand white rabbits of 3 to 9 months. A total of 0.5 mg of peptide was used per immunization. After bleeding the rabbits, the blood was allowed to clot and serum collection was performed with centrifugation and stored at 20°C. Sera from two immunized rabbits (#88841 and #88857) were collected at different stages; 1st immunization boost (2 weeks), first bleed (4 weeks), 2nd boost (6 weeks), second bleed and 3rd boost (8 weeks) and third bleed (10 weeks). Based on serum antibody titer, rabbit #88841 serum at 8 weeks was chosen as the source for the primary antibody used for our immunoblot studies (See Section 5.15. SDS-PAGE, Western Blot Analysis). The synthetic peptide and preimmune bleed serum was also obtained by Research Genetics and used to perform control immunoblots. Serum was prepared for immunoblotting by heating aliquoted amounts at 55°C for 30-45 minutes and stored at -20°C until needed.

5.11.2. The Anti-hPIP Antibody

Polyclonal antibodies to the human homologue, human prolactin-inducible protein (hPIP) was previously generated by Shiu & Iwasiow (1985). Briefly, the hPIP was obtained from the human breast cancer cell line, T-47D and purified by excising the appropriate sized bands from a Coomassie Blue stained SDS-PAGE gel. Three isoforms were obtained with the molecular weights of 16, 14 and 11 kD. Ten μg of each isoform was emulsified with complete Freund's adjuvant and injected intradermally at multiple sites in a rabbit. This procedure was repeated two and four weeks later in incomplete Freund's adjuvant and the rabbits were bled 2 weeks after the final injection. Antisera were obtained after removing the clots and cells. Preimmune sera were also obtained by bleeding the rabbits prior to immunization.

5.11.3. The Anti-rPIP Antibody

Polyclonal antibodies to the rat homologue, rat prolactin-inducible protein (rPIP) were provided by the laboratory of Dr. Lily Mirels (Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA). The rat protein homologue was designated IIG4 and a glutathione-S-transferase-IIG4 fusion protein was prepared as described by Mirels et al. (1998). Antibodies were isolated from rabbit antiserum raised against this prepared protein and affinity purified. Aliquots of this affinity purified antibody as well as preimmune serum were received.

5.12. Saliva Collection

Human saliva was collected directly into centrifuge tubes (Nalgene, Rochester, NY, USA) and centrifuged in an RC-5C Automatic Superspeed Refrigerated Centrifuge

(Sorvall Instruments) with SM-24 rotor size at 12,350 g for 8 minutes at 4°C. The clear supernatant was then incubated with several bacterial strains.

Four 4-6 week old female CD1 mice were used for saliva collections. Mice were anaesthetized for approximately 45 minutes by injecting 0.35-0.40 ml of Avertin intraperitoneally. Saliva was collected according to the protocol of Benarde et al.'s (1956). We constructed a homemade multi-unit animal holder with cardboard, similar to the model described by Benarde et al. (1956) for rat saliva collection, but to a smaller scale (Figure 5). The angle of incline of the apparatus was approximately 10-20 degrees to prevent the mice from swallowing or inhaling their own saliva. The anaesthetized mice were then administered subcutaneously with 0.30 ml of pilocarpine (~5mg/kg). The mice were secured onto the apparatus by their tails with tape. As salivation continued, the drops were collected into Fisherbrand clear glass screw threaded 5.5 ml vials (Fisher Scientific) positioned below each mouth. Collection ceased as salivation slowed down, a period varying between 15 to 45 minutes depending on each individual mouse. Saliva samples were either immediately analyzed or stored at -20°C until further analysis. When possible, saliva was collected from the same mice on more than one occasion.

Fractions of saliva were obtained from two adult female mice in 50 µl increments and kept on ice. Collection of these fractions continued until salivation ceased for each mouse. Subsequently, five fractions from the first mouse and 6 fractions from the second mouse were retrieved. The concentration of mSMGP in each fraction was immediately analyzed by Western blot analysis.

5.13. Protein Extraction from Tissues

Tissues were placed in 2 ml polypropylene tubes and their volume was estimated. Based on the estimation, the appropriate volume of 4X SDS Sample Buffer (1X SDS sample buffer = 1.25 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.01% w/v bromophenol blue, 0.05 mM DTT) and ddH_2O was added to obtain a final concentration of 1XSDS in the total tissue sample. Tissues were homogenized using the Brinkmann Polytron Homogenizer and then sonicated at medium power for 15 to 30 seconds using the Megason Ultrasonic Disintegrator (Ultrasonic Instruments International, Farmingdale, NY, USA). Samples were boiled for 5 minutes and centrifuged at 12,000 g at 4°C for another 5 minutes. The supernatant was transferred to a clean polypropylene tube and the protein concentration was determined (See Section 5.14. Determination of Protein Concentration). Protein was extracted from rat SMG, mouse SMGs (male and female), brain, adrenal, lacrimal and mammary glands and analyzed by Western blot analysis (See Section 5.15. SDS-PAGE, Western Blot Analysis) along with mouse saliva. The samples were electrophoresed in duplicate and the transferred proteins were either detected with the anti-rPIP or anti-mSMGP antibodies (See 5.11. Preparation of Antibodies).

5.14. Determination of Protein Concentration

The protein concentrations of the collected tissue samples were determined by the Bio-Rad Protein Microassay procedure (Bio-Rad). Protein samples in 1X SDS were diluted 1:3 in ddH_2O. One µl of each diluted sample was added to 0.8 ml of PBS, while 1 µl of a 1:3 dilution of 1X SDS was added for each BSA standard and blank. Next, 0.2 ml of Bio-Rad's Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories,

Mississauga, ON, Canada) was added to every tube. The tubes were mixed by inverting several times, followed by incubation at room temperature for 20 minutes. The BSA standard concentrations used for optical density measurement were 1.25 μg/ml, 2.5 μg/ml, 5.0 μg/ml, 7.5 μg/ml, 10 μg/ml, 15 μg/ml and 20 μg/ml. This assay is based on the principle that when a dye reagent containing Coomassie Brilliant Blue G-250 is added to the test samples, the dye will bind to the protein and an absorbance shift from 465 nm to 595 nm occurs. Therefore, absorbance was read at a wavelength of 595 nm and a BSA standard curve was plotted using the software GraphPad Prism™ Version 2.00 (GraphPad Software Inc., San Diego, CA, USA). The slope (m) and y-intercept (b) was obtained and used to calculate the unknown concentration of the protein samples according to the following equation with a dilution factor of 3.

Equation 3:

$$[(OD_{595}\text{-}b)/m] \text{ X Dilution Factor} = \text{Protein } \mu g/\mu l$$

5.15. SDS-PAGE, Western Blot Analysis

Samples were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 0.5 ml polypropylene tubes with 1X SDS sample buffer and boiled for 5 minutes at 100°C. Bio-Rad Kaleidoscope prestained standards and Horse Radish Peroxidase (HRP) Biotinylated Protein Marker (NEB) were used as a standard size marker. For radioactive detection, a ^{14}C protein molecular weight marker was used (Amersham Pharmacia). SDS-PAGE was performed using the EC120 mini vertical gel system (E-C Apparatus Corporation, Holbrook, NY, USA) and samples were electrophoresed in a 4% polyacrylamide stacking gel and 15% polyacrylamide separating

gel. The separation of proteins was either performed with the Tris-Glycine buffer system (5X Running Buffer = 125 mM Tris, 960 mM Glycine, 0.5% SDS, pH ~8.3) or the Tris-Tricine buffer system (5X Cathode Running Buffer = 500 mM Tris, 500 mM Tricine, 0.5% SDS, pH ~8.25; 5X Anode Running Buffer = 1 M Tris, pH 8.9). After electrophoresis, proteins were transferred onto 0.2 μm nitrocellulose membranes (Bio-Rad) using Bio-Rad's Trans-Blot Electrophoretic Transfer cell with plate electrodes. Blocking was carried out with 5% BSA in 1X Tris Buffered Saline containing Tween® 20 (1X TBST = 20 mM Tris, 137 mM NaCl, pH 7.6 and 0.05% Tween® 20, Fisher Scientific) for 2 hours at room temperature, followed by incubation with primary antibody (diluted in the buffers outlined in Table 6) overnight on a Vari-mix Aliquot Mixer (Barnstead/Thermolyne, Dubuque, IA, USA) at 4°C. Following membrane washes with 1X TBST (9X for minutes, 2X for 15 minutes), incubation with the secondary antibody was performed in the conditions outlined in Table 6. Each membrane was incubated with the secondary antibody for 2 hours on the Vari-mix at room temperature and washed again with 1X TBST (12X for 5 minutes, 2X for 15 minutes) before visualization. Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) was used for detection with equal parts of Luminol/Enhancer solution and Stable Peroxide solution according to manufacturer's instructions. Membranes were exposed onto Kodak BioMax MR film (InterScience) for various periods.

Stripping of nitrocellulose membranes for reuse was performed by immersing the membrane in 100 ml of stripping solution (2% SDS, 62.5 mM Tris pH 6.8, 0.05 M DTT) at 50°C for 20 minutes followed by 2 washes with 1X TBST for 10 minutes each. To

confirm the membrane was stripped successfully, Supersignal West Pico Chemiluminescent Substrate (Pierce) was applied and the membrane was exposed to Kodak BioMax MR film (InterScience) for a minimum of 15 minutes. After developing this film, protein bands should no longer be present. The stripped membrane was then washed again with 1X TBST, twice for 10 minutes each, before blocking and incubation with the primary antibody.

5.16. Plasmid Preparation

The mSMGP cDNA was inserted into the pcDNA3.1/His B (Figure 6; Invitrogen, Carlsbad, CA, USA) vector and transfected into *E. coli* DH5α bacteria. Glycerol stocks of the *E. coli* transformants were prepared and stored at -70°C. An overnight culture of the bacteria was grown by placing a loopful of the glycerol stock into 5 ml of Luria-Bertani (LB) Broth (1% Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl, Difco Laboratories, Detroit, MI, USA) containing 100 μg/ml of ampicillin. The cultures were incubated with constant agitation at 37°C overnight. The following day, 2 ml from each culture tube was decanted into 2 ml polypropylene flat top microcentrifuge tubes (Fisher Scientific) and centrifuged at 13,000 RPM for 1 minute at room temperature. Plasmids were isolated using the Ultraclean™ Mini Plasmid Prep Kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions. The final plasmid sample was eluted with 50 μl of sterile ddH_2O. The concentration of the isolated plasmid was determined by spectrophotometric analysis using an ultraviolet wavelength of 260 nm. The following equation was applied to determine the DNA concentration:

Equation 4:

$$\frac{\text{(Optical Density)(50)(Dilution Factor)}}{1000} = \text{DNA } \mu g/\mu l$$

The restriction enzyme, Xba1 (Boehringer Mannheim) was used to linearize the mSMGP plasmid. Digestion was performed in a 20 µl reaction mixture containing 1 µl Xba1 enzyme, 2 µl of 10X React 2 Buffer (GibcoBRL) and the mSMGP plasmid for an incubation period of 1 ½ hours at 37°C. The linearized plasmid was extracted with an equal volume of Phenol/Chloroform/Isoamyl Alcohol 25:24:1 solution, pH 6.7 (Fisher Scientific). The tube was inverted several times to mix thoroughly and then centrifuged at 13,000 RPM for 5 minutes at room temperature. The top aqueous layer was carefully collected and transferred into a new 1.5 ml polypropylene tube. Sodium acetate (3M NaOAc) and 95% ethanol was used to precipitate the linearized plasmid. The following equations were applied to determine the volumes of NaOAc and ethanol needed:

Equation 5:

Volume of 3M NaOAc = 1/15 (estimated volume of aqueous layer)

Equation 6:

Volume of 95% Ethanol = 2.5 (estimated volume of aqueous layer + volume of 3M NaOAc)

The mixture was inverted several times and placed at -70°C for 1 hour. This step was followed by centrifugation of the mixture for 30 minutes at 13,000 RPM at 4°C. The supernatant was carefully poured out and the pellet was washed with 100 µl of 70% ethanol. The ethanol was then poured out and the pellet was allowed to dry at room temperature for 5 to 10 minutes. The pellet was redissolved in ddH_2O and the DNA concentration was determined according to Equation 4 after the spectrophotometric reading.

5.17. *In-Vitro* Transcription/Translation

In vitro transcription and translation was performed using the TnT® Coupled Reticulocyte Lysate System (Promega) or the TnT® Coupled Wheat Germ Extract System (Promega) with 1 μg of circular pcDNA3.1 plasmid (Invitrogen) containing the T7 promoter and target cDNA insert. To prepare proteins for Western blot analysis, the provided amino acid mixture, minus methionine and amino acid mixture, minus cysteine were added to ensure that a full complement of amino acids was present for the translation reaction. For ^{35}S detection, transcription/translation reactions were prepared with only the amino acid mixture, minus methionine. The amino acid complement was then completed by adding the EasyTag™ Methionine, L-[^{35}S] (NEN™ Life Science Products, Boston, MA, USA). The reaction components were assembled according to the protocol provided by Promega for a 50 μl reaction in a 0.5 ml polypropylene microcentrifuge tube. This reaction was incubated at 30°C for 90 minutes and then stored at -20°C until needed. The translated protein was analyzed by aliquoting 5 μl of the reaction mixture into 20 μl of 2X SDS sample buffer. Five μl of this diluted sample was boiled and electrophoresed on a 15% polyacrylamide gel and Western blot analysis was performed as described in Section 5.15. SDS-PAGE, Western Blot Analysis. For the ^{35}S radioisotope detection, the gel was incubated in fixing solution (50% methanol, 10% glacial acetic acid, 40% ddH_2O) on a shaker for 30 minutes at room temperature. The gel was then soaked in a solution containing 7% glacial acetic acid, 7% methanol and 1% glycerol for 5 minutes at room temperature to prevent the gel from cracking during the drying procedure. The gel was placed on a piece of Whatman® chromatography paper and dried using a slab gel dryer (Savant) for 1 hour at 65°C. Immediately after drying,

the gel was exposed to Kodak BioMax MR film (InterScience) overnight at room temperature with an intensifying screen. Further exposures were performed if necessary.

5.18. Culturing and Maintenance of Bacterial Isolates

All the isolates were stored freeze-dried. Freeze dried bacteria were reconstituted in broth containing 0.1% Nutrient Broth No.2 (OXOID), 0.5% glucose, 0.05% L-cysteine hydrochloride, 0.4% tryptone (Difco Laboratories) and 0.4% yeast extract with 0.6% KH_2PO_4 (w/v), 0.9% K_2HPO_4 (w/v), 0.1% $(NH_4)_2SO4$, 0.02% $MgSO_4$, 0.001% CaCl. Using a sterile glass Pasteur pipette, the freeze dried sample was suspended in the broth, spread onto blood agar plates and incubated in a candle jar overnight at 37°C. Single colonies were then subcultured the following day. Bacteria cultures were maintained on blood agar (Base No. 2, OXOID, Nepean, ON, Canada) and incubated at 37°C in candle jars with an anaerobic environment containing CO_2.

Two female mice were sacrificed by exposure to CO_2 and their heads removed with a sterile scalpel and placed in a sterile petri dish. A sterile disposable type 1 Calgiswab® (Spectrum Laboratories, Dallas, TX, USA) was used to swab the inside of the mouse oral cavity, including under the tongue, carefully ensuring that the swab was not contaminated from the lips. The swab was then placed into a sterile polystyrene falcon culture tube (12x75mm, Fisher Scientific) containing 1 ml of reduced transport fluid (RTF= 0.045% KH_2PO_4, 0.045% K_2HPO_4, 0.09% NaCl, 0.09% $(NH_4)_2SO_4$, 0.018% $MgSO_4$, 0.038% EDTA, 0.04% Na_2CO_3 and 0.02% DTT) and the aluminum shaft of the swab was then broken off to allow the tube to be capped. To disintegrate the calcium alginate, each tube was vigorously mixed with a vortex. Serial dilutions of this

suspension in RTF buffer were made in the following ratios: 1:10, 1:100, 1:1000, 1:5000. Forty µl of each dilution was automatically spirally plated onto blood agar plates with a Spiral Plater Model C (Spiral System Instruments, Cincinnati, OH, USA). Each dilution was plated in duplicate, one set was incubated at 37°C in a candle jar for two days while the second set was incubated in an anaerobic chamber (Coy, Ann Arbor, MI, USA) containing 80% N_2, 10% H_2, 10% CO_2 at 37°C for the same period. Single colonies were selected from these plates, assigned a number and subcultured on blood agar plates. Isolates were coded by the animal origin; either mouse 1 or mouse 2 (M1 or M2), followed by a number that identified the individual isolate. The lower case letter 'a' following some species identifications indicate that the organism was initially subcultured from plates incubated under anaerobic conditions in an anaerobic chamber.

5.19. Identification of Bacteria

5.19.1. Gram Stain

A smear of the organism was made on a clean slide, heat fixed and flooded with Crystal Violet. After approximately 10 seconds, the stain was washed off with tap water and flooded with Lugol's iodine, again letting it stand for 10 seconds. The slide was then flooded once with 100% acetone for decolorization and then immediately rinsed with tap water. The final counterstain was performed with carbol fuchsin (1:200), letting it stand for approximately 10 seconds before rinsing with tap water. The water was removed by blotting the slide with a paper towel and the slide was briefly flamed to dry before examination using an oil immersion lens on a binocular microscope. The cell shape and

color was noted; dark purple cells indicated Gram-positive cells, while red cells were Gram-negative (See Table 7).

5.19.2. Catalase Test

A catalase test was performed to determine whether the organism produced catalase, an enzyme that hydrolyzes hydrogen peroxide. Briefly, a drop of hydrogen peroxide was placed on a clean glass slide and a sample of the organism was placed into the hydrogen peroxide using a standard loop. Oxygen production, indicated by bubble formation, revealed a positive reaction (See Table 7).

5.19.3. Commercial Identification Systems

Further biochemical tests on Gram-positive and Gram-negative organisms were performed using the **rapid ID 32 Strep** and **ID 32 E** systems (bioMérieux, St. Laurent, PQ, Canada), respectively (Table 7). For the rapid ID 32 Strep system, bacteria were added to the suspension medium (bioMérieux) to produce a cloudy suspension (McFarland Scale 4). Fifty-five µl of this suspension was pipetted into each cupule and the provided lid was placed over the strip. Each strip was then incubated aerobically at 37°C for 4 hours. After incubation, the 32 biochemical reactions on each strip were read for positive and negative results and recorded on the result sheets provided. For the ID 32 E system, the cells were suspended to the same density in NaCl 0.85% medium (bioMérieux) and 55 µl of this suspension was pipetted into each cupule. A lid was then placed over the strip and incubated aerobically at 37°C overnight in a sealed container containing a small amount of water to prevent the strips from drying out. The strips were analyzed the next day and the results were recorded on the sheets provided. Both systems consisted of 32 cupules testing for different biochemical reactions as outlined in Table 7.

Standard tables of biochemical characteristics of known bacterial species were provided by the manufacturer. These standard tables show the percentage of positive reactions given for each test by several strains of known species of *Streptococcus* and enteric rods. Comparisons were made between the standard tables and the test results for each mouse isolate in order to identify the test strain. To simplify comparisons, tests on the standard tables that gave <10% positive results were regarded as negative.

5.19.4. Glucose Fermentation

Glucose fermentation was also tested on one Gram-positive isolate thought to be a *Staphylococcus* species. Briefly, the tested organism was subcultured on two glucose agar plates and incubated at 37°C, one aerobically and the other anaerobically. A color change of the medium from pink to yellow indicated glucose fermentation, a positive reaction (Table 7).

5.19.5. Identification of *Lactobacillus* Species

Lactobacillus species are commonly found within the oral cavities of mice (Trudel et al., 1986, Gadbois et al., 1993) and are non-hemolytic. Thus, to determine whether the non-hemolytic unknown organisms were possibly in this genus, organisms were cultured on Rogosa agar plates (1% tryptone, 0.5% yeast extract, 0.6% KH_2PO_4, 0.2% ammonium citrate, 0.5% salt solution B, 2% glucose, 0.1% tween 80, 2.5% sodium acetate, 1.5% agar and 0.132% glacial acetic acid) and incubated in a candle jar at 37°C (Table 7). The lower pH of these agar plates (pH 4.8) selects for acidophilic organisms such as *Lactobacillus spp.*

5.19.6. Cytochrome Oxidase Test

All Gram-negative organisms were also tested for cytochrome oxidase activity. A sterile filter paper in a petri dish was wetted with sterile water and a bacterial smear was made on a section of the paper. A drop of OX reagent (1 % tetramethyl-p-phenylenediamine in isoamyl alcohol, w/v) (bioMérieux) was placed over the bacteria and allowed to sit for 1 to 2 minutes. A color change to violet/purple indicated a positive reaction while a negative reaction produced no color change (Table 7).

5.20. Bacteria Binding Studies

5.20.1. Incubation of Bacteria with Saliva

One standard 4 mm loopful of the bacterial organism (Table 8) was added to either 200 µl of saliva (human or mouse) or 200 µl of 10 mM Hepes (Sigma) buffer, pH 7.5. The tubes were mixed vigorously until the solution appeared homogenous. Tubes were then incubated at 37°C on an Orbit Shaker (Lab-Line Instruments) for 20-30 minutes. Samples were centrifuged at 10,350 g in an IEC minicentrifuge (IEC) for 1½ minutes at room temperature. The supernatant was discarded and replaced with 200 µl of 10 mM Hepes buffer as a wash solution. Tubes were mixed vigorously for 10-15 seconds and centrifuged again under the same conditions. Two more washes were performed for a total of three washes for each sample. After the final wash, the supernatant was discarded and the pellet was redissolved in 6-30 µl of 10 mM Hepes buffer, depending on each pellet size, and 2-10 µl 4X SDS sample buffer for a final concentration of 1X SDS. Samples were stored at -20°C until analyses by immunoblotting.

5.20.2. Incubation of Bacteria with *In-Vitro* Translated Protein

One standard loopful of the bacterial organism was added to 200 µl of 10 mM Hepes buffer, pH 7.5 and vortexed until homogenous. Fifty µl of this suspension was transferred to a fresh 1.5 ml polypropylene tube and 5 µl of the final ^{35}S-labeled *in-vitro* translated reaction product (See Section 5.17. *In Vitro* Transcription/Translation) was added. Fifty µl of the bacteria suspension without the added *in-vitro* translated reaction product was carried out in parallel as a negative control. Tubes were tapped gently to give an even suspension of bacteria and protein solution. The mixture was subjected to the same incubation and washing conditions described for the incubation of bacteria with saliva (Section 5.20.1. Incubation of Bacteria with Saliva).

5.21. Densitometric Analysis of Bacteria Binding Affinities

The amount of bacteria-bound ^{35}S-labeled *in vitro* translated mSMGP and Psoriasin in each bacterial suspension was determined by analyzing the intensity of the bands developed on x-ray film (Figure 27). Using the MCID M4 software, the density (in pixels) of each appropriate band was measured after baseline subtraction (to eliminate measurements of varying backgrounds or non-specific density variation). A portion (1/5) of each *in vitro* translated protein, without bacterial incubation, was also electrophoresed and exposed to film for ^{35}S detection. The intensity of the bands developed from these samples represented the amount of protein initially available to bind to bacteria (1_o, 2_o; Figure 27). The intensity of all other bands was compared to these initial values to compare the amount of protein bound to the amount originally available. This comparison was expressed as a percentage. Each bacteria cell pellet was

resuspended in various volumes of 1X SDS (20 µl, 32 µl or 40 µl), depending on the pellet size (See Section 5.20.1. Incubation of Bacteria with Saliva). However, 10 µl of each sample was used for SDS-PAGE. Therefore the dilution factor (2, 3.2 or 4) of each sample was taken into account during the calculations and the original versus final protein concentration relationship was expressed as a ratio of pixel densities. The following calculation was applied:

Equation 7:

$$\frac{\textbf{(Densitometric value of final product)(Dilution Factor)}}{\textbf{(Densitometric value of initial product)(Dilution Factor)}} \times 100 = \textbf{\% of Protein Bound}$$

These percentage values were compared to assess the binding capabilities of each protein (Figure 28).

5.22. Wilcoxon Signed Rank Sum Test

Statistical analysis was performed using the Wilcoxon Signed Rank Sum Test from the software program GraphPad Prism™ Version 2.00 (GraphPad Software Inc). P values < 0.05 were considered significant.

6. RESULTS

6.1. mSMGP Gene Expression

6.1.1. Tissue Specific Gene Expression in the Adult Mouse

To determine the tissue specific expression of the mSMGP gene in the adult mouse, a commercially available RNA Master Blot™ (Clontech) was screened with a ^{32}P-labeled mSMGP cDNA probe. The blot consisted of poly A^+ RNA derived from a wide variety of tissues and whole mouse embryos. mSMGP gene expression was detected in the submandibular and prostate gland. The level of gene expression in the submandibular glands was considerably higher than mSMGP gene expression in the prostate gland (Figure 7). Gene expression was not found in the whole mouse embryos.

6.1.2. Tissue Specific Gene Expression in the Mouse Embryo

mSMGP gene expression was examined in a variety of tissues collected from a day 18 mouse embryo (E18). Following RNA extraction, the RNA was electrophoresed on a 1.3% agarose gel. Figure 8 shows that high quality intact RNA was obtained, with the exception of RNA from the skin, which showed a small degree of degradation. Using RT-PCR amplification followed by Southern blot analysis, mSMGP gene expression was only detected in the submandibular glands of the E18 embryo. All other tissues examined were negative for mSMGP gene expression (lungs, heart small (sm.) intestine, stomach, liver, skin, paws and tail) (Figure 9).

6.1.3. mSMGP Gene Expression in the Developing Submandibular Gland

Since our results showed that the mSMGP gene is highly expressed in the embryonic submandibular glands (SMGs), we investigated how early in development the gene is first expressed. Tissues were collected at 1-day intervals from E14, the approximate age at which the submandibular gland (SMG) begins development, until

birth. To ensure that a negative result was not due to 'poor' RNA quality, once again the integrity of the RNA extracted from all tissues was analyzed on a 1.3% agarose gel. High quality intact RNA was obtained, similar to the results seen in Figure 8. .

The upper body sections, including their SMGs, of E14, E15 and E16, and the isolated SMGs of E17 to E21, were then analyzed for mSMGP gene expression by RT-PCR, Southern and Northern blot analyses. RNA was analyzed for integrity and all RNA samples were observed to be of high quality. By Northern blot analysis, the single mSMGP mRNA transcript of approximately 700 bases long was only detected in isolated SMGs of E20 and E21 embryos (Figure 10). By RT-PCR, mSMGP gene expression was only detected in the E18 to E21 embryonic SMGs as indicated by the 570 bp band (Figure 11A). However, further analysis of RT-PCR products by Southern blot analysis showed mSMGP gene expression as early as E14 (Figure 11B). In addition, total RNA from the upper section with intact SMGs of an E19 embryo was analyzed to compare the detectable levels of gene expression with that of the isolated SMG. The level of gene expression observed in the entire upper section was lower than what was seen in the isolated E19 SMG.

To determine whether the mSMGP gene was expressed in any tissue other than the SMG, the SMG was dissected from the embryos prior to RNA extraction and gene expression analysis of their entire bodies. mSMGP gene expression was not detected by either RT-PCR or Southern blot analysis (Figure 12)

6.1.4. Localization of mSMGP Gene Expression in the Embryonic Submandibular Gland

In situ hybridization analysis was carried out on the SMGs of an E18 embryo. As mSMGP gene and protein expression has previously been examined in the adult SMGs (Myal et al., 1994, Mirels et al., 1998), an adult female mouse SMG was used as a positive control in this study. ^{35}S-labeled sense and antisense mSMGP probes

were generated as described in Section 5.10. "*In Situ* Hybridization". The sense probe was used as a negative control. The hematoxylin and eosin (H&E) staining of the SMGs was used to identify the main cell types: acinar (ac) and ductal cells (dc) (Figure 13A and Figure 14A). Our results showed that the mSMGP gene was only expressed in the acinar cells of the adult and embryonic SMGs (Figure 13B, Figure 14B). No radioactive binding was observed with the ^{35}S-labeled sense mSMGP probe (Figure 13C and Figure 14C).

6.2. The mSMGP in Mouse Saliva

6.2.1. Specificity of the mSMGP Antibody

A synthetic peptide coding for a short sequence in the mSMGP was custom made (Research Genetics) and used to generate polyclonal antibodies (Research Genetics) to the mSMGP protein. To examine the specificity of the antibody, the mSMGP synthetic peptide was blotted onto two nitrocellulose membranes in decreasing amounts of 1000 ng, 500 ng, 100 ng, 10 ng and 1 ng. One membrane was incubated with the preimmune serum alone while the second membrane was incubated with the anti-mSMGP antibody. The antibody to the mSMGP reacted to all concentrations of the synthetic peptide (Figure 15B), whereas incubation with preimmune serum did not reveal any non-specific binding (Figure 15A).

6.2.2. Analysis of Mouse Saliva Fractions

As we were uncertain whether the use of pilocarpine increased the amount of protein secreted when salivation was stimulated, the concentration of the mSMGP in different saliva fractions collected overtime was analyzed. The results showed that there was little difference in mSMGP levels between any of the saliva fractions over time (Figure 16).

6.2.3. Fresh versus Frozen Mouse Saliva

To determine whether freezing the mouse saliva before Western blot analysis affected the integrity of the mSMGP, freshly collected and frozen mouse saliva samples (20 μl) were compared by Western Blot analysis (Figure 17). Human saliva was used as a positive control. The expected size band (14 kD) was found in the mouse saliva samples, although the band intensity from the fresh saliva appeared to be slightly stronger. Frozen or fresh human saliva samples did not appear to differ in reactivity with the anti-hPIP antibody.

6.2.4. Identification of the mSMGP in Saliva

To determine whether the mSMGP was present in mouse saliva, Western blot analysis was carried out using the antibody to the mSMGP (See Section 5.11.1. The Anti-mSMGP Antibody). Incubation with the preimmune (PI) serum was carried out first to determine the level of nonspecific binding with the rabbit serum alone. The results showed that there was no cross-reactivity with the preimmune serum (Figure 18). The anti-mSMGP antibody, however, recognized two salivary proteins of approximately 14 kD and 40 kD in size.

To assess whether there was any cross-reactivity between the rabbit anti-human PIP and the rabbit anti-mouse SMGP antibodies with human and mouse salivary proteins, human and mouse saliva were collected and electrophoresed in duplicate. The anti-mSMGP antibody bound weakly to the glycosylated forms (14kD, 16 kD) of the human homologue and to some larger proteins (approximately 25 kD and 50 kD) in human saliva (Figure 19A). Although the anti-hPIP antibody recognized a larger mouse salivary protein of approximately 16 kD, it did not bind to the 14 kD mSMGP (Figure 19B). These results indicated that there was minimal

cross-reactivity between the immunogenicity of the protein homologues from the two species.

6.3. Analysis of Mouse Oral Bacteria

The next objective of this study was to determine whether the SMGP is capable of binding to certain oral bacteria. We therefore characterized some bacterial isolates obtained from the mouse oral cavity before examining whether they interacted with the mSMGP. The results from the biochemical tests on the isolates are shown in Table 9 and Table 10 and these results were compared to those of known bacterial species according to the method outlined in Section 5.19.3 "Commercial Identification Systems". Tables 11, 12 and 13 lists the bacterial species with highest similarity to the unidentified organisms. The boxes shaded in gray indicate the mismatches between the biochemical characteristics of the unidentified organism and the known species. The number of matches out of the 32 biochemical tests were calculated and a percentage of similarity (%) is indicated at the bottom of the table. Four of the nine organisms were found to have a high degree of similarity to the species *Aerococcus viridans*, *Streptococcus parasanguis*, *Streptococcus oralis* and *Streptococcus intermedius* (Table 11). Four other isolates were found to be similar to *Sphingomonas paucimobilis*, *Vibrio metschnikovii*, the *Aeromonas hydrophila* group and *Pseudomonas aeruginosa* (Table 12). The final organism, M1/8, was a Gram-positive coccus and was most likely a member of the genus *Staphylococcus* (Table 13).

6.4. Incubation of Bacteria with Human and Mouse Saliva

6.4.1. Human Oral Bacteria with Human Saliva

The hPIP appeared to bind selectively to different bacterial strains with varying affinities as indicated by different band intensities. Figure 20 shows a representative Western blot, while Table 14 summarizes the results for each bacterial strain. Incubation with the preimmune serum demonstrated that the antibody to the hPIP was specific.

6.4.2. Mouse Oral Bacteria with Human Saliva

To determine whether the hPIP can bind to bacteria from the mouse oral flora, human saliva was incubated with bacteria obtained from the mouse oral cavity. Western blot analysis results showed that the hPIP specifically bound to several of the mouse bacteria tested. However, there was more specific binding (as assessed by band intensities) to some strains as compared to others. Interestingly, M2/9a and M1/9a, the strains identified as Gram-negative rods in the *Pseudomonas* spp. (See Section 6.3. Analysis of Mouse Oral Bacteria) consistently failed to bind to the hPIP. Figure 20 shows a representative Western blot, while Table 15 summarizes the results for each mouse bacterial strain.

6.4.3. Human and Mouse Oral Bacteria with Mouse Saliva

To determine whether the mSMGP can bind to certain human and mouse oral bacteria, strains were tested for their binding affinities to the mSMGP by incubating each strain with mouse saliva. We were unable to demonstrate any binding of the mSMGP in mouse saliva with either human or mouse oral bacteria (Figure 21).

6.5. The Use of the Rat Prolactin-Inducible Protein Antibody to Detect the mSMGP

As the anti-rPIP antibody was made against the native protein as opposed to a short synthetic peptide, we wanted to examine whether this antibody was more specific and effective than the anti-mSMGP antibody. Protein was extracted from the SMG of the rat and the SMG, adrenal gland, brain, lacrimal and mammary glands of the mouse. With the exception of the SMGs and the mouse lacrimal glands, all the tissues used for analysis are known not to express the mSMGP and thus were used as negative controls. The lacrimal gland has been reported to express the mSMGP and was included in this study as another positive control. Duplicate Western blot analyses were performed using the anti-rPIP and anti-mSMGP antibodies to test for the specificity of each antibody to the mSMGP and rPIP. Preimmune serum obtained from each original immunized rabbit was also used. Figure 22A and Figure 22B show the results from the preimmune and anti-rPIP antibody incubation, respectively. This antibody strongly recognized the target protein in the rat SMG and only weakly recognized the mouse homologue in mouse saliva (Figure 22B, black arrows). Using the anti-mSMGP antibody, the mSMGP was detected in the saliva and lacrimal glands of the mouse (Figure 22D, black arrows).

6.6. *In Vitro* Transcription/Translation Studies

6.6.1. The TnT® Coupled Reticulocyte Lysate System (Promega)

The composition of saliva is made up of a variety of proteins. Since we were unable to detect any specific binding between bacteria and the mSMGP in mouse saliva, we attempted to incubate bacteria directly with the mSMGP generated by *in vitro* translation. The mSMGP, Psoriasin and Estrogen receptor-alpha (ER-α)

proteins were generated using the TnT® Rabbit Reticulocyte Lysate and analyzed by Western Blot analysis. Translation was initiated at an AUG site on the pcDNA3.1 vector and continued until a stop codon was reached at the end of the target protein sequence. With the inclusion of this short segment of the plasmid that is translated and the intact signal peptide, the *in vitro* translated mSMGP was estimated to be approximately 19 kD. The expected sizes of the translated products for Psoriasin and ER-α were 11 kD and 67 kD respectively. The results after incubation with the anti-mSMGP or anti-Psoriasin antibodies revealed similar banding patterns for all samples, indistinguishable from the control "empty" vector (pcDNA3.1 vector alone without a cDNA insert). However, with the anti-ER-α antibody, a single band was detected in the third lane that corresponded to the 67 kD ER-α protein (Figure 23).

6.6.2. The TnT® Coupled Wheat Germ Extract System (Promega)

Similarly, protein synthesized by the TnT® Coupled Wheat Germ Extract System (Promega) was analyzed by Western blot as described in Section 6.6.1. "The TnT® Coupled Reticulocyte Lysate System". The results are shown in Figure 24. Again, there was no difference between the experimental and control lanes. However, the anti-Psoriasin antibody recognized the 11 kD Psoriasin protein (Figure 24B). Incubation with anti-ER-α antibody did not produce any positive results. A second Western blot analysis was performed using higher concentrations of the extracts containing the mSMGP translated protein or the "empty" pcDNA3.1 vector. Mouse saliva was included as a positive control. The same banding pattern was seen among the *in vitro* translated products, indistinguishable between the control pcDNA3.1 vector and the mSMGP product. Although the intensity of the bands was greater when the concentration of the samples increased, a unique 19 kD band was not detected (Figure 25).

6.6.3. *In Vitro* Transcription/Translation Using ^{35}S-methionine

Circular and linear mSMGP plasmids, circular Psoriasin plasmid and the pcDNA3.1 vector alone were *in vitro* transcribed and translated with ^{35}S-methionine using the rabbit reticulocyte lysate and wheat germ extract systems followed by SDS-PAGE on a 15 % polyacrylamide gel. The predominant translated product with the mSMGP plasmid was of approximately 19 kD while the predominant product with the Psoriasin plasmid was of approximate 11 kD in size (Figure 26). As expected, transcription and translation of the "empty" pcDNA3.1 vector did not result in any translated products. Fewer non-specific bands were observed with the use of the wheat germ extract as compared to the rabbit reticulocyte lysate. In addition, transcription of the linear plasmid compared to the circular plasmid did not result in a greater yield of the mSMGP translated product.

6.6.4. Incubation of *In Vitro* Translated mSMGP with Human and Mouse Oral Bacteria

The TnT® Coupled Wheat Germ Extract System (Promega) and ^{35}S-methionine was used for all future experiments. Incubation was performed as described in Section 5.20.2 "Incubation of Bacteria with *In-Vitro* Translated Protein" and the final samples were analyzed by SDS-PAGE (Figure 27). The results showed that the mSMGP and Psoriasin had varying affinities to each organism. The degree of binding was measured by densitometric analysis using the MCID M4 software and values were analyzed as a ratio of initial to final concentration of translated products (See Section 5.21. Densitometric Analysis of Bacteria Binding Affinities). Graphical representation of the calculated results is shown in Figure 28. Overall, the mSMGP (Median = 7.52, Mean = 7.39 Standard Deviation = 6.428) demonstrated significantly higher specificity (P = 0.0313; Wilcoxon Signed Rank Sum Test) in bacteria binding than the control protein, Psoriasin (Median = 0.79, Mean = 0.99, Standard Deviation

= 0.68). Binding of the mSMGP was strongest to the human streptococci, SK145 and SK100. The mouse oral streptococci, M2/7a, M1/23 and the *Aerococcus* sp., M1/6 (See Section 6.3. Analysis of Mouse Oral Bacteria), also showed high degrees of specificity in binding. The two bacterial strains, M1/8 (*Staphylococcus* sp.) and M2/9a (*Pseudomonas* sp.) showed the lowest binding interaction with the mSMGP.

7. DISCUSSION

mSMGP Gene Expression

The mouse submaxillary gland protein (mSMGP) is a 14 kD protein that is abundantly expressed in the mouse salivary (submandibular and parotid) and lacrimal glands (Myal et al., 1994). The function of the mSMGP is unknown. However, the conservation of the SMGP sequences across several species suggests an important role for this protein (Myal & Shiu, 2000). The mSMGP shares a 56% nucleotide and an 80% amino acid homology with a rat homologue, the rat prolactin-inducible protein (rPIP; Mirels et al., 1998). The human homologue, human PIP (hPIP; Myal et al., 1994) shares a 51 % amino acid homology with the mSMGP. The hPIP is found in saliva, tears, sweat and seminal plasma. Although the function of the rat and human homologues are also not known, several functions for the hPIP have been proposed: the hPIP is thought to play a role 1) in the protection of the organism from HIV infection (Autiero et al., 1991, 1995 & 1997) 2) in fertilization (Bergamo et al., 1997), 3) as a survival factor by inhibiting apoptosis (Gaubin et al., 1999), 4) as a mitogen (Cassoni et al., 1995), and 5) in non-immune host defense (Schenkels et al., 1993). To address the importance of the mouse SMGP function, we first examined mSMGP gene expression in the developing mouse embryo.

It has been previously demonstrated, by Northern blot analysis using total RNA extracted from various tissues of the adult mouse, that expression of the mSMGP gene was restricted to the salivary and lacrimal glands (Myal et al., 1994). Since total RNA was used, it is possible that low gene expression in other tissues may have gone undetected. In order to examine expression in the adult tissue more thoroughly, a commercially available RNA Master Blot™ (Clontech) containing poly A^+ RNA from a variety of adult tissues and mouse embryos was screened for mSMGP

gene expression (Figure 7). Once again, a high level of gene expression was detected in the submandibular gland, confirming previous observations. Interestingly, a low level of gene expression was detected in the prostate gland. This observation has not been previously reported. However, no mSMGP gene expression was detected in the whole mouse embryos on this Master Blot™ (Figure 7).

It has been reported that the rPIP gene is expressed as early as E20 (Mirels et al., 1998). In this study mSMGP gene expression was observed at age E18 of the developing mouse embryo by RT-PCR and Southern Blot analysis. Compared to all other tissues examined, gene expression was only found in the submandibular glands (Figure 9). At this early stage, it was impossible to dissect and examine the other major salivary glands (sublingual and parotid) individually. Therefore, we were unable to determine whether these glands specifically expressed the mSMGP gene, as in the adult mouse.

Since we had now established that the mSMGP gene was highly expressed in the submandibular glands, we then proceeded to examine whether this expression was present earlier in embryonic development. A high level of gene expression was clearly evident in the isolated submandibular glands of embryos E18 to E21. However, when the entire upper section containing the intact submandibular gland was analyzed compared to the gene expression in the isolated gland of the E19 embryo, a much lower level of gene expression was observed (Figure 11). This observation demonstrated that the high gene expression detected in the submandibular gland alone was 'diluted' by other tissues. Therefore, it was difficult to accurately quantitate expression of the mSMGP gene. With Southern blot analysis, following RT-PCR, we were able to detect mSMGP gene expression as early as E14. This age coincided with the approximate time at which the submandibular glands begin to

appear in the developing embryo. These results suggest that the mSMGP may be important during normal submandibular gland development. The fact that we did not detect mSMGP gene expression in the embryonic samples on the RNA Master Blot™ was not surprising. This was because whole embryos aged E7, E11, E15 and E17 were used to obtain the poly A^+ RNA, thus the 'dilution' effect was again a factor. In addition, at E7 and E11, the submandibular glands have not yet begun to develop.

To determine whether the mSMGP gene was expressed in any embryonic tissues other than the submandibular glands, the submandibular glands were removed and the embryos were sectioned as described in Section 5.2. "Tissue Collection". Although mSMGP gene expression was not detected elsewhere in the embryos from E14 to birth (Figure 12), the 'dilution' effect may again have contributed to the results observed.

In situ hybridization studies were carried out in order to localize mSMGP gene expression in the mouse embryonic submandibular gland. Our laboratory previously reported that, in the adult submandibular gland, gene expression was localized to the acinar cells (Myal et al., 1994). We therefore wanted to determine whether the same cell type expressed the mSMGP gene in the embryo. The results showed that in the embryonic submandibular gland (E18), gene expression was observed in the proacinar cells but not in the few ductal cells present (Figure 13). During submandibular gland development, the cells of the "terminal bulb" in the E15 mouse embryo already have multiple, well-formed Golgi units and few strands of dilated rough endoplasmic reticulum (Cutler & Chaudhry, 1974). The presence of such structures in the embryonic submandibular gland and our finding that mSMGP gene expression was present in the proacinar cells, demonstrated that this developing gland was already biologically functional.

Therefore, our results suggest that the mSMGP may play an important role during the development of the mouse embryo. Salivary peroxidase, another protein known to protect the oral mucosa, was first detected in the submandibular gland at E17 (Yamashina & Barka, 1973; Osugi, 1977). In addition, the first mature T-cells are not detected until E18 (Ema et al., 1997) and the first detectable sIg-bearing B-lymphocytes have been reported to occur at E17 (Delassus et al., 1998). Since the mSMGP is expressed as early as E14, it is possible that the mSMGP may aid in the protection of the developing mouse embryo through non-immune host defenses, prior to the maturation of the immune system.

Analysis of the mSMGP in Mouse Saliva

Since the analysis of the anti-mSMGP antibody demonstrated that it was both sensitive and specific (Figure 15), it was used in the Western blot analyses to identify the mSMGP in mouse saliva. Administration of pilocarpine was used to increase the volume of saliva normally produced by the mouse. However, we were concerned that this artificial increase in saliva volume may have resulted in a dilution of the overall protein concentration in our saliva samples. To address these concerns, we collected and analyzed both early and late saliva fractions and measured the mSMGP concentrations. However, our results showed that there was no considerable difference in the mSMGP levels between different saliva fractions collected (Figure 16), suggesting that the levels of mSMGP secreted in saliva did not decrease throughout the collection period.

Saliva samples collected from each mouse were routinely stored at -20°C for further uses to minimize the frequency of drug applications and the stress on the animals. To determine whether the freezing had any effect on the stability of the

mSMGP, we compared the mSMGP in frozen and fresh saliva samples from both human and mouse. When equal sample volumes were analyzed, there appeared to be no overall difference in protein concentrations in the fresh and frozen samples (Figure 17). We concluded that using saliva that was freeze-thawed did not compromise the stability of the mSMGP and our ability to detect the protein.

When the mouse saliva samples were analyzed for the mSMGP, the anti-mSMGP antibody recognized two proteins; a 14 kD protein, the expected size of the secreted mSMGP, and a 40 kD protein in mouse saliva (Figure 18). It is possible that the 40 kD protein represents a multimeric form of the 14 kD mSMGP, since the human homologue, hPIP, has been shown to form dimers in saliva (Rathman et al., 1989) and tetramers in breast cyst fluid (Haagensen et al., 1979). However, this was unlikely since a denaturing gel was used. Denaturing gels dissociates subunits and eliminates the presence of any linkages. It was also unlikely that the identified 40 kD protein was a glycosylated form of the mSMGP since no glycosylation sites have been identified by database analysis (Windass et al., 1984). It is possible that this larger protein was another salivary protein with some homology to the mSMGP and thus was recognized by the anti-mSMGP antibody.

The mSMGP shares a 51% homology on the amino acid level with the hPIP. Studies were carried out to determine whether the mSMGP antibody would cross-react with the hPIP and whether the hPIP antibody would cross-react with the mSMGP. The anti-hPIP antibody recognized a broad protein band of approximately 14-16 kD and a smaller band approximately 11 kD in human saliva (Figure 19B). The larger band most likely represented both the 14 kD and 16 kD glycosylated forms of the hPIP. The lower band was the 11 kD unglycosylated form of hPIP. However, the anti-hPIP antibody did not recognize the 14 kD mSMGP in mouse saliva, while

the anti-mSMGP antibody only weakly recognized the glycosylated forms of the hPIP in human saliva (Figure 19). The 11 kD form of hPIP was not recognized at all by the anti-mSMGP antibody (Figure 19A). Therefore, these results indicated that the antibodies were species specific.

Analysis of Mouse Oral Bacteria

Prior to examining the binding interactions between the mSMGP and oral bacteria, we wanted to characterize the bacterial isolates obtained from the mouse oral cavity. Gram staining was performed on the 48 isolates collected. Since this was a large number to use in our study, we randomly selected nine isolates to analyze further. These nine isolates were characterized using commercial test systems and then compared to the biochemical characteristics of known bacterial species to assign possible species identifications. The bacterial isolate, which we referred to as M1/6, appeared to have highest similarity to the species *Aerococcus viridans* (*A. viridans)*, an organism that normally inhabits the skin, ear and eye of humans (Murray, 1998), not the oral cavity of mice. Its presence in the mouth may be attributed to the grooming habits of the mice, since they can pick up the bacteria by licking themselves or others. Three other isolates, M1/23, M1/7a and M2/7a, were identified as streptococci. *Streptococcus* species are normal inhabitants of mucosal membranes and many are found on the tooth surfaces of humans and several animals (Kilian, 1998). M1/1, a Gram-negative organism, had a high degree of similarity to *Sphingomonas paucimobilis*. This species is related to the genus *Pseudomonas* and is often isolated from the environment (i.e. water) and saline (Pitt, 1998). Likewise, the organisms in the *Vibrio* and *Aeromonas* genera (M1/6a, M2/1a) are normal inhabitants of both marine and freshwater. Aeromonads have been isolated from

several water sources such as wells and drinking fountains and numerous foods (Janda, 1998) and it was possible that the M1/1, M1/6a and M2/1a isolates entered the mouse oral cavity through the drinking water provided to the mice. The isolate M2/9a showed a high similarity to *Pseudomonas aeruginosa* (*P. aeruginosa*), an extremely ubiquitous organism (Costerton & Anwar, 1994). *P. aeruginosa* has been isolated from a diversity of sources including water and disinfectant solutions, due to its ability to use many different organic compounds and survive in almost an absence of nutrients (Pitt, 1998). There are indeed many opportunities for *P. aeruginosa* to enter the mouse oral cavity as mice frequently nibble all over their immediate environment, including their bedding and cages. The ninth isolate, M1/8, was a Gram-positive organism that did not show alpha-haemolysis like the *Streptococcus* species. Although M1/8 was able to grow under acidic conditions (Rogosa agar plate), like species in the genus *Lactobacillus*, this isolate was found to be a catalase-positive coccus. Since *Lactobacillus* species are catalase-negative rods, this genus was ruled out as a possible candidate for M1/8. However, a high degree of similarity existed between the biochemical characteristics of known *Staphylococcus* species and those of M1/8. Species in this genus normally inhabit the skin, skin glands and mucous membranes of warm-blooded animals (Kloos & Schleifer, 1986). Such an organism may have entered the mouse oral cavity during grooming. Interestingly, although *Lactobacillus murinis* (*L. murinis*) was previously found to be a major species in the mouse oral cavity (Table 2 and Table 3), it was not one of the identified species in this study. The ingredients of the rodent chow provided for these animals had low protein content (RMH 3200, Agway PROLAB). It has been reported that the proportion of *L. murinis* declines with lowered dietary protein levels (Blais and Lavoie, 1990) and thus may explain why this species was not found in our cultures. In addition, *L. murinis*,

along with the other predominant species previously isolated from the CD1 mouse, *S. sanguis* (Table 3), may have been among the bacterial isolates that we did not select for further characterization.

Protein Interactions with Bacteria in Human and Mouse Saliva

Human PIP has been shown to bind to certain bacterial species that reside in the human mouth, skin and ear canal (Schenkels et al., 1993, 1997). In the present study, we analyzed this binding relationship between the hPIP and oral bacterial strains from our laboratory. We determined that the hPIP in saliva could bind to at least one strain of all the species analyzed (Figure 20 and Table 14). The hPIP showed positive binding to almost all the bacterial strains, with the exception of the *S. mutans* strain, CH168A. Previous studies did not observe binding of the hPIP with this species as well (Schenkels et al., 1993). In addition, Schenkels and colleagues (1993, 1997) reported negative binding of the hPIP with *S. sanguis*, *S. milleri*, *A. naeslundii* and *S. mitis*. Although the current results showed that the hPIP could indeed bind to these species, the strains tested in these studies were not identical to the strains tested in the previous studies. Strain differences within species may have been a possible explanation for the varying results. Therefore, we have confirmed that the hPIP can bind to bacteria and identified new oral bacterial strains that bind to this protein.

To establish whether the hPIP was capable of binding to bacterial strains isolated from the mouse oral cavity, mouse bacterial isolates were individually incubated with human saliva and analyzed by Western blotting. The hPIP bound to 43 of the bacterial isolates with varying affinities (Table 15). Therefore, again for the

first time, these results demonstrated that the hPIP could selectively bind to bacteria that originate from the mouse (Figure 20).

Interactions between these mouse oral bacteria and the mSMGP were then examined. After incubation of these bacteria with mouse saliva, the mSMGP was detected by Western blot analysis using the anti-mSMGP antibody raised against the synthetic peptide. Our results showed that the mSMGP in mouse saliva failed to bind to the bacteria tested (Figure 21).

One possible explanation for this observation was that the bound bacterium inhibited the antibody from recognizing the protein. Since the anti-mSMGP antibody was generated against only a short sequence of the mSMGP, the antibody binding site may have been blocked or hidden after the bacterium had bound to the protein.

Several other possible scenarios may explain why the mSMGP from saliva did not bind to bacteria. For instance, the level of protein in saliva was too low for binding of bacteria to occur. Salivary components can either adhere to oral surfaces and contribute to the formation of the pellicle, or remain in the solution phase (Prakobphol & Fisher, 1993). It is possible that the mSMGP must first adhere to the tooth enamel, accumulate and become a constituent of the pellicle before it can efficiently bind to bacteria. As saliva continually flows and replenishes the oral cavity, accumulation of the mSMGP can occur on the pellicle, despite its low concentration in saliva. This may then result in a greater concentration of the protein in the oral cavity, attaining a critical level that is required for binding bacteria. Furthermore, it has been found that proline-rich proteins (PRPs) do not bind to bacteria in solution but are capable of promoting bacterial attachment when they are adsorbed as part of the pellicle (Ligtenberg et al., 1992). PRPs are also major salivary proteins that have a high affinity for hydroxyapatite and have been shown to bind to

bacteria, like the human homologue of the mSMGP, hPIP (Lamkin & Oppenheim, 1993). Although our results with hPIP suggested that these situations were not necessary to show binding with bacteria, it is possible that there is a difference between species.

The Use of The Rat Prolactin-Inducible Protein Antibody to Detect the mSMGP

Using a polyclonal antibody to the hPIP, we have demonstrated the binding of bacteria to the hPIP in saliva, confirming the previous observation of Schenkels et al. (1993, 1997). However, we were unable to demonstrate bacteria binding to the mSMGP in mouse saliva. This could be due to the fact that the anti-mSMGP antibody was raised against a short synthetic peptide, whereas the anti-hPIP antibody was generated against the entire hPIP (See Section 5.11.2 The Anti-hPIP Antibody). Recently, Mirels et al., (1998) had generated antibodies to the reactive rPIP (rat homologue of mSMGP). We reasoned that a) an antibody generated to the rPIP may sufficiently cross-react with the mSMGP, since the rPIP has a 56% overall identity and 80% similarity in amino acid sequence to the mSMGP, and b) since the polyclonal anti-rPIP antibody was generated to the entire native rPIP, it may therefore be more specific. We found that the anti-rPIP antibody only weakly recognized the mSMGP in mouse saliva (Figure 22). Alternately, the anti-mSMGP antibody did not cross-react with the rPIP in the rat SMG. Although the anti-mSMGP antibody recognized the mSMGP in mouse saliva and the lacrimal glands (which also expresses the mSMGP gene in the adult mouse; Myal et al., 1994), it failed to recognize the mSMGP in the mouse SMG. One reason for this observation may be that the mSMGP was readily secreted into mouse saliva upon synthesis.

The use of the anti-rPIP antibody did not improve our ability to detect the mSMGP in the mouse SMG or saliva and thus was not used further for our bacteria binding studies.

Interactions Between Bacteria and the *In Vitro* Translated mSMGP

It is estimated that the hPIP constitutes only about 1% of total protein in human secretions, including saliva (Schenkels et al., 1997). The proportion of total protein that represents the mSMGP in mouse saliva is not known. Since we were unable to detect the mSMGP after incubating mouse saliva with bacteria, it was possible that the level of mSMGP was too low for a binding interaction to take place. Therefore, to determine whether the mSMGP directly interacts with bacteria, we elected to use an *in vitro* transcription/translation strategy. By this method, we expected a translated product of 19 kD when the signal sequence and the plasmid DNA were taken into consideration. Using a rabbit reticulocyte lysate system, we failed to obtain any translated mSMGP product. However, an ER-α protein (67 kD), which was used as a control, was successfully translated (Figure 23). Numerous non-specific bands were also present in all other lanes. The rabbit reticulocyte lysate system is generally the most efficient system to generate mammalian proteins. However, when small proteins (12-15 kD) such as the mSMGP and Psoriasin are the target proteins, another system using wheat germ extract may be more efficient. In the wheat germ system, the ubiquiton-degradation pathway is not as active as in the rabbit reticulocyte lysate system, which can readily mistake small proteins for truncated proteins and degrade them. Another consideration was that the anti-mSMGP and anti-Psoriasin antibodies were generated in rabbits, while the anti-ER-α antibody was a mouse antibody. Therefore, it was possible that the anti-rabbit secondary antibodies used for Psoriasin and mSMGP detection, cross-reacted with

rabbit globins in the rabbit reticulocyte lysate used for the transcription/translation reaction. Endogenous globin is abundant in the reticulocyte lysate and co-migrates with synthesized proteins 12-15 kD in size (Wilkinson, 1999). It can mask the presence of the true product if it is within the same size range. Thus, the 14 kD bands observed in all the samples incubated with the anti-rabbit secondary antibody was possibly masking the 11 kD Psoriasin protein band. Since the ER-α protein was detected with a goat anti-mouse (not anti-rabbit) secondary antibody, cross-reaction with rabbit reticulocyte lysate components was not a factor and the 67 kD product was easily detectable. Added to that, was the fact that the anti-ER-α antibody was a monoclonal antibody. Monoclonal antibodies result in less non-specific binding than polyclonal antibodies like the anti-mSMGP and anti-Psoriasin antibodies, thus further enhancing the detection of ER-α. We subsequently employed a TNT® Coupled Wheat Germ Extract System (Promega) for our studies. With this system, we were able to identify our control product, the 11 kD Psoriasin, but we were still unable to detect the mSMGP by Western blot analysis (Figure 24). The wheat germ extract system also did not successfully translate the ER-α protein.

Since the plasmid construct was previously sequenced and verified to be in frame, the lack of detectable translated mSMGP was not due to poor construction, but may be attributed to an insufficient amount of the translated product. To investigate this possibility, we analyzed increasing concentrations of the *in vitro* translated product by Western blotting. We were still unable to detect the 19 kD *in vitro* translated mSMGP in any concentration (Figure 25).

However, we were able to visualize a translated product of approximately 19 kD, the expected size of the *in vitro* translated mSMGP by using ^{35}S-labeled methionine. The fact that we were able to identify the protein by this strategy was not

surprising, since radioactive methods are usually more sensitive than Western blotting. As with the rabbit reticulocyte lysate system, no translated products were observed in the control lane (pcDNA3.1 plasmid alone) with the wheat germ extract system. The utilization of linearized or intact plasmid for *in vitro* transcription did not make any difference in the intensity of the bands observed (Figure 26). Therefore, we chose the wheat germ extract system (since it was shown to have less non-specific bands) utilizing ^{35}S-labeled methionine and the circular mSMGP plasmid to generate the 19 kD *in vitro* translated mSMGP for binding to various bacterial strains.

The greatest degree of binding was observed between the mSMGP and the *Streptococcus* species (Figure 27, Figure 28). Members of the genus *Streptococcus* are predominant components of the oral flora. Therefore, the fact that the mSMGP binds to these bacteria may suggest that this protein is involved in regulating the population of this genus in the mouth. Our results showed that the 19 kD *in vitro* translated mSMGP bound to different strains with varying degrees (Figure 27). The mSMGP was selective and specific in its interaction with these species, as the control protein, Psoriasin, did not display the same level of interaction (P=0.0313, Wilcoxon Signed Rank Sum Test).

The results from our *in vitro* transcription/translation studies showed that the mSMGP specifically binds to certain bacterial strains. However, we were unable to demonstrate this observation in mouse saliva using our present antibody to the synthetic peptide. It will therefore be necessary to carry out further bacteria binding studies with mouse saliva using a more efficient antibody, made against the entire protein.

Future Studies

The mSMGP antibody made to an 18mer amino acid peptide may not have been specific enough to react with the target protein in our bacteria binding studies with mouse saliva. The generation of an antibody made to the native protein may lead to more conclusive results in future analyses. A recombinant mSMGP is currently being synthesized and will be used to generate a rabbit polyclonal antibody for our studies.

Future studies could also involve examining the binding interactions between the mSMGP in solution and bacteria versus the mSMGP bound to hydroxyapatite as part of the enamel pellicle. Although we showed that the mSMGP could bind selectively to certain oral bacterial strains in this study, the mechanism of this binding is not known and needs further investigation. Studies on the mSMGP binding to bacteria having modified surface receptors (using proteolytic enzymes) can provide information regarding the type of surface receptors involved.

To further examine the function of the mSMGP, gene "knockout" transgenic experiments will be carried out. Since we have determined that the mSMGP gene is expressed as early as E14 of embryonic development, these transgenic models may reveal the consequence of the loss of this gene in the developing submandibular gland and the entire animal. The effects observed on the mouse oral flora and the overall health of the "knockout" mice would contribute significantly to understanding the function of this novel protein.

Further bacteria-binding studies of the mSMGP with the remaining 39 isolates obtained from the mouse oral cavity, currently preserved in freeze-dried stocks, will also be performed. Our studies involving the identification of all the bacteria isolated will contribute to the limited literature on the resident oral flora of CD1 mice.

Summary

1. mSMGP gene expression in the developing mouse embryo was tissue specific. Gene expression was detected in the submandibular glands as early as E14, and as in the adult, the mSMGP gene was highly expressed in these glands of the developing embryo. In addition, we have made the novel observation that the mSMGP gene was expressed in low levels in the prostate gland.
2. As in the adult submandibular glands, this expression was localized within the acinar cells of the embryonic mouse submandibular gland.
3. The mSMGP was found to be a secreted protein, as it was identified in mouse saliva.
4. We have identified interactions between the hPIP and certain bacterial strains that have not been previously reported.
5. We have characterized various bacterial strains isolated from the CD1 mouse oral cavity.
6. The *in vitro* translated mSMGP has been found to bind to various oral bacterial strains isolated from the human and mouse.

Significance

Our observation that the mSMGP gene expression correlated with the onset of submandibular gland development at embryonic age 14 (E14) suggests that the mSMGP may be integral to embryonic development in the mouse. During this early period when the immune system is not fully developed and other molecules, such as salivary peroxidase, are not active, the mSMGP may already be involved in providing protection for the embryo against foreign organisms.

Our results demonstrated that the mSMGP protein specifically bound to several strains of bacteria. This observation suggests a potential function of the mSMGP in non-immune host defense in the mouse oral cavity.

At the present time, the literature on the indigenous oral flora of CD1 mice is limited. This study contributes additional information on our current knowledge of the resident flora of these animals.

Since the function of the mSMGP is still undetermined, this present study suggests an important role for this novel protein during embryonic development and provides insight into its possible involvement in host defense. The discovery of the biological role of the mSMGP in the submandibular gland will further increase our understanding of this complex organ.

FIGURES

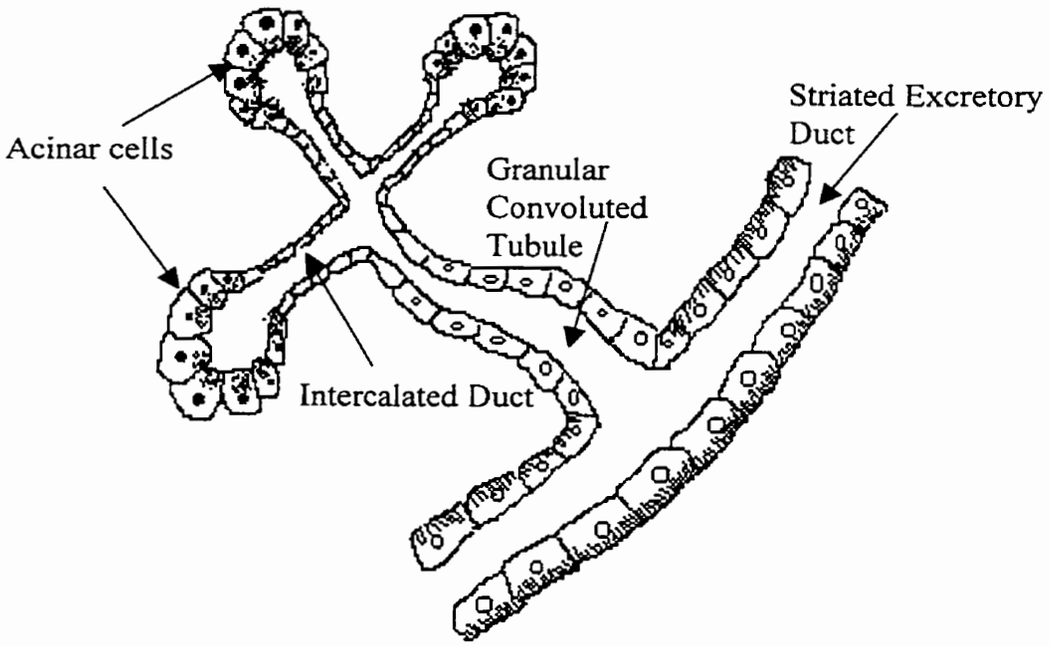


Figure 1. A schematic representation of the submandibular gland structure

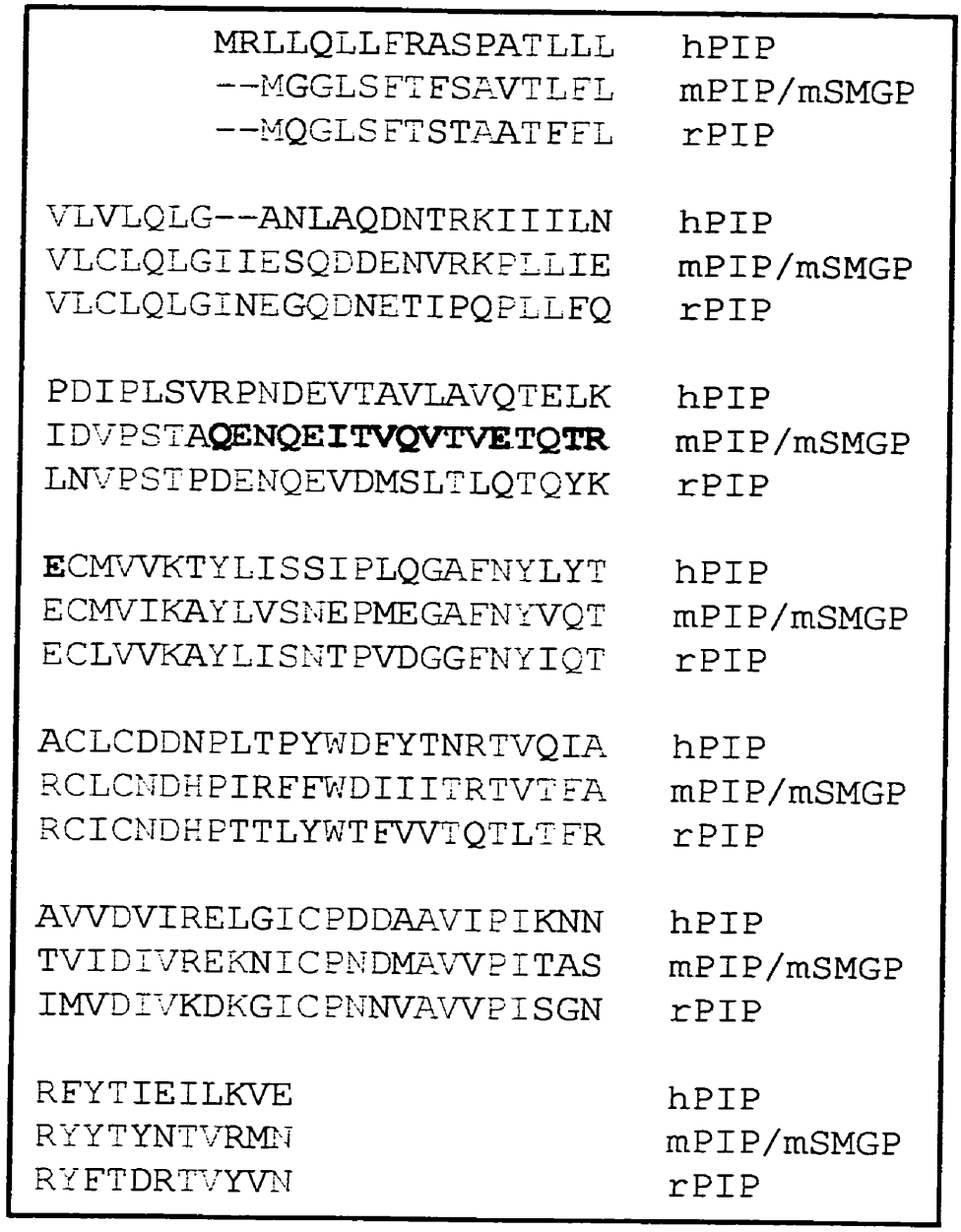


Figure 2. Comparisons between the amino acid sequences of the human PIP (hPIP), the mouse SMGP/PIP and the rat PIP (rPIP). The colored amino acids indicate homology between species: blue=human/mouse, red=mouse/rat, green=human/mouse/rat. The 18 amino acids highlighted indicate the peptide sequence used to generate the polyclonal antibody to the mSMGP (See Section 5.11.1. The Anti-mSMGP Antibody).

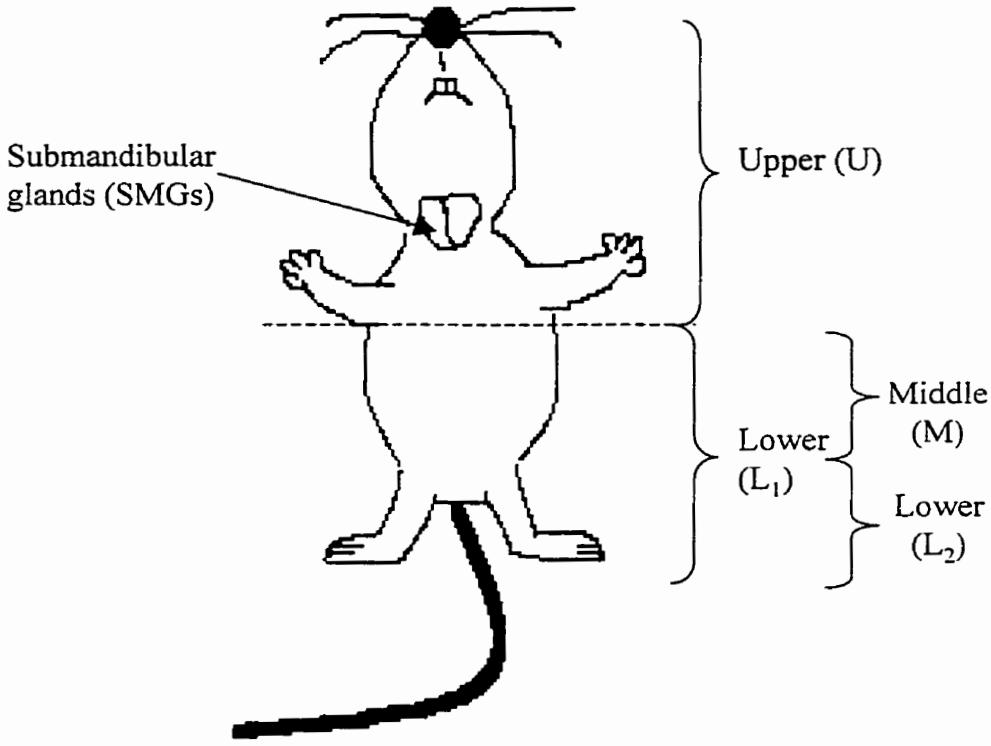


Figure 3. A schematic illustration of the mouse embryo sections analyzed for mSMGP gene expression. For the embryos E14 to E18, the body was analyzed in two separate sections: the upper (U) and lower (L_1) section. For the larger embryos, E19, E20, and E21, the body was separated into 3 parts: the upper (U), middle (M) and lower (L_2) sections. The submandibular glands (SMGs) were either left intact or removed from the upper section for separate analysis.

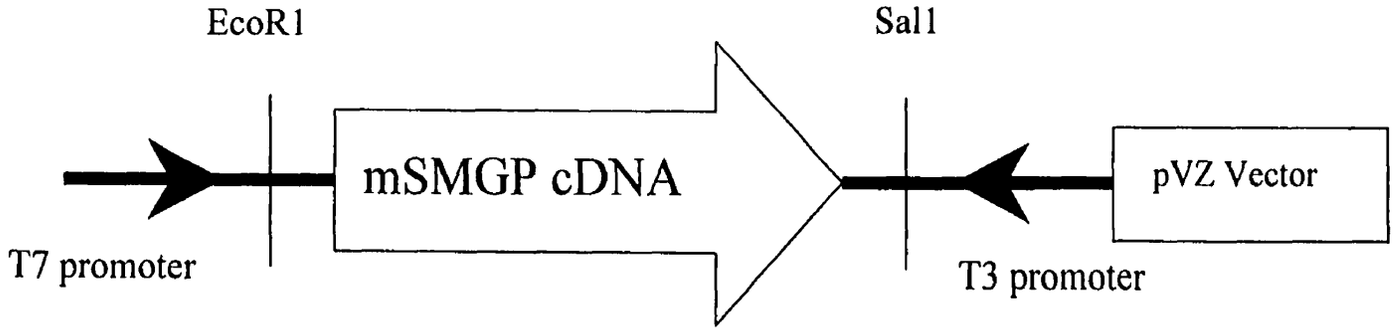


Figure 4. A schematic representation of the plasmid used to generate ^{35}S-labeled riboprobes. The large arrow represents the mSMGP cDNA insert oriented in the natural direction of transcription that was subcloned in the pVZI vector. Black arrows indicate the orientation of the promoters used for *in vitro* transcription (T7, T3). The restriction enzymes used to linearize the plasmids before transcription are indicated.

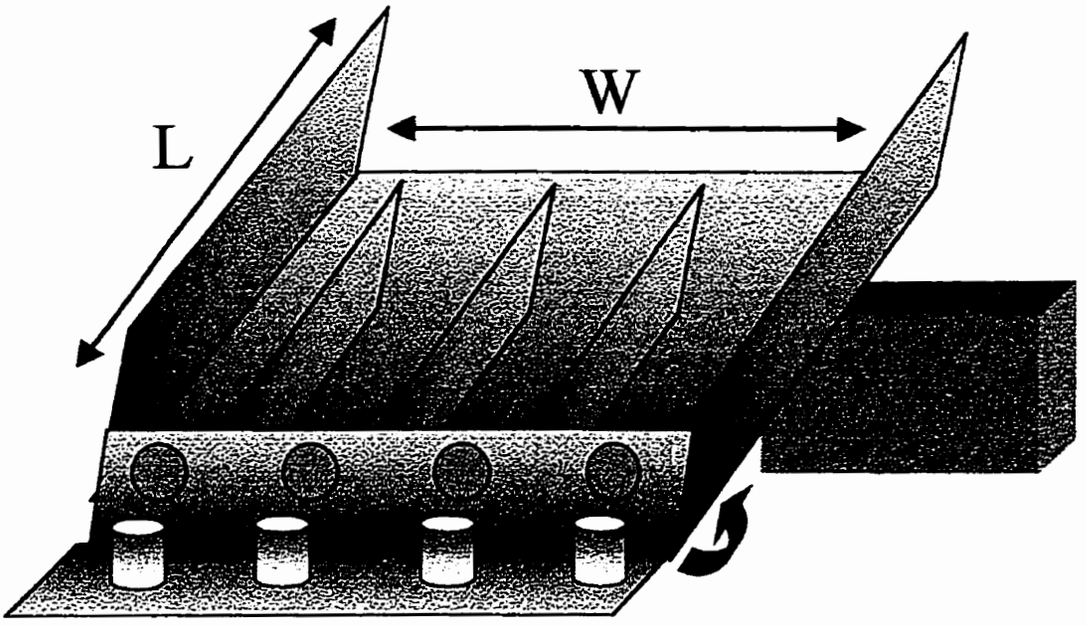


Figure 5. A schematic representation of the homemade multi-unit animal holder used for mouse saliva collection. The unit was constructed out of extra-strength cardboard with a length (L) of 32.5 cm, a width (W) of 42 cm and a diameter of 3.5 cm for each window. The saliva was collected into the glass vials placed directly underneath each window. A small box was used to elevate the apparatus at a 10-20° angle (blue arrow).

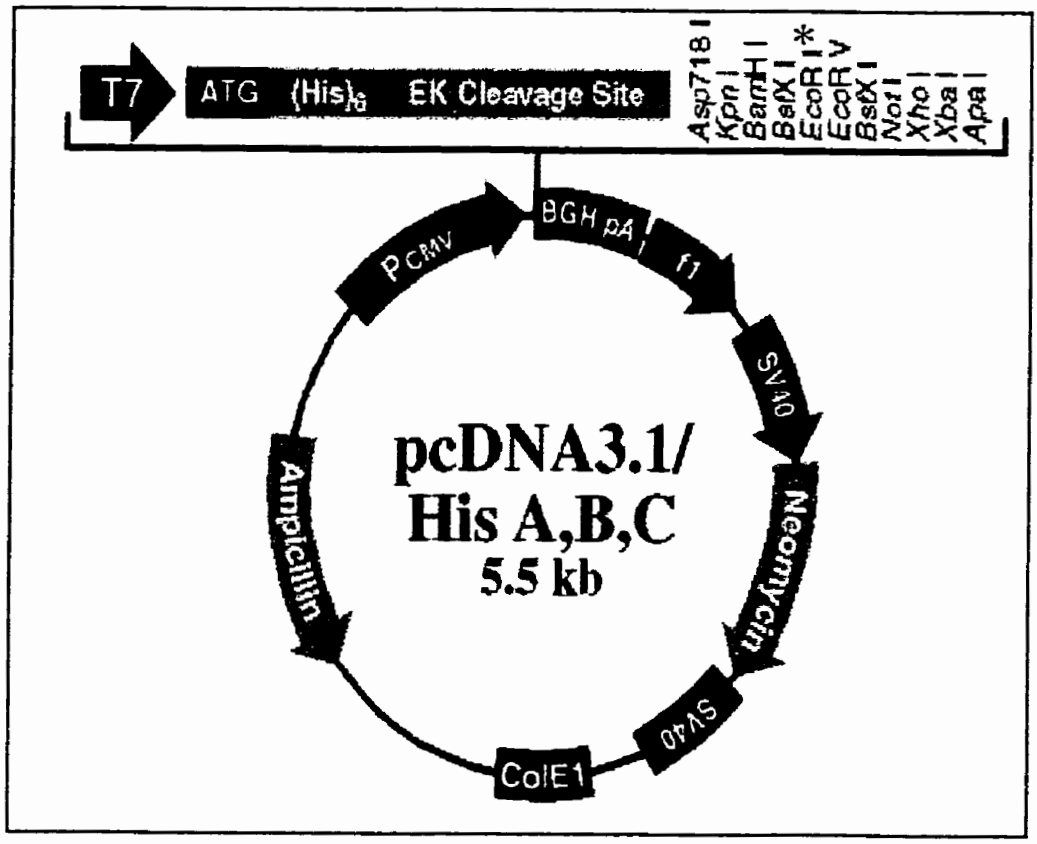


Figure 6. The pcDNA3.1 vector used for *in vitro* transcription/translation. The mSMGP cDNA insert was subcloned into the pcDNA3.1 vector shown above at the EcoRI site (*). After sequencing of the plasmid, the His B form was determined to contain the mSMGP cDNA in the correct frame and was chosen for further studies. (Reproduced from the website of Invitrogen, Carlsbad, CA, USA; http://www.invitrogen.com/vectordata/index.html)

A

	1	2	3	4	5	6	7	8
A	brain	eye	liver	lung	kidney			
B	heart	skeletal muscle	smooth muscle					
C	pancreas	thyroid	thymus	submax. gland	spleen			
D	testis	ovary	prostate	epididy-mus	uterus			
E	embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days				
F	yeast total RNA	yeast tRNA	*E. coli* rRNA	*E. coli* DNA				
G	poly r(A)	C_0t 1 DNA	mouse DNA	mouse DNA				
H								

B

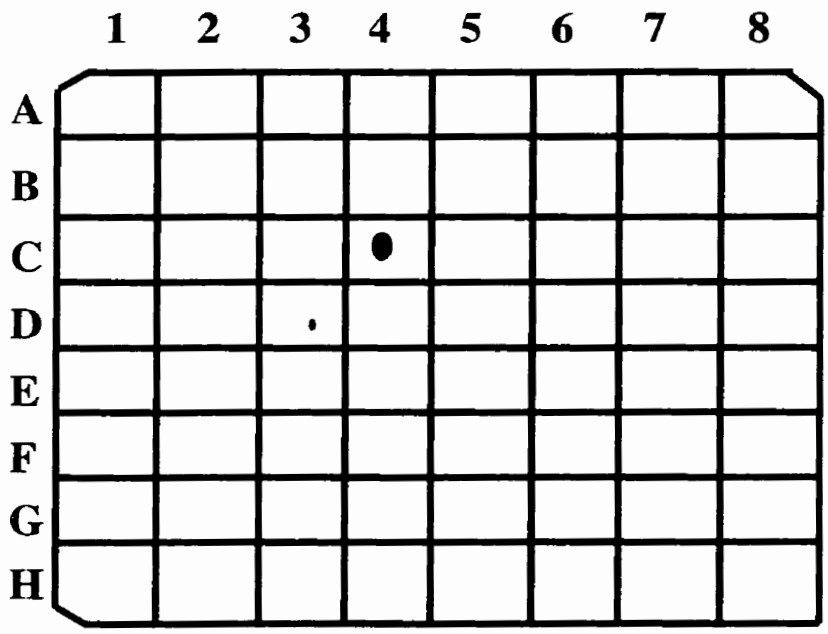


Figure 7. Tissue specific gene expression of mSMGP in the adult mouse. An RNA Master Blot™ (Clontech) containing poly A^+ RNAs (2μg) from different mouse tissues (Panel A) was screened with ^{32}P-labeled mSMGP cDNA. The mSMGP transcript (Panel B) was expressed in the mouse submandibular (C4) and prostate gland (D3).

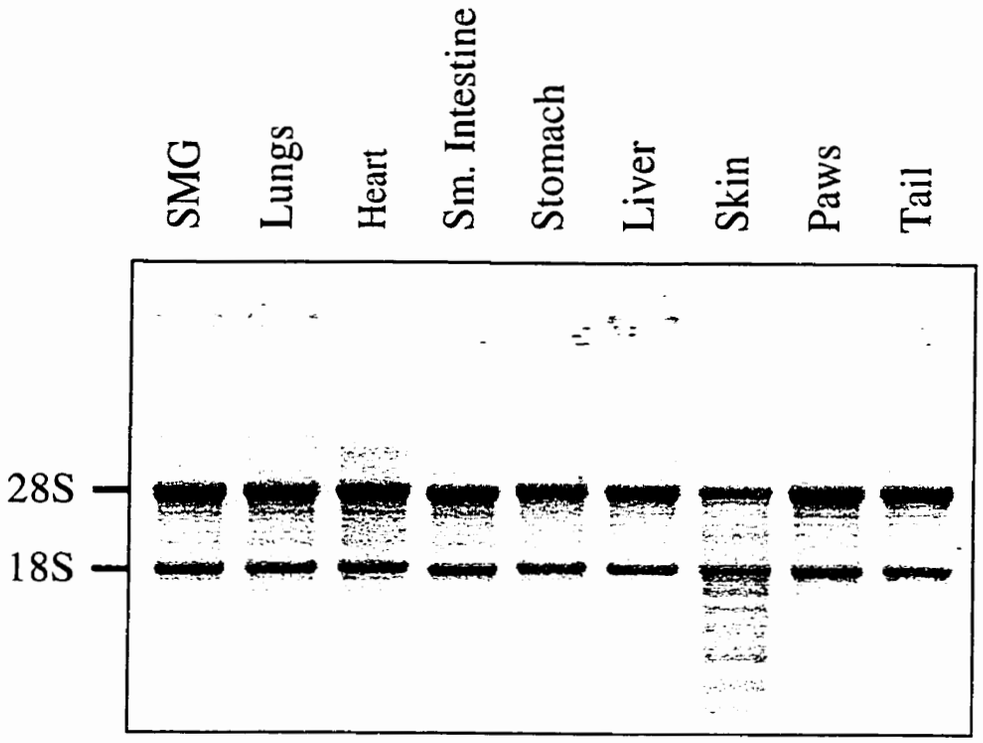


Figure 8. Analysis of RNA quality and integrity. Total RNA (1 μg) from several different E18 mouse tissues were electrophoresed on a 1.3% agarose gel after RNA extraction. Ribosomal bands were visualized by ultraviolet light.

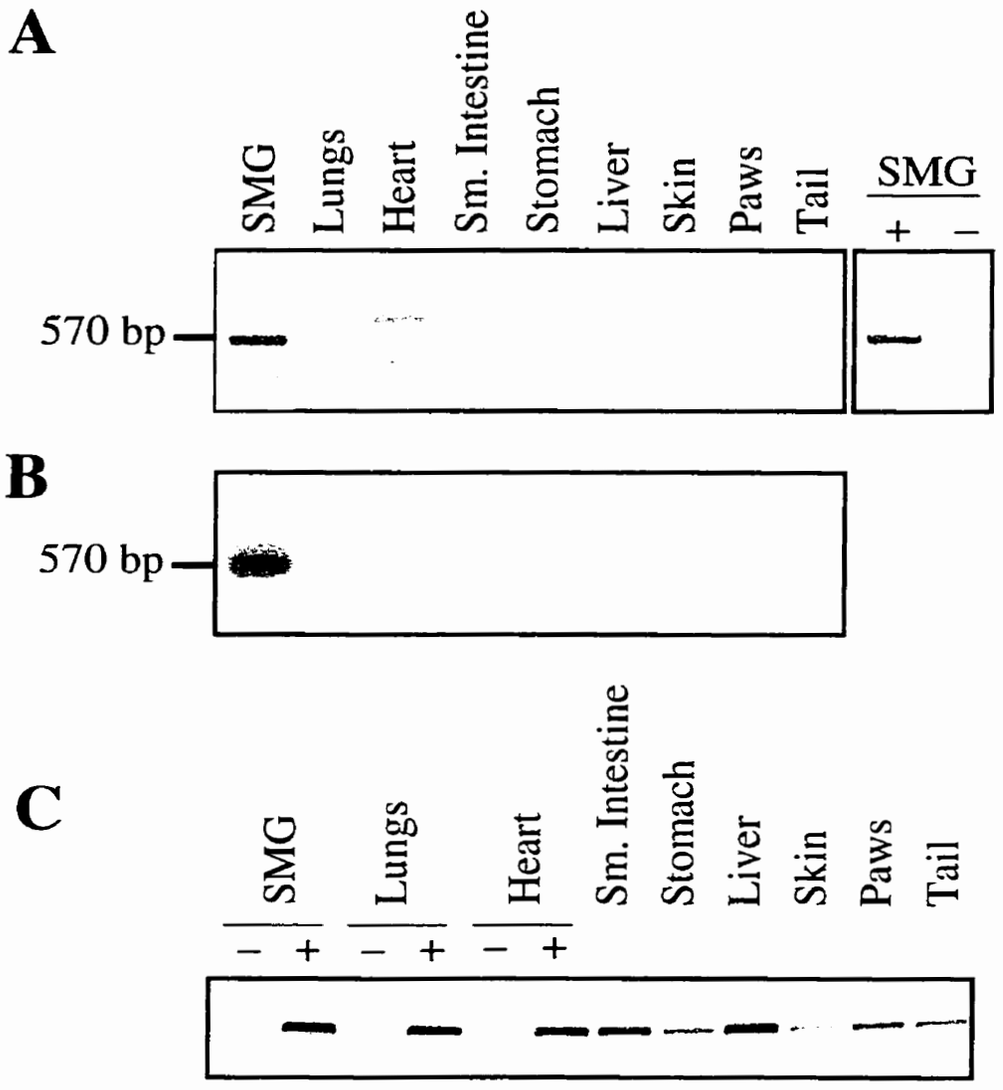


Figure 9. Tissue specific mSMGP gene expression in the mouse embryo. Various tissues were collected from a single E18 embryo and total RNA was analyzed by RT-PCR using mSMGP specific primers (see Table 4). PCR products were electrophoresed (Panel A) and Southern blot analysis was performed (Panel B). RT minus (-) and the expression of the housekeeping gene, mGAPDH (Panel C), were used as controls.

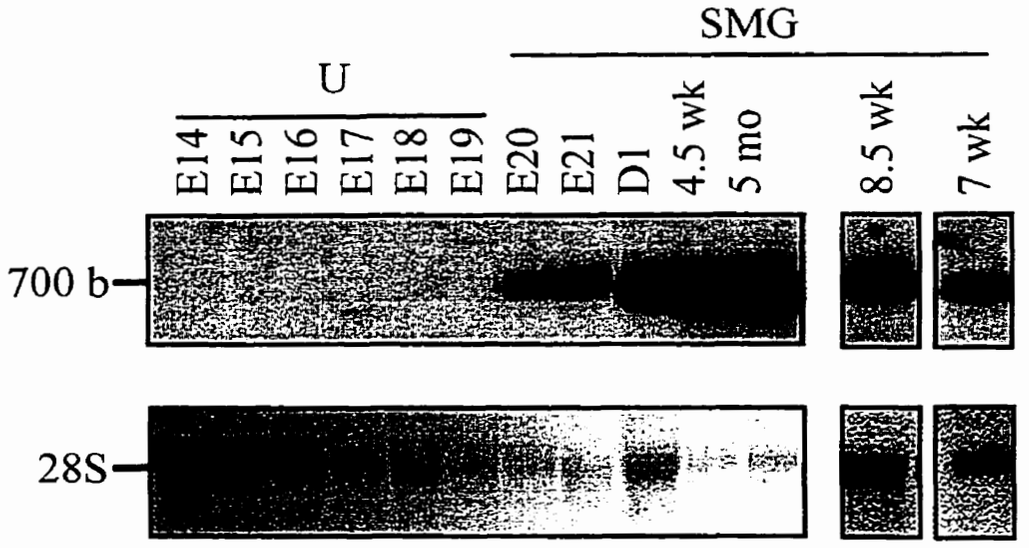
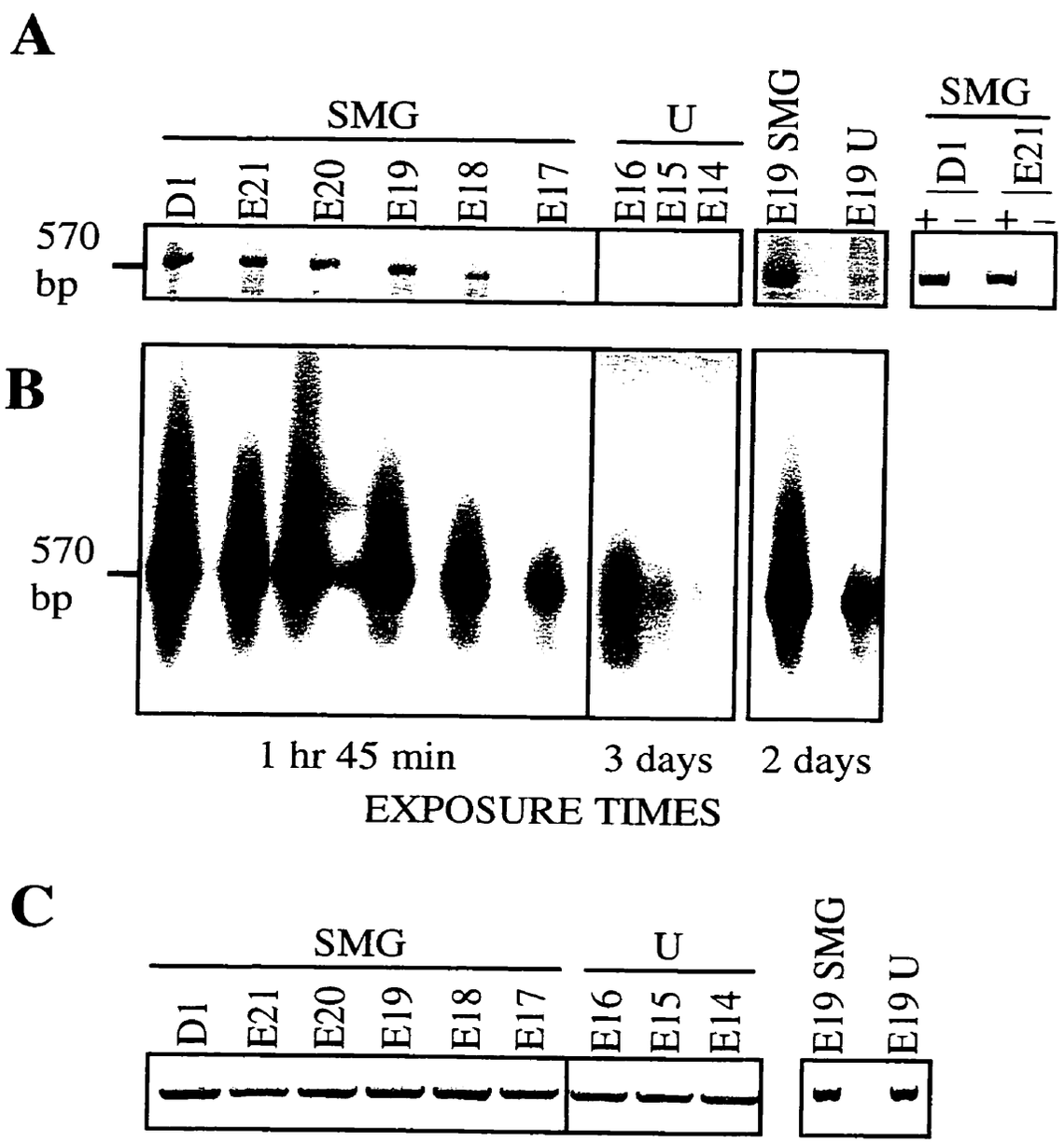


Figure 10. Northern blot analysis of mSMGP gene expression in embryonic SMGs. Northern blot analysis was performed on 10 μg total RNA of upper embryo sections (U) with intact SMGs of E14 to E19 and isolated submandibular glands (SMG) of E20 and E21. The top panels show the 700 bases long mSMGP transcript hybridized to the mSMGP cDNA probe. The bottom panels contain the ethidium bromide-stained 28S ribosomal RNA in the Northern gels. Day 1 (D1), 4.5, 7, 8.5 weeks and 5 month old SMGs were used as positive controls.

Figure 11. mSMGP gene expression in submandibular glands of developing embryos. Upper sections (U) with intact SMGs of E14 to E16 embryos and isolated SMGs of E17 to postnatal day 1 (D1) were analyzed for mSMGP gene expression. Total RNA was extracted and RT-PCR was carried out using mSMGP specific primers (see Table 4). RT-PCR products were electrophoresed (Panel A), transferred to nitrocellulose and analyzed by Southern blotting (Panel B). mSMGP expression at E14-E17, observed by RT-PCR analysis, was low and not visible by exposure to UV light but was detectable by Southern blot analysis after 3 days exposure to x-ray film. The entire upper section and the isolated SMG from an E19 embryo were compared (E19 U and E19 SMG) to observe the difference in gene expression detectable in both preparations. Panel C shows the results obtained after RT-PCR amplification with mGAPDH primers.


A
SMG
U
E19 SMG
SMG
D1
E21
E20
E19
E18
E17
E16
E15
E14
E19 U
D1
E21
+
−
+
−
570
bp
B
570
bp
1 hr 45 min
3 days
2 days
EXPOSURE TIMES
C
SMG
U
E19 SMG
D1
E21
E20
E19
E18
E17
E16
E15
E14
E19 U

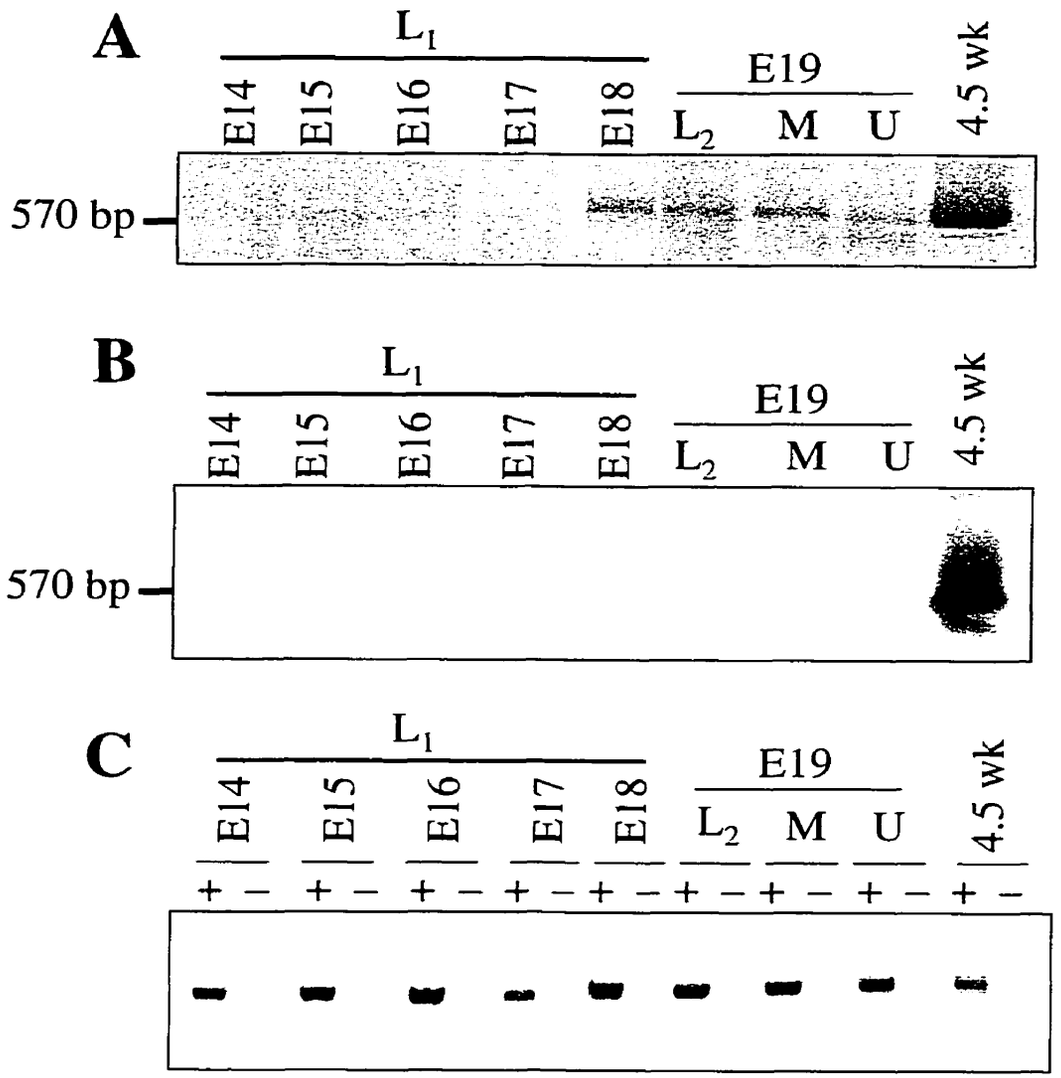
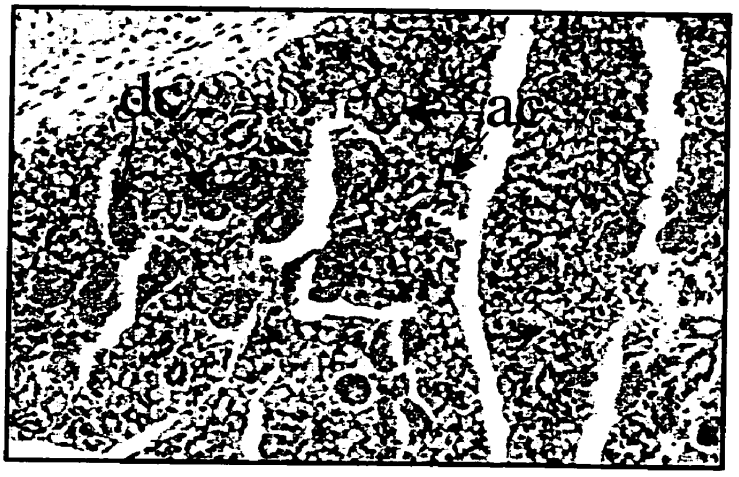


Figure 12. RT-PCR and Southern blot analysis of mSMGP gene expression in the mouse embryo. Panel A shows the RT-PCR results from the lower (L_1) sections of embryos E14 to E18 and lower (L_2), middle (M) and upper (U) sections without the SMGs of the E19 embryo. The RT-PCR products were then transferred onto nitrocellulose and analyzed by Southern blot analysis (Panel B). RT minus (-) and the expression of the housekeeping gene, mGAPDH (Panel C), were used as controls. A 4.5 week male mouse SMG was used as a positive control.

Figure 13. Localization of mSMGP gene expression in the adult mouse submandibular gland. Panel A: Hematoxylin & Eosin stain of an adult female submandibular gland (SMG). Panel B: *In situ* hybridization of the SMG to a ^{35}S-labeled antisense probe. Panel C: *In situ* hybridization of the SMG to a ^{35}S-labeled sense probe (control). ac, acinar cells; dc, ductal cells; 400X magnification.

A

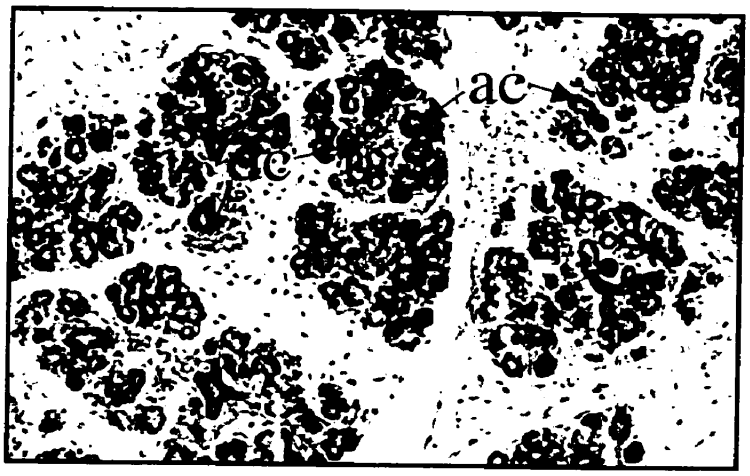


B

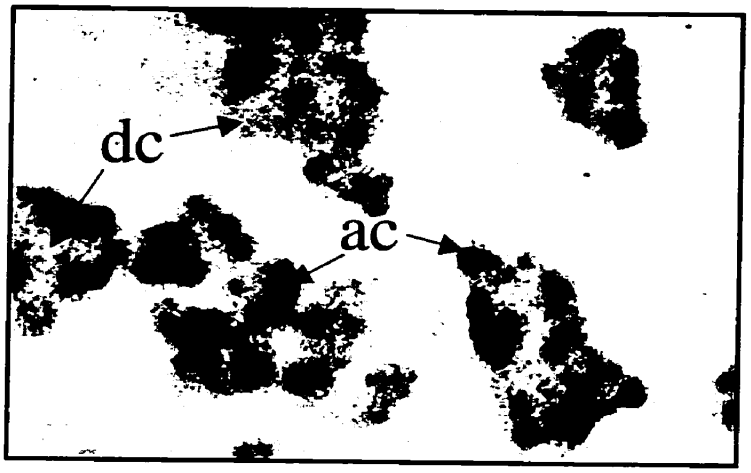
C

Figure 14. Localization of mSMGP gene expression in the E18 mouse submandibular gland. Panel A: Hematoxylin & Eosin stain of an E18 embryonic submandibular gland (SMG). Panel B: *In situ* hybridization of the SMG to a ^{35}S-labeled antisense probe. Panel C: *In situ* hybridization of the SMG to a ^{35}S-labeled sense probe (control). ac, acinar cells; dc, ductal cells; 400X magnification.

A



B



C

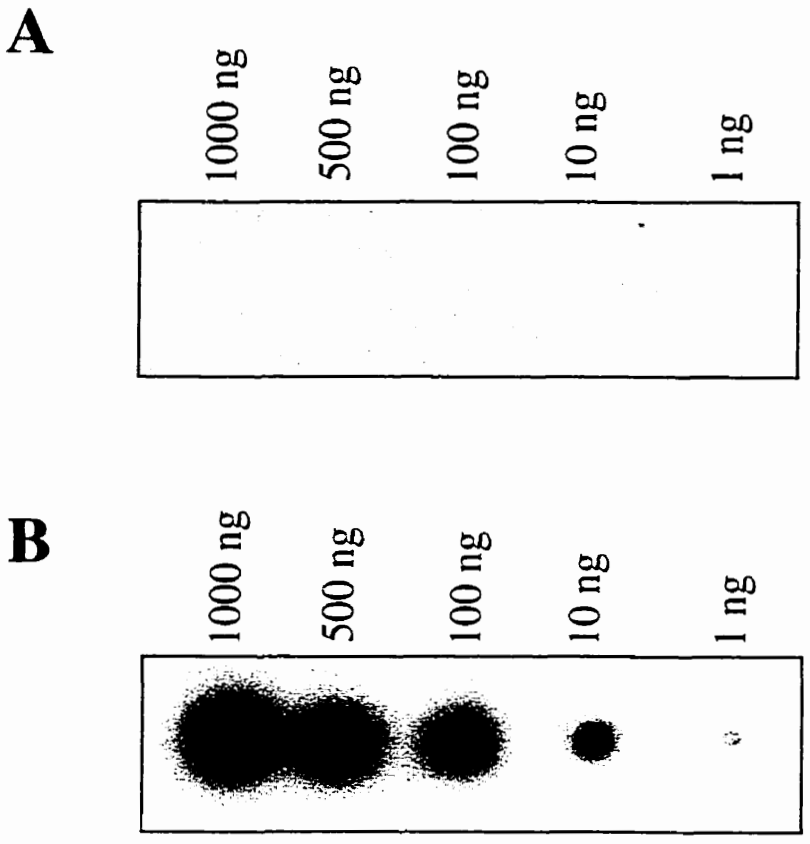
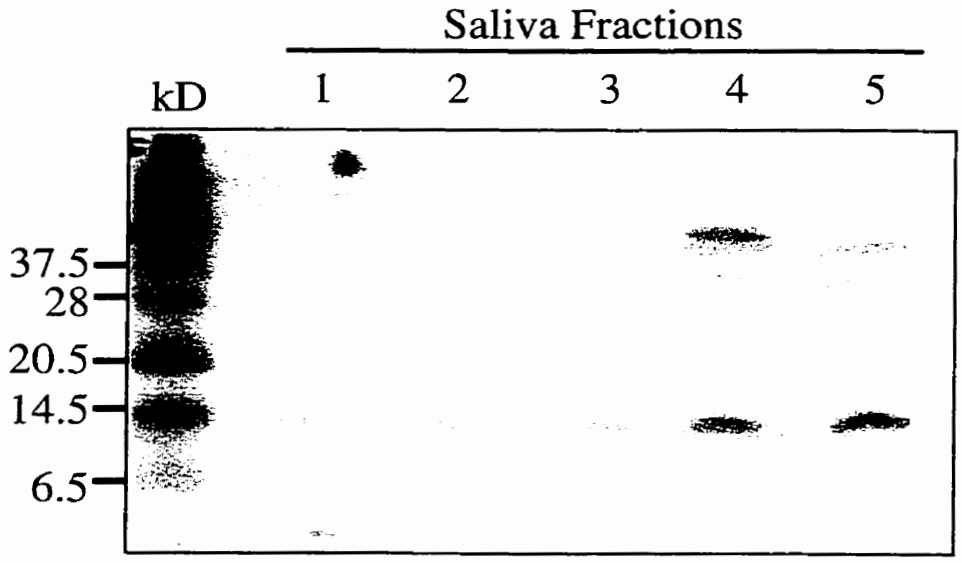


Figure 15. Dot blot analysis of the mSMGP synthetic peptide. Different concentrations of the 18 amino acid mSMGP synthetic peptide were blotted onto nitrocellulose membranes and incubated with preimmune rabbit serum (Panel A) or anti-mSMGP antibody (Panel B).

Mouse 1



Mouse 2

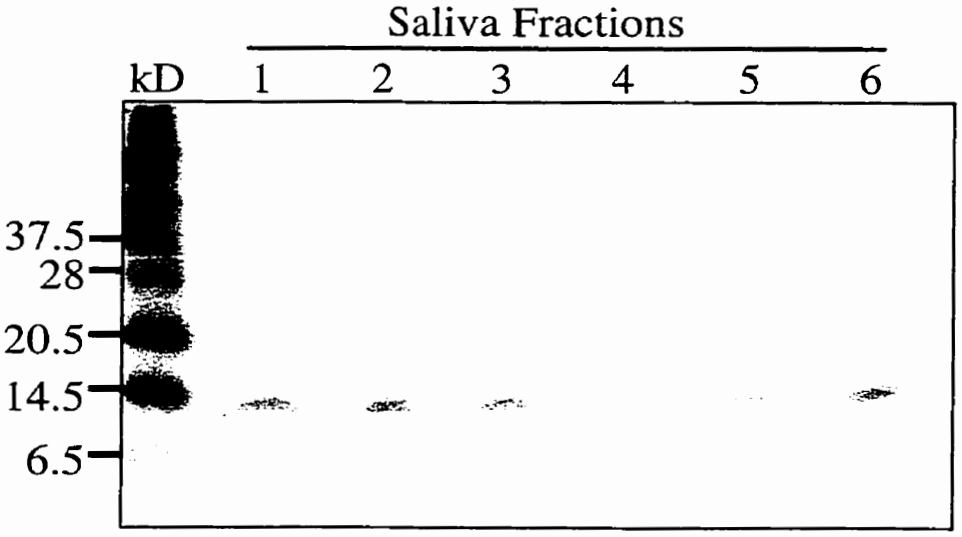


Figure 16. Analysis of mouse saliva fractions. Mouse saliva was collected from two different female mice in 50 µl increments. (fraction 1 to 5 (Mouse 1) or fraction 1 to 6 (Mouse 2)). Each fraction (9 µl) was electrophoresed in a 15% polyacrylamide gel under the Tris-Glycine buffer system, transferred onto 0.2 µm nitrocellulose and incubated with the anti-mSMGP antibody.

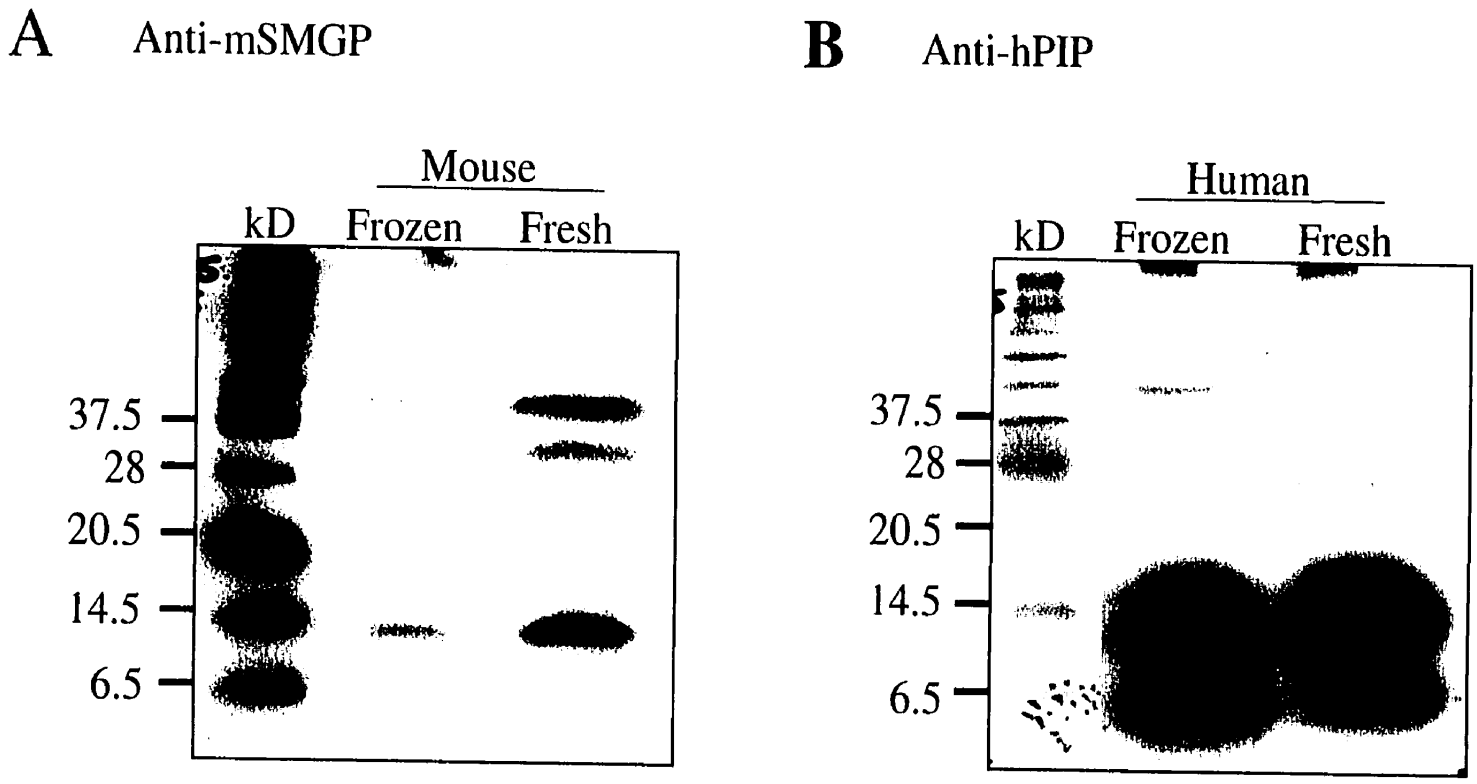


Figure 17. Comparison of frozen and fresh samples of mouse and human saliva. Fresh and previously collected frozen samples of mouse and human saliva (9 µl of each) were electrophoresed in a 15% polyacrylamide gel under the Tris-Glycine buffer system. Western blot analysis was performed using either the anti-mSMGP antibody (Panel A) or the anti-hPIP antibody (Panel B).

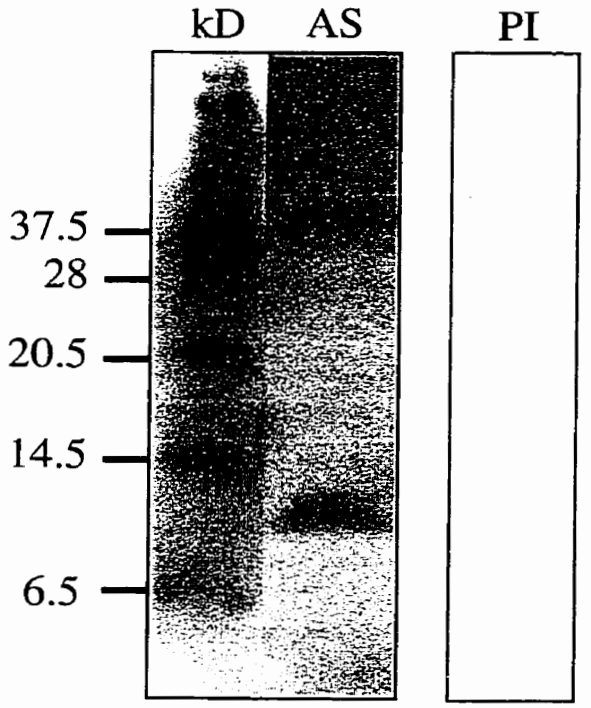


Figure 18. The mSMGP in mouse saliva. Nine μl of mouse saliva was electrophoresed in a 15 % polyacrylamide gel under the Tris-Tricine buffer system and transferred onto 0.2 μm nitrocellulose membrane. The membrane was first incubated with preimmune rabbit serum (PI), developed, then stripped and reincubated with the anti-mSMGP antibody (AS).

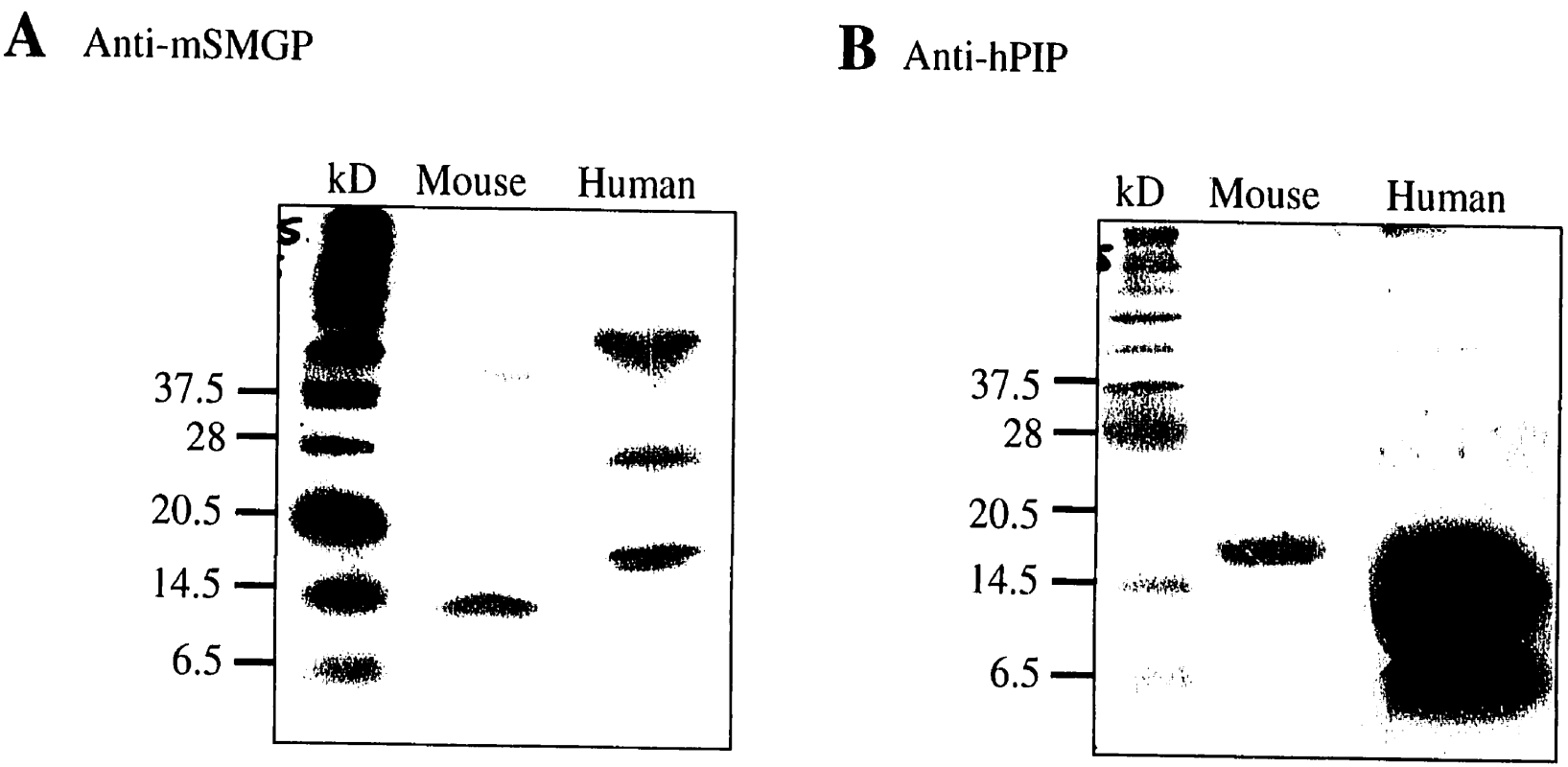


Figure 19. Cross-reactivity of the mSMGP and hPIP antibodies with proteins in human and mouse saliva. Mouse and human saliva were electrophoresed in a 15% polyacrylamide gel under the Tris-Glycine buffer system followed by transfer onto nitrocellulose membranes. The membranes were either incubated with the anti-mSMGP antibody (Panel A) or the anti-hPIP antibody (Panel B).

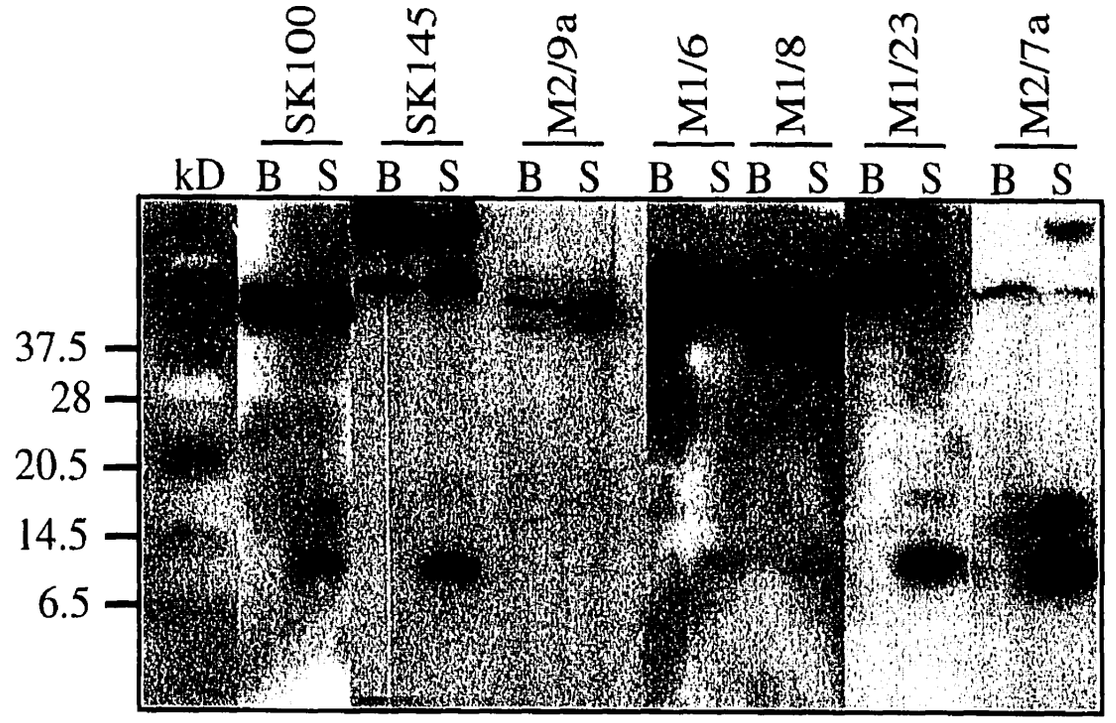


Figure 20. Binding of oral bacteria with the human prolactin-inducible protein (hPIP). Human saliva (S) was incubated with human (SK145, *Streptococcus mitis*; SK100, *Streptococcus oralis*) and mouse oral bacteria (M2/9a, M1/6, M1/8, M1/23, M2/7a). Protein binding was then examined by Western blot analysis using the anti-hPIP antibody to detect the 14 kD hPIP. As a control, bacterial strains were incubated in 10 mM Hepes Buffer pH 7.5 (B).

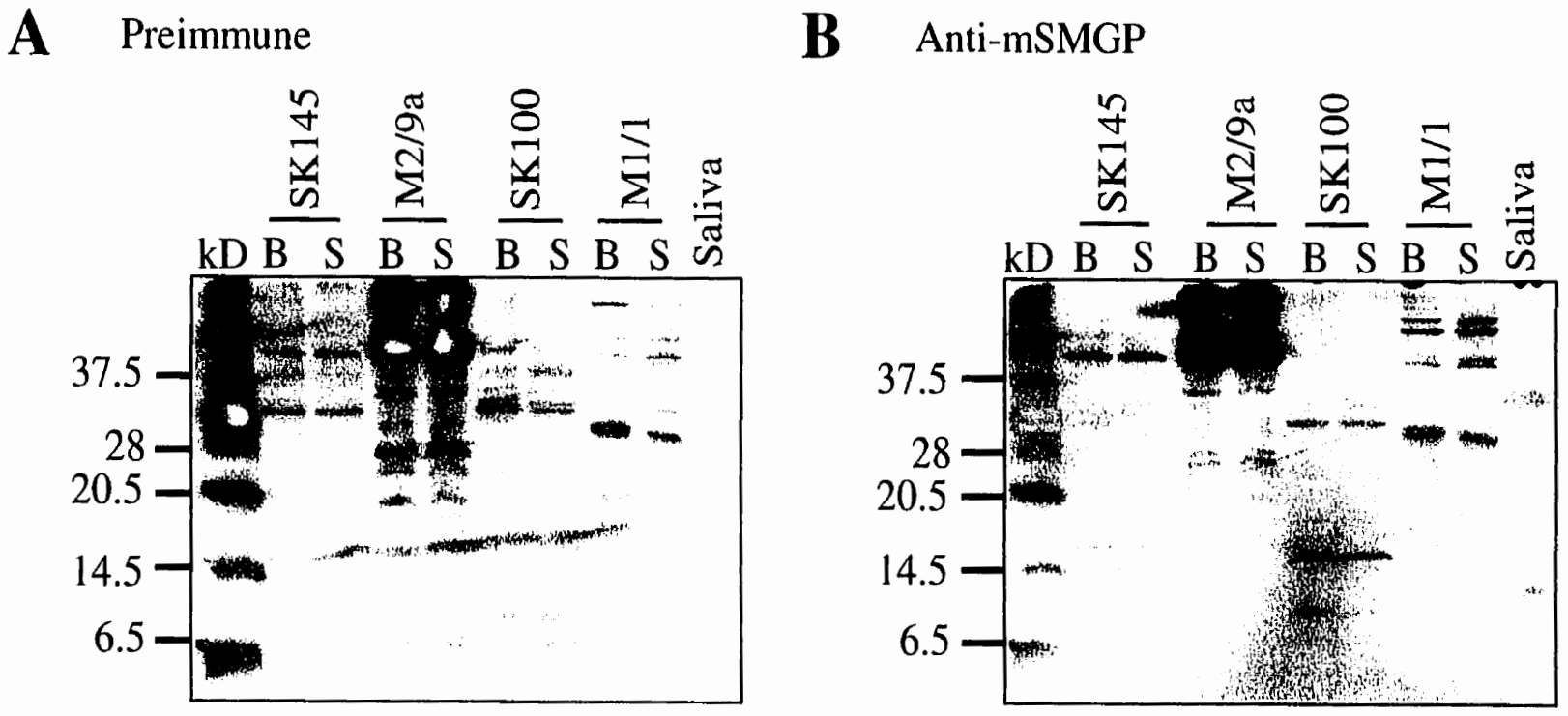
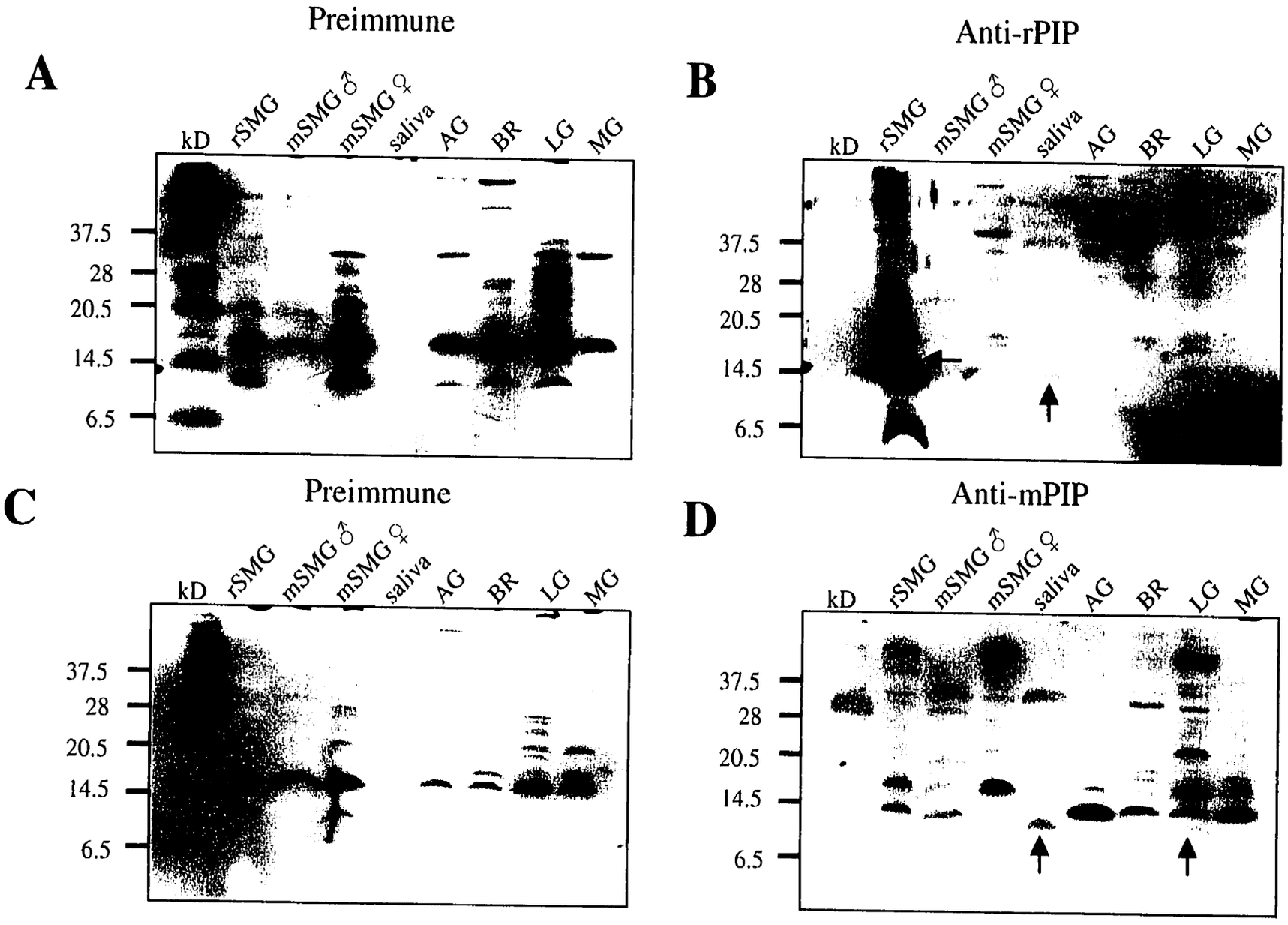


Figure 21. Binding of oral bacteria with the mSMGP in mouse saliva. Mouse saliva (S) was incubated with human (SK145, *Streptococcus mitis*; SK100, *Streptococcus oralis*) and mouse oral bacteria (M2/9a, M1/1). Binding was then examined by Western blot analysis. As a control, bacterial strains were incubated in 10 mM Hepes Buffer pH 7.5 (B). The nitrocellulose membrane was first incubated with preimmune serum and developed (Panel A) before it was stripped and reincubated with the anti-mSMGP antibody (Panel B).

Figure 22. Cross-reactivity of the antibodies anti-rPIP and anti-mSMGP with rat and mouse proteins. Western blot analysis using a 15% polyacrylamide gel under the Tris-Tricine buffer system was carried out to analyze the following samples: male rat submandibular gland, rSMG (25 μg); male mouse submandibular gland, mSMG ♂ (25 μg); female mouse submandibular gland, mSMG ♀ (25 μg); female mouse saliva (9 μl); female mouse adrenal gland, AG (25 μg); female mouse brain, BR (25 μg); female mouse lacrimal glands, LG (25 μg); female mouse mammary glands, MG (25 μg). Panel A and Panel B show the results after incubation with the preimmune serum and the anti-rPIP antibody for the rat, respectively. The black arrows indicate the 17 kD rPIP band (rSMG lane) and the 14 kD mSMGP band (saliva lane). Panel C and Panel D show the results after incubation with preimmune serum and the anti-mSMGP antibody for the mouse. The 14 kD mSMGP bands are designated by the blue arrows in the saliva and lacrimal gland lanes.


A
Preimmune
kD
rSMG
mSMG ♂
mSMG ♀
saliva
AG
BR
LG
MG
37.5
28
20.5
14.5
6.5
B
Anti-rPIP
C
Preimmune
D
Anti-mPIP

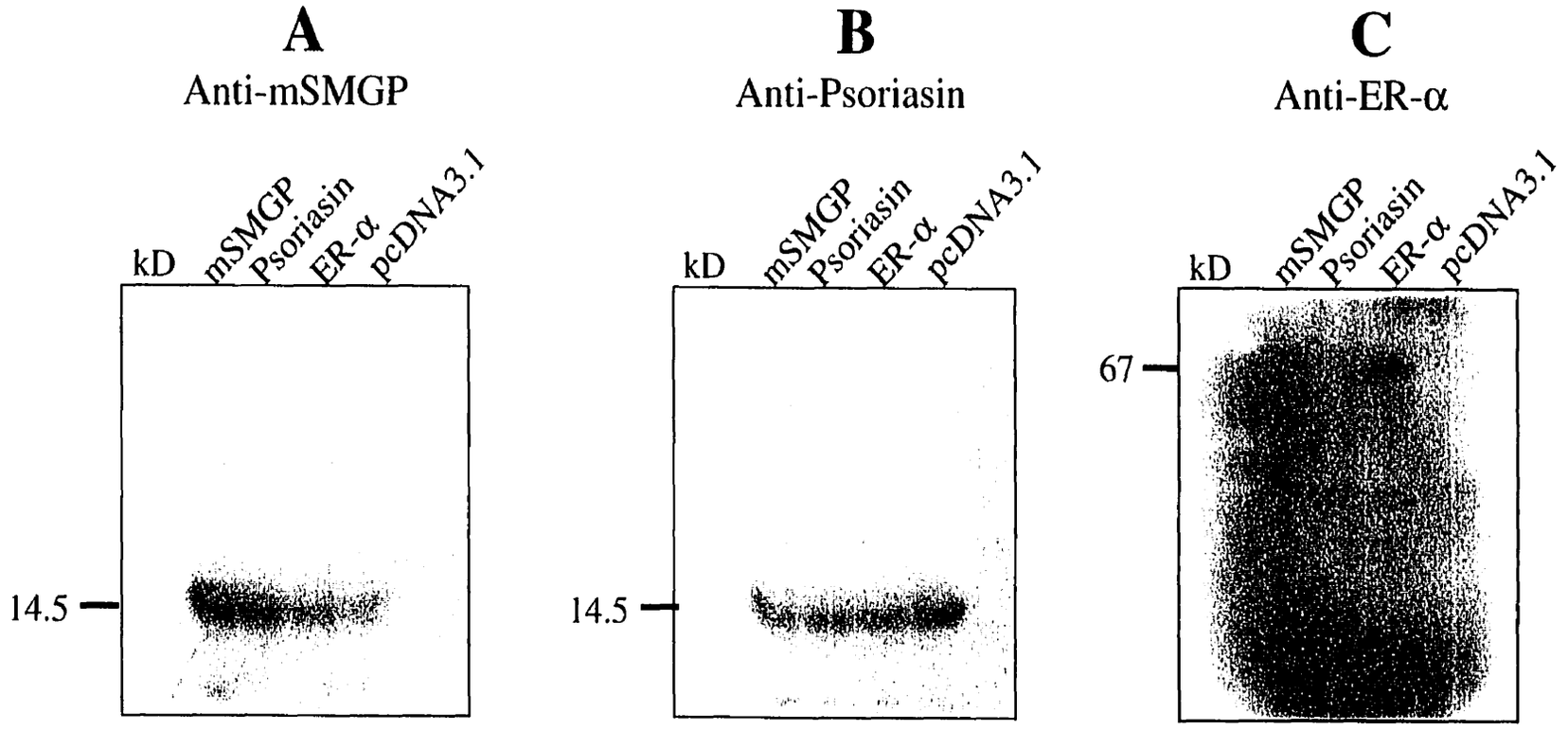


Figure 23. *In vitro* transcription/translation of proteins using rabbit reticulocyte lysate from the TNT® Coupled Reticulocyte Lysate System (Promega). *In vitro* translated proteins were generated from the pcDNA3.1 plasmids containing the mSMGP, Psoriasin or ER-α cDNA inserts. The pcDNA3.1 vector without a cDNA insert was also *in vitro* transcribed and translated as a negative control. The translated products were electrophoresed in a 15% polyacrylamide gel and analyzed by Western blot analysis under the Tris-Glycine buffer system. After transfer of the proteins onto 0.2 μm nitrocellulose membranes, each membrane was incubated with the anti-mSMGP antibody (Panel A), anti-Psoriasin antibody (Panel B) or anti-ER- α antibody (Panel C).

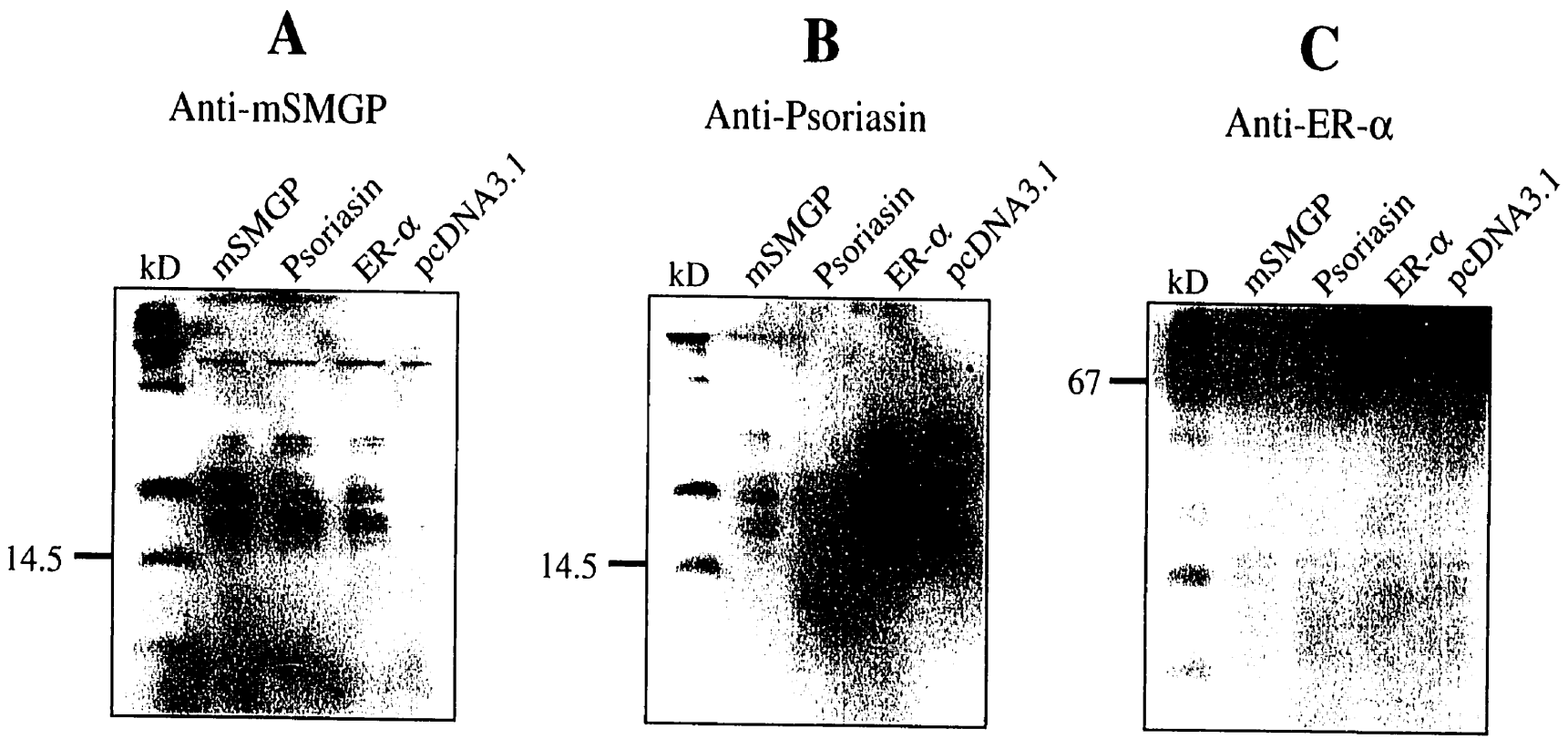


Figure 24. ***In vitro*** **transcription/translation of proteins using wheat germ extract from the TNT® Coupled Wheat Germ Extract System (Promega).** *In vitro* translated proteins were generated from the pcDNA3.1 plasmids containing the mSMGP, Psoriasin or ER-α cDNA inserts. The pcDNA3.1 vector without a cDNA insert was also *in vitro* transcribed and translated as a negative control. The translated products were electrophoresed in a 16.5% polyacrylamide gel and analyzed by Western blot analysis under the Tris-Tricine buffer system. After transfer of the proteins onto 0.2 μm nitrocellulose membranes, each membrane was incubated with the anti-mSMGP antibody (Panel A), anti-Psoriasin antibody (Panel B) or anti-ER- α antibody (Panel C).

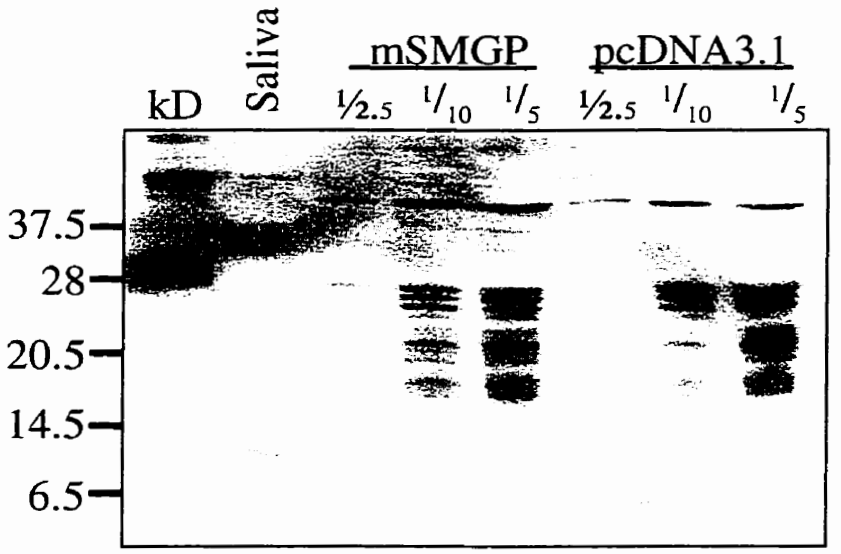


Figure 25. Western blot analysis of different volumes of *in vitro* translated mSMGP. *In vitro* translated proteins were generated from the pcDNA3.1 vector containing the mSMGP cDNA insert using the TNT® Coupled Wheat Germ Extract System (Promega). The pcDNA3.1 vector alone was also *in vitro* transcribed and translated as a negative control. Mouse saliva was included as a positive control. Different volumes of the translated products (1/2.5, 1/10 and 1/5 of the original 50 µl reaction volume) were electrophoresed in a 15% polyacrylamide gel and analyzed by Western blot analysis under the Tris-Tricine buffer system. After transfer of the proteins onto a 0.2 µm nitrocellulose membrane, the membrane was incubated with the anti-mSMGP antibody.

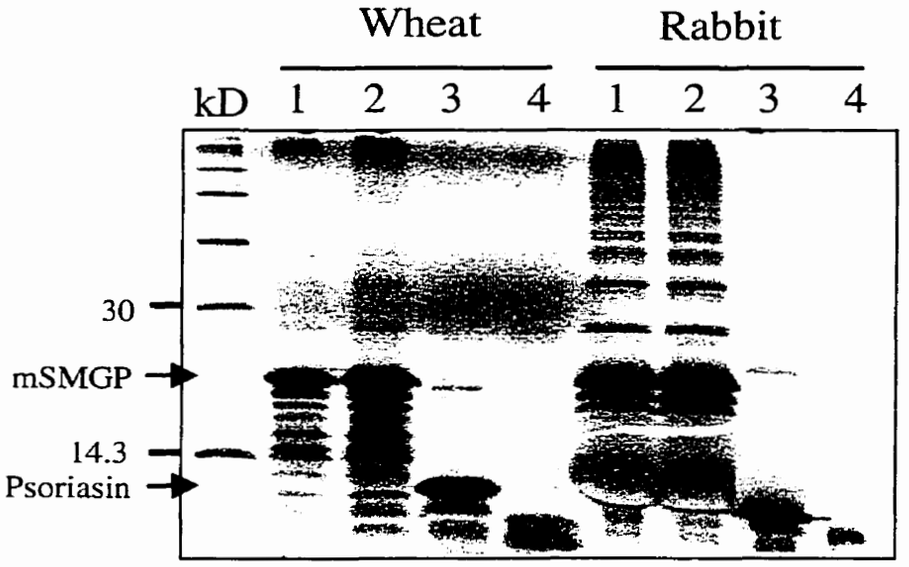


Figure 26. SDS-PAGE of ^{35}S-labeled *in vitro* translated protein products. ^{35}S-labeled *in vitro* translated proteins were generated using either the wheat germ extract system (Wheat) or the rabbit reticulocyte lysate system (Rabbit). Lanes 1) intact mSMGP plasmid; 2) linearized mSMGP plasmid; 3) intact Psoriasin plasmid; 4) the pcDNA3.1 vector alone. The translated products were electrophoresed in a 15% polyacrylamide gel and analyzed by SDS-PAGE under the Tris-Tricine buffer system. The gel was fixed and dried and exposed to x-ray film overnight for ^{35}S detection.

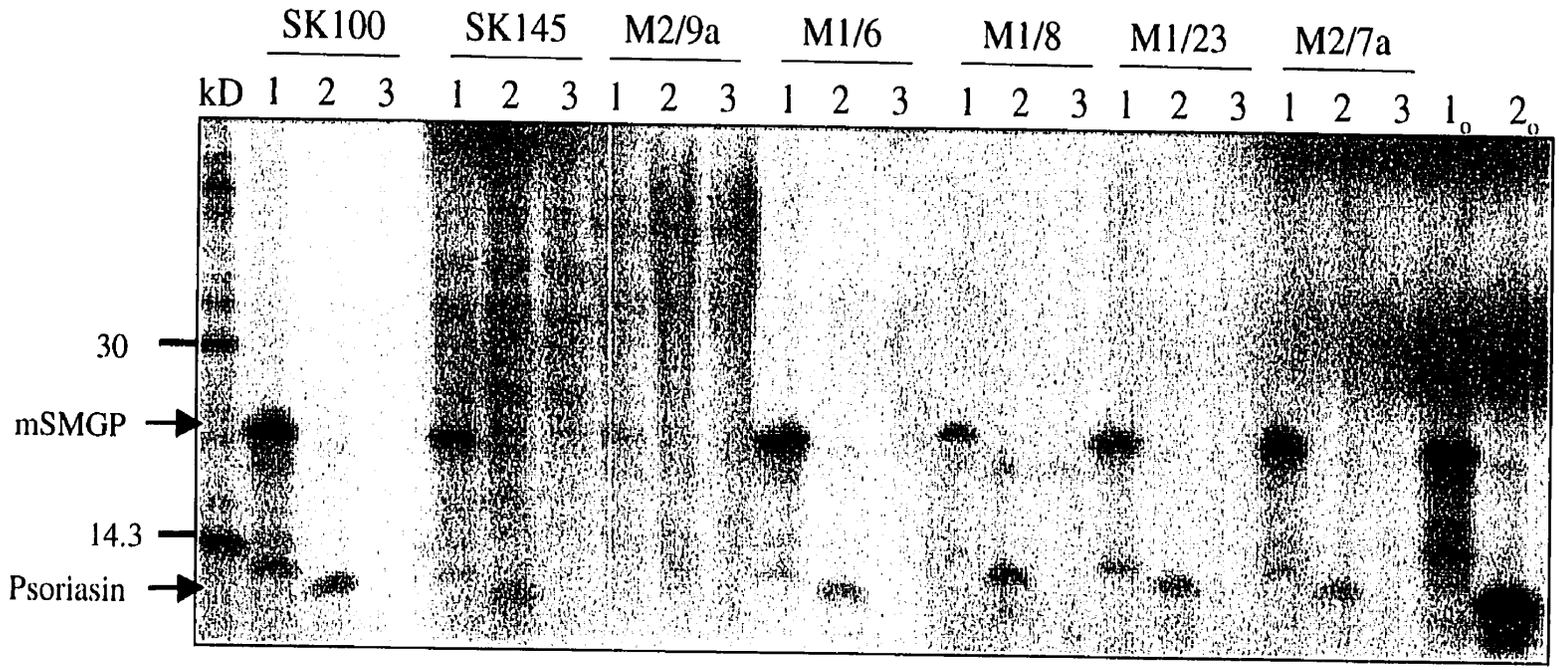
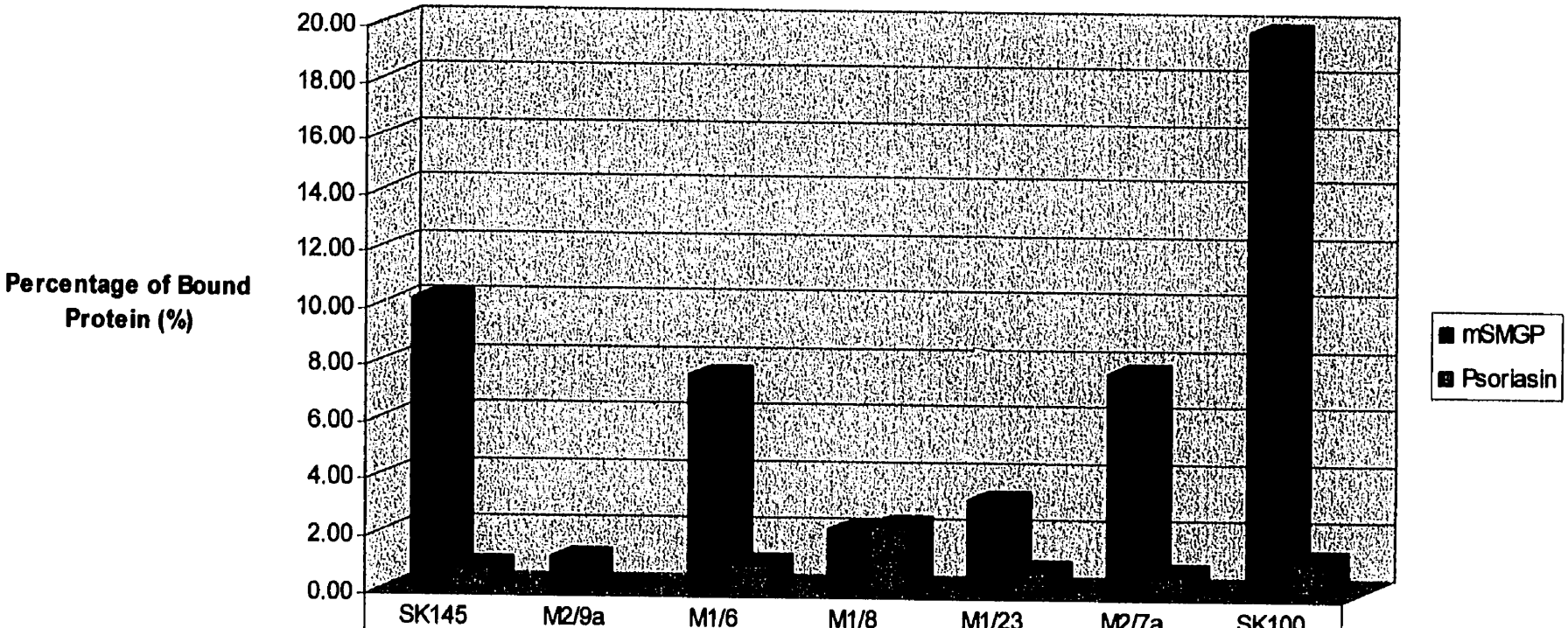


Figure 27. SDS-PAGE of bacteria-bound ^{35}S-labeled *in vitro* translated proteins. The mSMGP (1) and Psoriasin (2) proteins were generated with the TNT® Coupled Wheat Germ Extract System (Promega) along with the pcDNA3.1 vector alone (3). Each translated product was incubated with human (SK145, *Streptococcus mitis*; SK100, *Streptococcus oralis*) and mouse oral bacterial strains (M2/9a, M1/6, M1/8, M1/23, M2/7a). Binding of the proteins to bacteria was visualized by SDS-PAGE in a 15% polyacrylamide gel under the Tris-Tricine buffer system. A portion of the mSMGP (1_o) and Psoriasin (2_o) *in vitro* transcription/translation reaction volumes, without incubation with bacteria, were also electrophoresed. The gels were fixed and dried and exposed to film overnight for ^{35}S detection.

Figure 28. Proportions of *in vitro* translated protein products bound to different oral bacteria. Each ^{35}S-labeled *in vitro* translated product was incubated with human (SK145, *Streptococcus mitis*; SK100, *Streptococcus oralis*) and mouse oral bacterial strains (M2/9a, M1/6, M1/8, M1/23, M2/7a). Binding of the proteins with bacteria was visualized by SDS-PAGE. The amount of *in vitro* translated products bound was determined by densitometric analysis using the MCID M4 image analyzer (Version 2.0). All values were compared in relation to the pixel densities of the bands corresponding to the mSMGP and Psoriasin reaction volumes without bacterial incubation (See Section 5.21. Densitometric Analysis of Bacteria Binding Affinities).



	SK145	M2/9a	M1/6	M1/8	M1/23	M2/7a	SK100
mSMGP	10.14	1.08	7.52	2.15	3.24	7.73	19.87
Psoriasin	0.77	0.17	0.97	2.35	0.79	0.68	1.18

TABLES

Table 1. Immune and Non-immune Defense Factors of the Oral Cavity (Marsh & Martin, 1992).

DEFENSE FACTOR	MAIN FUNCTION
IMMUNE	
Intra-epithelial lymphocytes	Cellular barrier to penetrating bacteria and/or antigens
sIgA	Prevents microbial adhesions and metabolism
IgG, IgA, IgM	Prevent microbial adhesion; opsonins; complement activators
Complement	Activates neutrophils
Neutrophils/macrophages	Phagocytosis
NON-IMMUNE	
Saliva flow	Physical removal of micro-organisms
Mucin/agglutinins	Physical removal of micro-organisms
Lysozyme-protease-anion system	Cell lysis
Lactoferrin	Iron sequestration
Apo-lactoferrin	Cell killing
Sialoperoxidase system	Hypothiocyanite production (neutral pH) Hypocyanous acid production (low pH)
Histidine-rich peptides	Antibacterial and antifungal activity

Table 2. Bacterial Species Isolated from the Oral Flora of BALB/c Mice (Trudel et al., 1986).

Species	Proportion (%)
Lactobacillus murinis	38%
Staphylococcus aureus	37%
Streptococcus faecalis	8%
Staphylococcus sciuri	4%
Escherichia coli	3%
Clostridium bifermentans	2.5%
Staphylococcus epidermis	2%
Staphylococcus xylosus	1.5%
Enterobacter cloacae	1.5%
Aerococcus viridans	Few
Bacillus sp.	Few
Proteus mirabilis	Few
Clostridium botulinum II	Few
Group B streptococci	Few
Streptococcus faecium	Few
Streptococcus intermedius	Few
Staphylococcus warneri	Few
Staphylococcus hominis	Few

Table 3. Distribution of Bacterial Species Isolated from the Oral Cavity of CD1 Mice (Gadbois et al., 1993).

Species	Upon Arrival (%)	1 Week later (%)
Lactobacillus murinus	15	53
Staphylococcus aureus	8	6
Staphylococcus cohnii	21	5
Staphylococcus sciuri	12	3
Streptococcus sanguis	34	27
Enterococcus faecalis	10	4

Table 4. Primer Sequences Used for RT-PCR.

Target		Sequence (5'-3')	Primer Position	Product size (bp)	Conc. used
mSMGP	Fwd	TGGTGTTCTGACTTCTCCAC	7-26	570	500 nM
	Rev	GACTCGAGTCGACATCGATT			500 nM
mGAPDH	Fwd	TCATCATCTCCGCCCCTTCTGC	396-417	623	200 nM
	Rev	GTCCACCACCCTGTTGCTGTAG	997-1018		200 nM

Table 5. PCR Profiles Performed for RT-PCR.

	mSMGP		mGAPDH	
Step	**Temp (°C)**	**Time (min)**	**Temp (°C)**	**Time (min)**
Initial	94	5	94	2
Denaturation } 1 cycle	94	1	94	0.5
Annealing } 1 cycle	50	2	52	0.5
Elongation } 1 cycle	72	1	72	0.5
Final	72	10	72	10
Number of Cycles	35		24	

Table 6. Western Blot Analysis Conditions for Each Antibody Used.

TARGET PROTEIN	PRIMARY ANTIBODY (1° Ab)	DILUTION OF 1° Ab USED*	SECONDARY ANTIBODY (2° Ab)*	DILUTION OF 2° Ab USED**
mSMGP	Polyclonal Rabbit anti-mouse[1] (or preimmune serum)	1:500	Polyclonal Goat anti-rabbit[6]	1:10,000
hPIP	Polyclonal Rabbit anti-human[2] (or preimmune serum)	1:500	Polyclonal Goat anti-rabbit[6]	1:10,000
rPIP	Polyclonal Rabbit anti-rat[3] (or preimmune serum)	1:200	Polyclonal Goat anti-rabbit[6]	1:20,000
Psoriasin	Polyclonal Rabbit anti-human[4]	1:5000	Polyclonal Goat anti-rabbit[6]	1:10,000
ER-α	Monoclonal Mouse anti-human[5]	1:1000	Polyclonal Goat anti-mouse[7]	1:5000

*The anti-biotin antibody conjugated to horseradish peroxidase antibody (NEB) in a 1 in 1000 dilution was combined with each 2° Ab for standard detection.

**Diluted in 1X TBST with 3% BSA

[1]See Section 5.11.1 "Preparation of Antibodies: The Anti-mSMGP Antibody"

[2] See Section 5.11.2 "Preparation of Antibodies: The Anti-hPIP Antibody"

[3] See Section 5.11.3 "Preparation of Antibodies: The Anti-rPIP Antibody"

[4] Provided by Dr. Peter Watson's Laboratory (Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada)

[5] NovoCastra, Burlington, ON, Canada; Provided by Dr. Leigh Murphy's Laboratory (Department of Biochemistry, University of Manitoba, Winnipeg, Manitoba, Canada)

[6] Bio-Rad EIA grade affinity purified goat anti-rabbit IgG (H+L) horse radish peroxidase conjugate secondary antibody (Bio-Rad)

[7]Bio-Rad; Provided by Dr. Leigh Murphy's Laboratory (Department of Biochemistry, University of Manitoba, Winnipeg, Manitoba, Canada)

Table 7. Physiological and Biochemical Tests for Bacterial Identification.

TEST	RESULT	
	Positive	**Negative**
Hemolysis (HEM)		No change in surrounding medium (Gamma; γ)
	Greenish halos in surrounding medium (Alpha; α)	
	Clear zone in surrounding medium (Beta; β)	
Gram Stain (G)	Dark purple cells	Pink cells
Catalase Test (CAT)	Bubble formation	No bubble formation
Lactobacillus Test (ROG)	Growth on ROGOSA plate	No Growth on ROGOSA plate
Cytochrome Oxidase Test	Violet Purple color change	No color change
Rapid ID 32 Strep Tests (bioMérieux)		
Arginine DiHydrolase (ADH)	Red/orange-red	Yellow
βGLUcosidase (βGLU)	Fluorescent pink/red-orange	Pale orange
βGALactosidase (βGAR)	Fluorescent pink/red-orange	Orange
βGlucURonidase (βGUR)		
αGALactosidase (αGAL)	Yellow	Colorless
Alcaline Phosphatase (PAL)	Yellow	Colorless/very pale yellow
RIBose (RIB; Acidification)	Yellow/orange	Red/red-orange
MANnitol (MAN; Acidification)		
SORbitol (SOR; Acidification)		
LACtose (LAC; Acidification)		
TREhalose (TRE)		
RAFfinose (RAF; Acidification)		
Acetoine Production (VP)	Pink	Colorless
Alanine-Phenylalanine-Proline Arylamidase (APPA)	Orange	Colorless/pale orange
βGALactosidase (βGAL)	Purple	Colorless/pale orange/pale purple
Pyroglutamic acid Arylamidase (PyrA)	Orange	Colorless/pale orange
N-Acetyl-β Glucosaminidase (βNAG)	Purple	Colorless/pale orange/pale purple
Glycyl-Tryptophane Arylamidase (GTA)	Orange	Colorless/pale orange
Hydrolysis of HIPpurate (HIP)	Blue	Colorless

Table 7 continued…

TEST	RESULT	
	Positive	**Negative**
Rapid ID 32 Strep Tests (bioMérieux) cont'd		
GLYcoGen (GLYG; Acidification)	Yellow/orange	Red/red-orange
PULlulan (PUL; Acidification)		
MALtose (MAL; Acidification)		
MELibiose (MEL; Acidification)		
MeLeZitose (MLZ; Acidification)		
SACcharose/Sucrose (SAC; Acidification)		
L-ARAbinose (LARA; Acidification)		
D-ARabitoL (DARL; Acidification)		
Methyl-B-D Glucopyranoside (MβDG; Acidification)		
TAGatose (TAG; Acidification)		
βMANnosidase (βMAN)		
CycloDEXtrin (CDEX; Acidification)		
UREase (URE)	Pink/red-violet	Yellow/beige-pink
ID 32 E Test (bioMérieux)		
Ornithin DeCarboxylase (ODC)	Red/orange	Yellow/yellow-orange
Arginine DiHyrdolase (ADH)		
Lysin DeCarboxylase (LDC)	Blue-violet	Yellow-green
UREase (URE)	Pink-violet	Yellow/yellow-orange
L-ARabitol (LARL; Acidification)	Yellow/green-yellow	Blue/blue-green
GalacturonaTe (GAT; Acidification)		
5 KetoGluconate (5KG; Acidification)		
LIPase (LIP)	Blue	Colorless
Phenol Red (RP; Acidification)	Yellow	Red/orange
β GLUcosidase (βGLU)	Yellow	Colorless
MANnitol (MAN; Acidification)	Yellow/green-yellow	Blue/blue-green
MALtose (MAL; Acidification)		
INDol (IND; Production)	Pink/red	Colorless/yellow/beige
N-Acetyl-β-Glucosaminidase (βNAG)	Blue	Colorless

Table 7 continued...

TEST	RESULT	
	Positive	**Negative**
ID 32 E Test (bioMérieux) cont'd		
β GALactosidase (βGAL)	Yellow	Colorless
GLUcose (GLU; Acidification)	Yellow/green-yellow	Blue/blue-green
SACcharose/Sucrose (SAC; Acidification)		
L-ARAbinose (LARA; Acidification)		
D-ARabitoL (DARL; Acidification)		
α GLUcosidase (αGLU)	Yellow	colorless
α GALactosidase (αGAL)		
TREhalose (TRE; Acidification)	Yellow/green-yellow	Blue/blue-green
RHAmnose (RHA; Acidification)		
INOsitol (INO; Acidification)		
ADOnitol (ADO; Acidification)		
PaLatinosE (PLE; Acidification)		
β GlUcuRonidase (βGUR)	Yellow	Colorless
MaloNaTe (MNT)		
CELlobiose (CEL; Acidification)	Yellow/green-yellow	Blue/blue-green
SORbitol (SOR; Acidification)		
α MALtosidase (αMAL)	Yellow	Colorless/very pale yellow
L-Aspartic acid Arylamidase (AspA)		

Table 8. Bacterial Strains Used for Binding Studies.

IDENTIFICATION #	SPECIES	SOURCE
Laboratory Strains:		
1395-4C	*Streptococcus parasanguis*	UK Parasanguis
139N-3A	*Streptococcus parasanguis*	UK Parasanguis
RW55898	*Streptococcus parasanguis*	UK Parasanguis
A8-2a	*Streptococcus parasanguis*	St. Boniface, Canada
SK 112	*Streptococcus sanguis*	Dr. M. Kilian, Aarhus, Denmark
SK 100	*Streptococcus oralis*	
SK 120	*Streptococcus oralis*	
SK 65	*Streptococcus milleri*	
SK 145	*Streptococcus mitis*	
SK 95	*Streptococcus mitis*	
SK 137a	*Streptococcus mitis*	
ATCC 12104	*Actinomyces naeslundii*	American Type Culture Collection, Rockville, MA
WVU 627	*Actinomyces naeslundii*	Dr. Ma Gerencser, West Virginia University
CH 168A	*Streptococcus mutans*	Dr. George Bowden, University of Manitoba, Winnipeg, MB.
DH 20	*Streptococcus mutans*	
	Staphylococcus epidermis	
Mouse Isolates:		
M1/6	*Aerococcus viridans*[◆]	Female CD1 mouse 1, Charles River Laboratories
M1/6a	*Vibrio metschnikovii*[♣]	
M1/8	*Staphylococcus warneri*[♠]	
M1/23	*Streptococcus parasanguis*[◆]	
M2/7a	*Streptococcus intermedius*[◆]	Female CD1 mouse 2, Charles River Laboratories
M2/9a	*Pseudomonas aeruginosa*[♣]	

[◆]See Table 11
[♣]See Table 12
[♠]See Table 13

Table 9. Gram-positive Mouse Oral Bacterial Strains and their Biochemical Characteristics.

ID	S	CAT	ROG	HEM	ADH	βGLU	βGAR	βGUR	αGAL	PAL	RIB	MAN	SOR	LAC	TRE
SK100	C	–	–	–	–	–	+	–	+	+	–	–	–	+	–
SK145	C	–	–	–	–	–	–	–	+	–	+/–	–	–	+	+
M1/6	C	–	–	–	–	+	–	+	–	+	–	+	–	–	+
M1/23	C	–	–	α	–	–	+	+	+	–	+/–	+	+/–	+	+
M1/7a	C	–	–	α	–	–	+	+	+	–	+/–	+	–	+	+
M2/7a	R	–	–	–	+	+	+	–	–	+	–	–	–	+	+
M1/8	C	+	+	α	+	–	–	+	–	–	–	–	–	–	+

ID	RAF	VP	APPA	βGAL	PyrA	βNAG	GTA	HIP	GLYG	PUL	MAL	MEL	MLZ	SAC	LARA
SK100	+	–	+	–	–	–	+	–	–	+	+	+	–	+	–
SK145	+/–	–	+	–	–	–	+	–	–	+	+	–	–	+	–
M1/6	+	–	–	–	+/–	–	+	–	–	+/–	+	–	–	+	–
M1/23	+	–	+	+	+	–	+	+	–	–	+	+	–	+	–
M1/7a	+	+	+	+	+	+	+	+	–	–	+	+	–	+	–
M2/7a	–	+	+	–	–	–	+	–	–	+	+	–	–	+	–
M1/8	–	+	–	–	+	–	–	+	–	–	+/–	–	–	+/–	–

ID	DARL	MβDG	TAG	βMAN	CDEX	URE
SK100	–	–	+/–	–	–	–
SK145	–	+/–	+	–	–	–
M1/6	–	–	–	–	–	–
M1/23	–	–	+	–	–	–
M1/7a	+/–	–	+	–	–	–
M2/7a	–	+	+/–	–	–	–
M1/8	–	–	–	–	–	+

+, positive; –, negative; +/–, intermediate; –w, negative to weak; ND, not determined; HEM, Hemolysis reaction; S, Colony Shape; C, cocci; R, Rods

Table 10. Gram-negative Mouse Oral Bacterial Strains and their Biochemical Characteristics.

ID	S	CAT	OXI	HEM	ODC	ADH	LDC	URE	LARL	GAT	5KG	LIP	RP	βGLU	MAN
M1/1	R	+	+	–	–	–	–	–	–	–	–	–	–	+	–
M1/6a	R	+	–	–	–	+	–	+	–	–	+	–	–	+	–
M2/1a	R	+	–	–	–	–	–	+	–	–	–	+	–	+	+
M2/9a	R	+	–	–	+	+	–	+	–	–	–	+	–	–	–

ID	MAL	IND	βNAG	βGAL	GLU	SAC	LARA	DARL	αGLU	αGAL	TRE	RHA	INO	ADO	PLE
M1/1	–	–	–	+	–	–	–	–	+	+	–	–	–	–	–
M1/6a	+	+	–	+	+	+	–	–	+	–	+	–	+	–	–
M2/1a	+	–	–	+/–	+	+	–	–	+	+	+	–	–	–	–
M2/9a	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–

ID	βGUR	CEL	SOR	αMAL	MNT	AspA
M1/1	–	–	–	+	–	–
M1/6a	–	–	–	+	–	+/–
M2/1a	+	+	–	–	–	–
M2/9a	–	–	–	–	+	+/–

+, positive; –, negative; +/–, intermediate; –w, negative to weak; ND, not determined; HEM, Hemolysis reaction; S, Colony Shape; C, cocci; R, Rods

Table 11. Gram-positive and Alpha-hemolytic Unidentified Bacteria and their Possible Identities Obtained from the Rapid ID 32 Strep Tests (bioMérieux).

	M1/6	*Aerococcus viridans*	*Leuconostoc spp.*	*Streptococcus acidominimus*	**M1/23**	*Streptococcus parasanguis*	*Streptococcus pneumoniae*	**M1/7a**	*Streptococcus oralis 1*	*Streptococcus parasanguis*	*Streptococcus pneumoniae*	**M2/7a**	*Streptococcus intermedius*	*Streptococcus gordonii*
ADH	–	1	2	1	–	72	26	–	2	72	26	+	86	95
βGLU	+	70	36	7	–	34	26	–	4	34	26	+	90	99
βGAR	–	3	44	1	+	100	88	+	98	100	88	+	99	90
βGUR	+	30	0	40	+	0	0	+	0	0	0	–	0	0
αGAL	–	60	72	1	+	65	84	+	93	65	84	–	0	28
PAL	+	0	5	1	–	89	1	–	93	89	1	+	100	100
RIB	–	28	25	10	+/–	0	0	+/–	0	0	0	–	4	0
MAN	+	75	36	40	+	0	0	+	0	0	0	–	2	0
SOR	–	25	0	1	+/–	1	0	–	0	1	0	–	0	0
LAC	–	79	44	50	+	94	99	+	99	94	99	+	100	85
TRE	+	91	55	75	+	50	95	+	40	50	95	+	99	99
RAF	+	42	50	1	+	65	84	+	93	65	84	–	0	1
VP	–	1	94	2	–	0	0	+	3	0	0	+	100	50
APPA	–	0	33	90	+	100	99	+	100	100	99	+	100	100
βGAL	–	10	91	10	+	27	2	+	23	27	2	–	89	1
PyrA	+/–	83	0	10	+	1	23	+	1	1	23	–	0	0
βNAG	–	0	0	0	–	10	74	+	26	10	74	–	97	0
GTA	+	0	0	1	+	39	90	+	99	39	90	+	51	35
HIP	–	92	2	100	+	10	1	+	1	10	1	–	0	0

Table 11 continued...

	M1/6	*Aerococcus viridans*	*Leuconostoc spp.*	*Streptococcus acidominimus*	**M1/23**	*Streptococcus parasanguis*	*Streptococcus pneumoniae*	**M1/7a**	*Streptococcus oralis 1*	*Streptococcus parasanguis*	*Streptococcus pneumoniae*	**M2/7a**	*Streptococcus intermedius*	*Streptococcus gordonii*
GLYG	–	10	0	4	–	0	0	–	3	0	0	–	0	0
PUL	+/–	10	0	100	–	0	74	–	99	0	74	+	97	02
MAL	+	95	80	100	+	100	79	+	100	100	79	+	97	99
MEL	–	1	61	0	+	55	22	+	80	55	2	–	0	1
MLZ	–	0	0	0	–	0	0	–	0	0	0	–	2	0
SAC	+	100	72	100	+	100	99	+	100	100	99	+	100	100
LARA	–	1	16	0	–	0	0	–	0	0	0	–	2	0
DARL	–	0	2	0	–	0	0	+/–	0	0	0	–	0	0
MβDG	–	65	13	90	–	20	14	–	1	20	14	+	27	74
TAG	–	0	0	26	+	31	1	+	40	31	1	+/–	1	1
βMAN	–	1	0	0	–	19	0	–	5	19	0	–	20	90
CDEX	–	1	19	0	–	0	0	–	3	0	0	–	0	0
URE	–	0	0	0	–	0	0	–	0	0	0	–	0	0
/32		**29**	**27**	**25**		**27**	**26**		**26**	**26**	**26**		**30**	**29**
%		**90.63**	**84.38**	**78.13**		**84.38**	**81.25**		**81.25**	**81.25**	**81.25**		**93.75**	**90.63**

+, positive; –, negative; +/–, intermediate reactions; values represent percentage of positive reactions, shaded areas indicate values that do not correspond with the reactions of the tested organism.

Table 12. Gram-negative Unidentified Bacteria and their Possible Identities Obtained from the ID 32 E Tests (bioMérieux).

	M1/1	*Sphingomonas paucimobilis*	*Agrobacterium radiobacter*	**M1/6a**	*Vibrio metschnikovii*	*Aeromonas sobria*	*Vibrio alginolyticus*	**M2/1a**	*Aeromonas hydrophila grp*	*Aeromonas sobria*	*Vibrio metschnikovii*	**M2/9a**	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*	*Pseudomonas putida*
ODC	–	0	0	–	0	0	75	–	0	0	0	+	1	0	0
ADH	–	0	0	+	3	25	0	–	40	25	3	+	99	35	99
LDC	–	0	0	–	1	1	1	–	0	1	1	–	0	0	0
URE	–	0	100	+	0	0	0	+	20	0	0	+	78	9	0
LARL	–	0	40	–	0	0	0	–	0	0	0	–	0	0	0
GAT	–	0	0	–	0	0	0	–	27	0	0	–	0	0	0
5KG	–	0	0	+	0	0	0	–	0	0	0	–	0	0	0
LIP	–	6	0	–	1	23	1	+	94	23	91	+	86	40	58
RP	–	0	0	–	44	28	46	–	72	28	44	–	0	0	0
βGLU	+	90	100	+	93	13	99	+	94	13	93	–	1	0	0
MAN	–	0	0	–	50	27	60	+	75	27	50	–	0	0	0
MAL	–	0	0	+	93	54	89	+	84	54	93	–	0	0	0
IND	–	0	0	+	50	59	99	–	68	59	50	–	0	0	0
βNAG	–	17	36	–	42	35	0	–	69	35	42	–	0	0	0
βGAL	+	68	100	+	71	100	0	+/–	99	100	71	–	0	0	0
GLU	–	0	0	+	96	83	100	+	98	83	96	+	69	14	15
SAC	–	0	0	+	93	61	100	+	94	61	93	–	0	0	0
LARA	–	0	0	–	0	6	0	–	84	6	0	–	5	0	0
DARL	–	0	0	–	0	0	0	–	0	0	0	–	0	0	0
αGLU	+	93	100	+	28	76	24	+	12	6	28	–	0	0	0

Table 12 continued...

	M1/1	*Sphingomonas paucimobilis*	*Agrobacterium radiobacter*	**M1/6a**	*Vibrio metschnikovii*	*Aeromonas sobria*	*Vibrio alginolyticus*	**M2/1a**	*Aeromonas hydrophila grp*	*Aeromonas sobria*	*Vibrio metschnikovii*	**M2/9a**	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*	*Pseudomonas putida*
αGAL	+	68	100	–	87	4	2	+	1	4	87	–	1	0	0
TRE	–	0	0	+	100	54	100	+	87	54	100	–	0	0	0
RHA	–	0	0	–	0	0	0	–	1	0	0	–	0	0	0
INO	–	0	0	+	50	0	0	–	0	0	50	–	0	0	0
ADO	–	0	0	–	0	0	0	–	0	0	0	–	0	0	0
PLE	–	0	0	–	0	36	0	–	8	36	0	–	0	0	0
βGUR	–	6	0	–	0	0	0	+	0	0	0	–	1	0	0
CEL	–	0	6	–	3	33	17	+	51	33	3	–	0	0	0
SOR	–	0	0	–	12	0	1	–	2	0	12	–	0	0	0
αMAL	+	43	75	+	60	27	97	–	50	27	60	–	1	0	0
MNT	–	0	0	–	1	0	0	–	0	0	1	+	81	22	50
AspA	–	85	0	+/–	3	6	0	–	45	6	3	+/–	41	12	0
/32		**32**	**31**		**29**	**28**	**27**		**29**	**28**	**28**		**31**	**30**	**30**
%		**100**	**96.88**		**90.63**	**87.50**	**84.38**		**90.63**	**87.50**	**87.50**		**96.88**	**93.75**	**93.75**

+, positive; –, negative; +/–, intermediate reactions; values represent percentage of positive reactions, shaded areas indicate values that do not correspond with the reactions of the tested organism.

Table 13. Gram-positive, Non-hemolytic Unidentified Bacteria and its Possible Identity.

	M1/8	*Staphylococcus warneri*	*Staphylococcus haemolyticus*	*Staphylococcus hominis*
Aerobic Growth	+	+	+	+
Oxidase test	–	–	–	–
D-Lactic Acid production	+	+	+	+
VP	+	+	d	d
LARA	–	–	–	–
RAF	–	–	–	–
SAC	+/–	+	+	(+)
MAL	+/–	(+)	+	+
MAN	–	d	d	–
TRE	+	+	+	d
LAC	–	ds	d	d
MLZ	–	ds	–	d
RIB	–	d	d	–
PAL	–	–	–	–
URE	+	+	–	+
ADH	+	d	+	d
Hemolysis	+	(ds)	(+)	–w
βGLU	–	+	d	–
βGUR	+	d	d	–
βGAL	–	–	–	–
/20		**19**	**19**	**18**
%		**95.00**	**95.00**	**90.00**

+, 90% or more strains positive; –, 90% or more strains negative; d, 11-89% strains positive; ( ), delayed reaction; w, weak reaction; –w, negative to weak reaction; ds, test differentiates subspecies (not separated out above in heading); shaded areas indicate values that do not correspond with the reactions of the tested organism.

Table 14. Human Bacterial Strains Tested for Binding with the hPIP.

SPECIES	STRAIN	BINDING
Streptococcus parasanguis	1395-4Ca	-/+
	1395-4Cb	++
	139N-3A	++
	RW55898	+
	A8-2a	+
Streptococcus sanguis	SK 112	+
Streptococcus oralis	SK 100	++
	SK 120	++
Streptococcus milleri	SK 65	+
Actinomyces naeslundii	ATCC 12104	-/+
	WVU 627	+
Streptococcus mitis	SK 145	++
	SK 95	+
	SK 137a	++
Streptococcus mutans	CH 168A	-
	DH 20	+
Staphylococcus epidermis		+

++, strong binding; +, moderate binding, -/+, weak binding, - no binding

Table 15. Mouse Bacterial Strains Tested for Binding with the hPIP.

Mouse Strain (M1)	Binding	Mouse Strain (M2)	Binding
M1/1	+	M2/1	–/+
M1/2	–	M2/2	+
M1/3	+	M2/3	+
M1/4	+	M2/4	+
M1/6	–	M2/7	+
M1/7	+	M2/8	+
M1/8	–	M2/9	+
M1/9	+	M2/10	+
M1/11	+	M2/11	+
M1/12	+	M2/12	+
M1/13	+	M2/13	+
M1/14	+	M2/14	+
M1/16	+	M2/16	+
M1/17	+	M2/17	+
M1/19	+	M2/18	+
M1/20	+	M2/21	+
M1/22	+	M2/22	+
M1/23	+	M2/24	–/+
M1/1a	+	M2/1a	+
M1/3a	+	M2/2a	+
M1/5a	+	M2/3a	+
M1/6a	+	M2/7a	+
M1/7a	+	M2/8a	+
M1/9a	–	M2/9a	–

++, strong binding; +, moderate binding, -/+, weak binding, - no binding

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