Interaction of *Helicobacter pylori* with Glycosylated Salivary Proteins

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Chapter 1

Introduction:

Helicobacter pylori

A Gastric Pathogen on its Way Through the Oral Cavity

Abstract

Helicobacter pylori (*H. pylori*) resides in the human stomach and is the primary cause for severe gastric diseases such as gastritis, peptic ulcers and even gastric cancer. It is generally accepted that *H. pylori* is acquired by oral ingestion and several studies provided evidence that *H. pylori* may be present in the human oral cavity. Therefore an interaction of this bacterium with components of saliva seems likely. The subject of this thesis was the "Interaction of *H. pylori* with glycosylated salivary proteins". It ought to be investigated whether the mechanisms of adhesin-receptor interactions known from the stomach are also found for interactions with salivary glycoproteins. Thus, this Introduction chapter describes the pathogenesis of *H. pylori* and, in particular, the interaction of *H. pylori* adhesins with corresponding receptor structures. Moreover, glycoproteins from saliva that could serve as putative receptors for these adhesins as well as the current knowledge about *H. pylori* in the oral cavity are summarized.

General Introduction to *Helicobacter pylori*

History

The story began at the end of the 1980s with a fortuitous observation by J. Robin Warren, an australian pathologist at the Royal Perth Hospital. While examining routine biopsies from dyspeptic patients he observed a large number of curved and spiral-shaped bacteria. Why had stomach acid not destroyed these organisms? One explanation became obvious. They were closely associated with the thick gastric mucus layer. J. R. Warren had observed these organisms only in inflamed tissues. This observation led him to study this question in more detail. With his colleague Barry J. Marshall he tried to culture the bacteria. Their attempts were not successful until they forgot some culture plates over Easter holidays. In these five days bacterial colonies had emerged (reviewed in [1]). They named these bacteria *Campylobacter pyloridis* because of their morphologic similarity to other bacteria of the *Campylobacter* genus and published their observations [2]. Because Warren and Marshall had found *C. pyloridis* in the majority of patients with gastric ulcers, they reasoned that peptic ulcer disease is associated with the occurrence of these bacteria in the stomach. As an experimental test Marshall himself drank culture material with *C. pyloridis* and, as expected, developed dyspeptic illness. In addition, the spiral-shaped bacteria could be detected in his gastric biopsies. The infection vanished without treatment. Some years later a successful antiobiotic therapy was possible, and, thus, eradication of *C. pyloridis*. Because *C. pyloridis* did not really fit into the *Campylobacter* genus, they renamed these microbes into *Helicobacter pylori* (*H. pylori*) in 1989 [3]. Approximately 20 years after their discovery of *H. pylori* and of its role in peptic ulcer diseases Warren and Marshall were awarded with the Nobel prize in Physiology or Medicine [4].

Microbiology

Three morphologic shapes have been described for *H. pylori*. Normally, this bacterium appears spiral-shaped or curved, but it assumes a rod-like shape on solid medium and coccoid forms after prolonged culture [5]. It is gram-negative and requires a microaerophilc atmosphere with about 5% O_2 and 5-10% CO_2. *H. pylori* cells are 2.5 to 5.0 µm long and 0.5 to 1.0 µm wide. They have four to six unipolar flagella (Fig. 1), which are essential for bacterial motility and thus for infection [5]. *H. pylori* survives in the acidic environment of the stomach because it produces high levels of urease, in order to buffer the pH in its immediate vicinity. Urease hydrolyses urea into NH_3 and CO_2, in this way creating a neutral layer around

the bacterial surface. Because urease-defective bacteria can not colonize the stomach, it is obvious that urease is indispensable as a colonization factor. Despite of its capability to survive in strong acid, *H. pylori* has to leave the stomach lumen, in order to prevent its transport into the intestine. Using its flagella *H. pylori* swims towards the viscous mucus layer that covers the epithelial lining of the gastric mucosa, guided by chemotactic factors including urea and bicarbonate. Like a corkscrew the bacterium bores into the mucus layer, finally reaching the surface of the gastric epithelium. This behavior is a second colonization factor, because non-motile mutants are not able to colonize the stomach (reviewed in [6]).

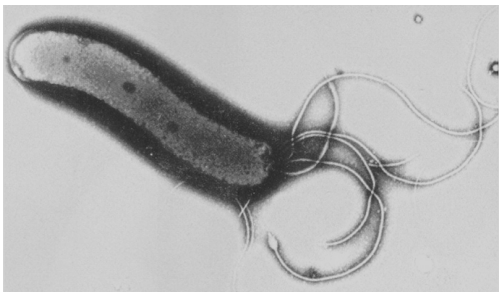

Fig. 1*: Curved Helicobacter pylori with unipolar flagella. Source: [7]*

H. pylori-associated diseases

Because of its very good adaptation to the hostile environment of the human stomach *H. pylori* establishes a lifelong chronic infection after a first infection early in life. The initial colonization with *H. pylori* causes an acute inflammatory response (acute gastritis) that is accompanied by an infiltration of the gastric mucosa by neutrophils. The degree of mucosal damage correlates with the neutrophil infiltration. Today it is accepted that a *H. pylori* infection is the primary cause of active chronic (type B) gastritis in humans [8].

Most infected people are asymptomatic initially but they may develop peptic ulcer diseases including gastric or duodenal ulcers with a risk of 10%. Almost all duodenal ulcers and 70% of gastric ulcers are attributable to *H. pylori* infection. This is supported by the fact that the recurrence rate of peptic ulcer diseases is noticeably reduced after the successful treatment of a *H. pylori* infection [9, 10].

A long-term consequence of gastric ulcer could be the development of adenocarcinoma [11] and mucosa-associated lymphoid tissue (MALT) lymphoma [12]. Therefore, *H. pylori* was designated as a class I carcinogen by the World Health Organization in 1994 [13]. Although the interrelation of *H. pylori* and gastric cancer is known, only a small fraction of colonized individuals develop this malignancy. This lead to the assumption that not only *H. pylori* genotype but also host genotypes influence the risk of carcinogenesis [11].

Virulence factors

Adhesion, invasion and toxin elaboration are three important factors of virulence that can be used by bacteria to cause disease in the host [10]. Although urease-production and flagella were recognized as important colonization factors of *H. pylori* early on, it was tried to identify the virulence factors of this organism, that finally cause gastroduodenal disease. The two best-characterized virulence factors of *H. pylori* are VacA and CagA.

The so called "vacuolating toxin", VacA inserts into the epithelial-cell membrane and forms hexameric, anion-selective, voltage-dependent channels. In addition, VacA forms pores also in the mitochondrial membrane [14]. A role of VacA in the pathogenesis of *H. pylori* is seen in the induction of apoptosis [15], in immune suppression [14] and in its proinflammatory activity [14]. However, the pathogenic role of this toxin is still under dispute, because of the extensive variability in the *vacA* gene in different geographic regions.

In contrast to VacA, the correlation between the expression of CagA (cytotoxin-associated gene A) and *H. pylori* virulence is well-documented. It is associated with both, duodenal ulcers and gastric cancer [16, 17]. CagA is translocated into the host cell by a type IV secretion apparatus, that is encoded by the *cag* pathogenicity island (*cag*-PAI). CagA is present only in virulent type I strains of *H. pylori* whereas type II strains do not express CagA [18]. CagA enters epithelial cells where it becomes tyrosine phosphorylated and initiates thus a series of events [19-21].

Although VacA and CagA constitute important virulence factors of *H. pylori*, the delivery of these proteins to host cells requires intimate contact between bacteria and gastric epithelial cells. Therefore, bacterial adhesion is considered to contribute as well to pathogenicity of *H. pylori*.

Adhesion Properties of *Helicobacter pylori* in Pathogenesis

Bacterial adherence

Bacteria have adapted to a great variety of ecological niches, including the human body. A key event in bacterial colonization of the host is adhesion mediated by bacterial adhesins or adhesive organelles, such as fimbria. These recognize either carbohydrates or peptide structures on host cell surfaces followed by adherence-associated events, such as bacterial uptake into cells or host signaling. By establishing a complex molecular host-pathogen crosstalk these events can finally lead to the disturbance of cellular function and the initiation of diseases [22-24]. Bacterial tools used for cell adhesion and invasion range from single

monomeric proteins like fibronectin-binding proteins [25] to highly sophisticated macromolecules like retractile type IV pili [26]. Because of fine-tuned specificities of bacterial adhesins and also by the tissue-specific distribution of receptors, only a restricted number of hosts and tissues are amenable to bacterial colonization. This is often referred to tissue-host tropism [27]. Tissue-host tropism is also well-known for *H. pylori* because it is a primate-host-specific pathogen that exhibits tropism for the gastric surface mucous cells in the lower part of the stomach [28]. The ability of *H. pylori* to adhere with its adhesins to specific receptors in the gastric mucosa is considered to be both, colonization and virulence factor. A lifelong infection of the stomach by *H. pylori* would not be able without adherence because of the rapid turnover of the gastric mucus and, thus, possible elution of the bacteria [29].

H. pylori adherence

Most *H. pylori* organisms are found motile in the mucus layer of the human stomach. Residual bacteria are found adherent to the epithelial surface (Fig. 2). The intimate attachment of *H. pylori* to gastric epithelial cells can be seen as the first step in infection, because it might facilitate bacterial colonization, the delivery of effector proteins such as CagA and VacA into host cells, or the gain of nutrients from cells that are damaged by the inflammation. Therefore, great efforts have been undertaken to identify adhesion mechanisms of *H. pylori*. Putative adhesins and related receptors of *H. pylori* have been investigated in several studies. Among the adhesins the *H. pylori* outer-membrane proteins (OMPs) BabA, SabA, AlpA and AlpB are very well-characterized now. Nevertheless, there are some more adhesins described in the literature, that have to be regarded also (reviewed in [28-30]).

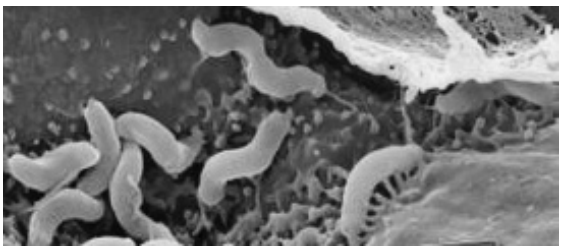

Fig. 2*: Adhesion of Helicobacter pylori to gastric epithelium. Source: [31]*

Blood group antigen-binding adhesin (BabA)

First indications for the interaction of *H. pylori* with the histo-blood group antigens was given by Falk *et al.* in 1993. He and its colleagues developed an *in situ* adherence assay to examine the nature of *H. pylori* binding to gastric epithelium. On the basis of three observations they suggested a role for fucosylated epitopes in the binding of *H. pylori* to surface mucous cells:

(i) fucosylated blood group antigens H and Lewis b blocked binding of *H. pylori* to epithelial cells, (ii) the lectin *Ulex europaeus* type I, that is specific for α-L-fucose, bound to the same cells that bound *H. pylori* and (iii) human colostrum secretory IgA inhibited adhesion in an α-L-fucosidase-sensitive fashion [32]. In the same year, Borén *et al.* supplied evidence, that the Lewis b antigen mediates the *H. pylori* attachment to human gastric mucosa. In addition, they showed that the Lewis b antigen substituted with a terminal GalNAcα1,3 residue (blood group A determinant), was not bound by *H. pylori*. They concluded, that the availability of *H. pylori* receptors might be reduced in individuals of blood group A and B phenotype, as compared with blood group 0 individuals [33]. In 1998, the respective blood group antigen-binding adhesin, BabA, could be purified by a novel technique called receptor-activity-directed affinity tagging (ReTagging). The molecular weigth of BabA is 78 kDa [34]. Analysis of the *babA* gene revealed, that there are *H. pylori* strains that carry two *babA* alleles (*babA1* and *babA2*) such as strain 17875 and strains that possess only one *babA2* allele, such as J99 [35, 36]. Deletion of the *babA2* allele resulted in loss of Lewis b binding activity, whereas deletion of *babA1* had no affect on binding. The *babA2* allele encodes consequently the Lewis b binding adhesin [34]. A recent study showed that the Lewis b-binding *H. pylori* strains subdivide into generalists and specialists. Generalists (more than 95% of all fucose-binding strains) recognize Lewis b irrespective of any terminal modification, i.e. they bind to A, B, and O antigens (see also next chapter "*H. pylori* Receptors: Histo-Blood Group Antigens"). Specialists (60% of adherent South American Amerindian strains) recognize only naked Lewis b, i.e. they bind blood group O antigens best. The authors concluded that cycles of selection have contributed to the *babA* diversity and that these cycles have led to a replacement of generalists by specialists in blood group O-dominant human populations [37].

The importance of BabA as virulence factor became obvious, when it could be correlated to the group of triple positive *H. pylori* strains. These disease-promoting strains (German clinical isolates) are characterized by the presence of *vacA* and *cagA* beside *babA2*. They were found significantly more frequent in patients with ulcer disease or gastric adenocarcinoma, than in patients with gastritis only [38]. However, these genetic markers have to be treated with caution, when estimating the risk of an infection outcome. Ilver *et al.* observed that a deletion of the *cag*-PAI resulted in no reduction of the Lewis b antigen-binding activity [34]. In addition, in a study with Japanese clinical *H. pylori* isolates, no significant correlation between the *babA2* genotype and an infection outcome could be observed [39].

Adherence-associated lipoproteins A and B (AlpAB)

Mutagenesis of *H. pylori* genes encoding surface-exposed proteins led to the identification of a genetic locus, that is involved in adherence to the gastric cells [40]. This operon was designated as *alpAB* (adherence-associated lipoproteins A and B). It encodes to homologous OMPs (AlpAB) that were observed to be essential for *H. pylori* binding to human gastric biopsy sections [41, 42]. Unpublished observations indicate that AlpAB proteins are involved in the functional folding and/or presentation of SabA and other putative adhesin of *H. pylori* and are no real adhesins [43].

Sialyl-Lewis a/x binding adhesin (SabA)

A persistent colonization of the human stomach by *H. pylori* is accompanied by a strong inflammation of the gastric tissue. This leads to the replacement of naturally produced Lewis antigens by sialylated Lewis antigens as part of complex gangliosides [44]. In this regard, it was searched for novel *H. pylori* receptors. It could be observed, that *H. pylori* preferentially bound sialylated gangliosides with multiple Lewis x motifs in the core chain. The corresponding sialyl-Lewis a/x binding adhesin SabA could be identified [45]. It was also isolated by the "ReTagging"-method and the underlying *sabA* gene was sequenced. The molecular weigth of SabA is 66 kDa. [45]. The role of SabA as virulence factor was supported by two observations. On the one, hand sialylated antigens are known to be elevated in gastric carcinoma [29]. On the other hand, SabA was shown to be important for the nonopsonic activation of human neutrophils [46].

N-Acetylneuraminyllactose-binding fimbrial hemagglutinin (HpaA)

The *H. pylori* interaction with sialylated binding sites was known for a long time. Evans *et al.* found that *H. pylori* possesses a cell-bound hemagglutinin detectable with human erythrocytes. Hemagglutination could be inhibited with rather 3'-sialyllactose, than 6'-sialyllactose [47]. The sialic acid-dependent hemagglutinin activity was attributed to fibrillar structures on the bacterial surface. The *hpaA* gene encoding the HpaA *N*-acetylneuraminyllactose-binding fimbrial hemagglutinin could then be cloned and sequenced [48]. Some years later, however, it could be shown, that HpaA is also located in the inner membrane and cytoplasm of *H. pylori.* Moreover it was demonstrated, that HpaA is not involved in hemagglutination, because a *hpaA* deletion mutant showed no reduced sialic acid-dependent hemagglutinin activity [49].

H. pylori neutrophil-activating protein (HP-NAP)

The *H. pylori* neutrophil-activating protein (HP-NAP) has been shown to induce adhesion of neutrophils to endothelial cells in culture. Therefore, it was suggested to play an important role in the activation of human neutrophils [50]. In addition, HP-NAP was shown to be responsible for binding of *H. pylori* to sulfated salivary mucin [51] and to sialylated neutrophil glycolipids [52]. In recent studies it could be shown that HP-NAP neither leads to the adherence of *H. pylori* to neutrophils nor induce nonposponic activation of neutrophils [46, 53].

Other adhesins

HopZ, also an *H. pylori* OMP, has been suggested to be involved in adherence of *H. pylori*. It was shown that *H. pylori* bound HopZ-mediated to gastric carcinoma cell lines AGS [54]. However, its role as adhesin is not yet proven. Besides the above mentioned adhesion properties further bacterial adhesin candidates have been described, suggesting an enrollment of additional interactions that are not fully explored yet. Specific binding of *H. pylori* to a phopholipid (phosphatidylethanolamine) in the antrum of the human stomach was reported [55]. The isolated adhesin candidate, however, was later shown to be a catalase [56]. Thus it seems unlikely that this was a true adhesin. Furthermore, adhesion of *H. pylori* to various extracellular matrix components such as lactoferrin and laminin was reported by many investigators [57-61]. Binding of *H. pylori* to lactoferrin was attributed to either a 60 kDa heat shock protein [58] or a 70 kDa lactoferrin-binding OMP of *H. pylori* [57]. For the binding of *H. pylori* to laminin also two explanations were proposed. A lectin-like interaction of the bacterium with terminal sialic acids on laminin [61] and an interaction of *H. pylori* lipopolysaccharides with laminin [62].

H. pylori Receptors: Histo-Blood Group Antigens

ABO blood group antigens are carbohydrates and represent terminal structures of glycan chains. They are found typically on erythrocytes, but they are also expressed on epithelial cell surfaces, such as pyloric and duodenal mucosa. Blood group A and B phenotypes are characterized by the presence of A and B antigens, respectively (Fig. 3). The product of the *O* gene was thought to be the O-Antigen, that was detectable with several lectins. However, it turned out that the "O-antigen" was present in not only O phenotypes, but also to a lesser extent in A, B and AB phenotypes. The "O-antigen" was in fact a precursor of the A and B antigens; it was then termed H-antigen. The *O* gene was thus a silent allele at the *ABO*-locus.

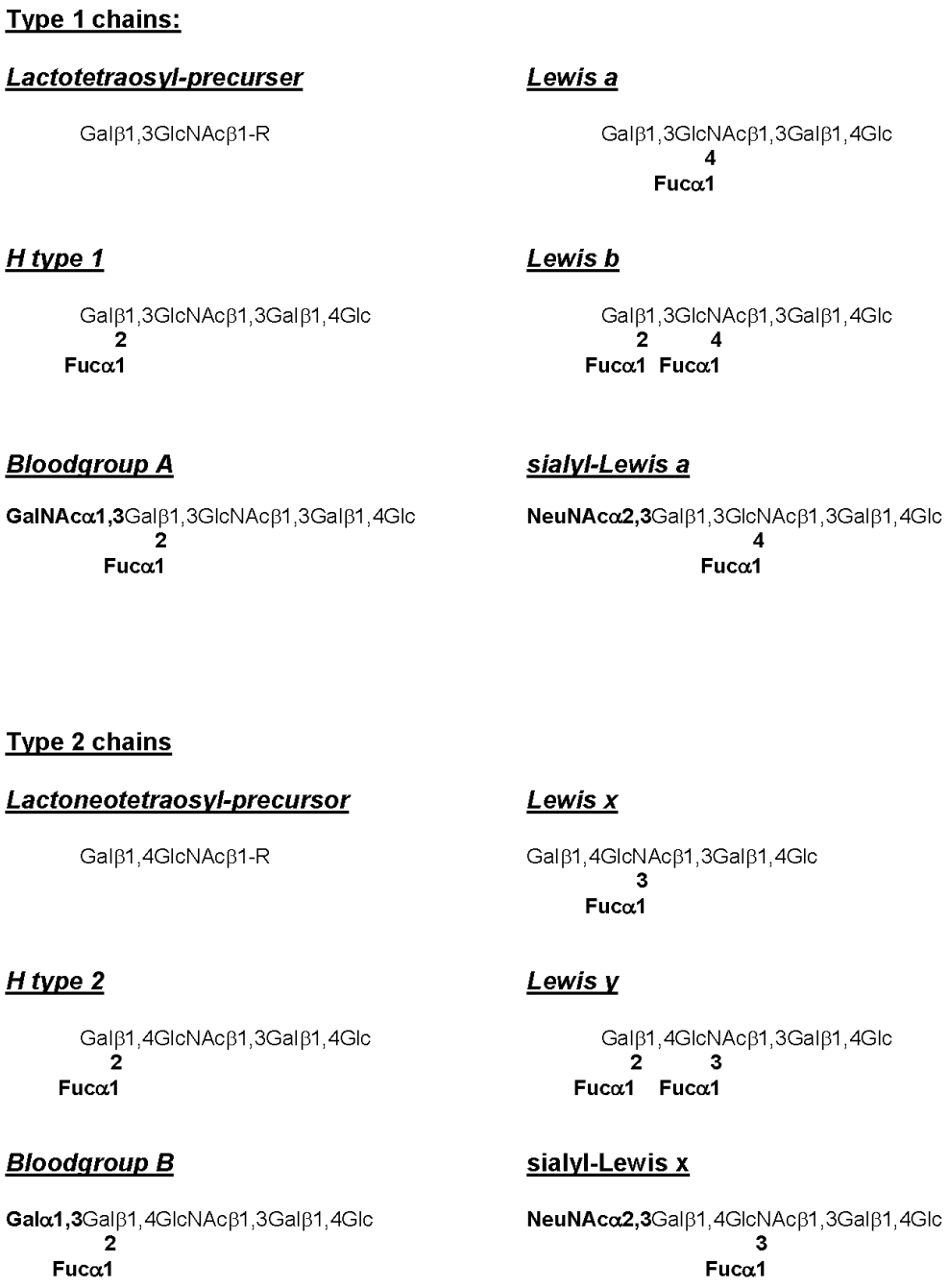

Fig. 3*: Structural relationships between* H. pylori*-relevant blood-group determinants*

The biosynthesis of ABH antigens proceeds from precursors by stepwise addition of monosaccharide units by glycosyltransferases. Two types of precursors are part of the lacto series (lactotetraosyl- and lactoneotetraosyl-precursor, Fig. 3) and are found amongst others in the gastrointestinal tract. Other types of precursors are found on *O*- and *N*-glycans or on various glycolipids. Starting from the lactotetraosyl- and lactoneotetraosyl-precursors the H type antigens (type I and type II, respectively) are formed by adding fucose to the terminal

galactose residue. The A and B antigens are formed by adding to the H antigens terminal *N*-acetylgalactosamine and galactose, respectively. The Lewis a and x antigens are formed directly from the precursors by adding a branched fucose. Lewis b and Lewis y are formed by adding branched fucose residues to the H antigens. The sialyl-Lewis antigens are obtained by addition of a sialic acid first, followed by addition of a fucose. (reviewed in [63-65])
In the ABO blood-group system the O phenotypes dominate with ~45% carriers among Caucasians, reaching 100% in some populations. Lewis b is the major blood group antigen in the Lewis system with more than 70% of individuals being positive. Some individuals were found to secrete "blood group substances" in secretions such as saliva or gastric mucosa. The expression of H antigens in secretions and the genes involved were named *Se* (secretor) and *se* (non-secretor). It turned out that individuals whose red cells typed as Le(a-b+) were always secretors, individuals who typed as Le(a+b-) were always non-secretors. Individuals who typed as Le(a-b-) could be either secretors or non-secretors [64].

The relevance of histo-bood group antigens as *H. pylori* receptors was supported by different observations: When the distribution patterns of blood group antigens in human gastric mucosa was mapped, it turned out that distribution of the A, B, H, Lewis a and Lewis b antigens matched the distribution of receptors for *H. pylori* completely. In addition, it was observed, that in secretor individuals, sialylated antigens are low in healthy gastric mucosa but are expressed strongly during gastritis [44, 45, 63].

The Oral Cavity as an Entry Pathway for *H. pylori*

Epidemiology and transmission

Up to one half of the world's population harbors *H. pylori* [1]. The prevalence varies greatly among countries and population groups even within the same country and is correlated with socioeconomic conditions. The prevalence among middle-aged adults is over 80% in developing countries, compared to about 20% in industrialized countries [66].

The origin of infections and the way of transmission are not entirely understood [67, 68]. The infection seems to be acquired almost always during early childhood by oral ingestion of the bacterium. Barry J. Marshall, who ingested *H. pylori* orally and, as a consequence, developed gastritis demonstrated that *H. pylori* must reach the stomach *via* the oral cavity [69]. It seems likely that in industrialized countries the direct transmission from person to person by vomitus, saliva, or feces predominates, whereas additional transmission routes, such as water, may be important in less developed countries [66, 68]. The intestinal

tract of humans and some non-human primates seems to be the sole reservoir for *H. pylori* [68].

It has been speculated whether the fecal-oral route or the oral-oral route is more likely or if both routes coexist. An argument for the fecal-oral transmission could be, that *H. pylori* is eliminated because of the constant turnover of the gastric mucosa after which the bacterium is transported into the intestine and eliminated into the environment. The survival of *H. pylori* in the external environment may be facilitated by the formation of the non-culturable but viable coccoid forms [70]. In fact, *H. pylori* could be isolated from human feces [71]. Feces contaminated drinking-water was regarded as potential infection source [72], although *H. pylori* could not be isolated from water. An argument for the oral-oral route was that *H. pylori* might be able to access the oral cavity from gastric juice by reflux from the stomach, and saliva might thus act as a vehicle for transmission. This latter hypothesis is supported by a previous study, that showed an oral-oral transmission in the case of an African women who premasticated food given to their infants [70].

Serological and DNA fingerprint analysis in several studies suggested that transmission of *H. pylori* occurs mainly within families, i.e. vertical instead of horizontal transmission takes place. In this regard transmission from mother to children seems more likely than transmission between siblings [68, 73-75].

Detection of H. pylori in the oral cavity

The oral cavity has a number of ecological niches, some of which may support the microaerophilic environment necessary for *H. pylori* survival. By bacterially induced loss of attachment between teeth and supporting bone a soft tissue-lined pocket is formed around the teeth. This provides an unique environment for bacterial colonization by a great number of bacterial species and probably also by *H. pylori* [76]. Therefore, the oral cavity was regarded as a transient or even permanent extra-gastroduodenal reservoir for *H. pylori* infection and transmission [77]. In many studies *H. pylori* DNA was detected in the mouth utilizing the polymerase-chain-reaction (PCR) technique (reviewed in [67]). However, this technique produced no consistent results about the number and frequency of occurrence of *H.* pylori in the oral cavity. Detection rates ranged from 0% to 100%, perhaps caused by the fact that studies differed in the primer sets used for PCR as well as in the choice of their study subjects that originated from different countries [67, 78-80]. In addition, contamination artifacts might have been a problem [81]. Therefore it was tried to culture *H. pylori* from oral samples. This failed in most cases [82-84]. However, there are a few reports that show the successful culture

of *H. pylori* from the oral cavity and a confirmation of these strains as *H. pylori* by hybdridization [70, 85, 86]. The low number of *H. pylori* organisms compared to a great number of other, faster-growing bacterial species in the oral cavity and the possible presence of non-culturable, but viable coccoid *H. pylori* organisms might complicate the culture of this bacterium further [87].

Interaction of Oral Bacteria With Salivary Proteins

Bacteria in the oral cavity

The Dutch glassmaker Antony van Leeuwenhoek can be regarded as the father of oral biology. By using very simple one-lens microscopes he discovered the first microbes in the mouth already 400 years ago, and recorded the diversity of these organisms [88]. The human body harbors a 10-fold greater number of microbial cells than human cells [89]. More than 700 bacterial taxa, of which over 50% have not been cultivated, have been detected in the oral cavity [90]. Van Leeuwenhoek stated:"...there's always been a sense that the community is what matters; the community is capable of doing things that individual [species] cannot" [88]. A guideline that is to keep at the back of one's mind when a certain bacterial species of the oral cavity ought to be investigated.

Numerous examples of specific interactions between oral bacteria and salivary components have been reported. The study of bacterial adhesion as the first step in infection had its origin in the examination of the interaction of oral bacteria with salivary proteins. The fact that the oral cavity is easily accessible, facilitated adhesion studies that would have been more difficult for other organ systems (e.g. respiratory or gastrointestinal). The fact that many human systemic pathogens probably first colonize the oral cavity before initiating diseases of target organs as it was suggested for the development of pneumonia or gastritis was another favourable condition [67, 91].

Salivary pellicle

The microbial community exists principally organized in biofilms on oral surfaces, on teeth, gums, tongue or prostheses when present [92]. Biofilm formation is initiated by the adhesion of primary microorganisms to components of the acquired enamel pellicle [93]. This term describes a thin (0.1 to 1.0 microns thick) organic film of proteinaceous nature that develops on a cleaned tooth surface within seconds to minutes [91, 94]. It was suggested that pellicle formation reaches an equilibrium between adsorption and de-sorption of protein within two hours [95]. This pellicle forms, however, not only on enamel, but also on cementum, on

dental restorative materials, on the mucosal epithelium and even on surfaces of oral microorganisms.

Attachment of salivary molecules to the tooth surface results from a combination of physical forces e.g. ionic or hydrophobic interactions, hydrogen bonding and van der Waals forces. Mainly negatively charged carboxyl-, phosphate-, sulfate- and sialic acid groups of theses salivary molecules interact with calcium ions of hydroxyapatite. Periodic acid schiff stain of extracted teeth led to the assumption of carbohydrates in the pellicle. A high content of glucose but also mannose, galactose, glucosamine and galactosamine could be identified. The high content of glucose was puzzling because this monosaccharide is rare in glycoproteins. Thus, it was suggested that the majority of glucose present in pellicle does not origin from salivary glycoproteins. Also the amino acid composition of the pellicle was investigated. One interesting aspect of these investigations was, that the composition of pellicle proteins is different from those of whole saliva. Pellicle proteins contain significantly more hydrophobic and less neutral amino acid than whole saliva. Major salivary proteins in the pellicle are mucin MUC5B, secretory immunglobuline A (S-IgA), lactoferrin, lysozyme, cystatin (type SA-I), acidic proline-rich proteins and amylase. Salivary carbonic anhydrase, albumin, IgM and IgG were found as well. In addition to salivary components, dental pellicles also contain constituents from crevicular fluid, microbial and cellular sources. The so-called early pellicle forms within 2 hours. It is assumed that the process of pellicle formation is generally similar on various surfaces. In contrast, the pellicle composition is influenced by surface modifications. The early pellicle undergoes by-and-by a transition to the so-called later pellicle, mainly caused by enzymatic degradation and/or addition of further components. Enzymes originate from bacteria, desquamated epithelial cells and leukocytes from crevicular fluid. This pellicle transition influences the pellicle composition, e.g. by a decrease of proline-rich proteins. Simultaneously a transition in bacterial attachment can be observed because receptors for one group of bacteria are destroyed and new receptors for others arise. (reviewed by [91, 95])

Oral biofilm

Bacteria in an aquatic environment show a tendency to interact with surfaces. Bacteria become first reversibly associated with tissue surfaces by electrostatic or hydrophobic forces of low specificity. However these forces are not sufficient to resist cleansing forces and to permit colonization. This reversible adherence can therefore only seen as a first approach of bacteria in the biofilm development. The real step of initial biofilm development is the

binding of proteinaceous components (adhesins) of bacteria to complementary molecules (receptors) on the tissue surface. The oral biofilm develops, thus, by binding of primary colonizers to proteins of the pellicle. This includes lectin-like recognition of oligosaccharide receptors or protein-protein interactions [96]. Amongst the earliest colonizers are more than 60% streptococci [93] as well as *Actinomyces* [97, 98], *Veillonella* and *Neisseria* [99]. These pioneer organisms then provide a new surface and appropriate metabolic conditions for the attachment of succeeding organisms (Fig. 4). Proteins bound by primary colonizers are, amongst others, proline-rich proteins and proline-rich glycoprotein, statherin, salivary mucins, salivary agglutinin gp-340, α-amylase, immunglobulins or lactoferrin [91]. Once bacteria have adhered to the oral surfaces, the development of the mixed species biofilm proceeds by cell growth and division as well as coaggregation of bacteria, such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* with the primary colonizers [100].

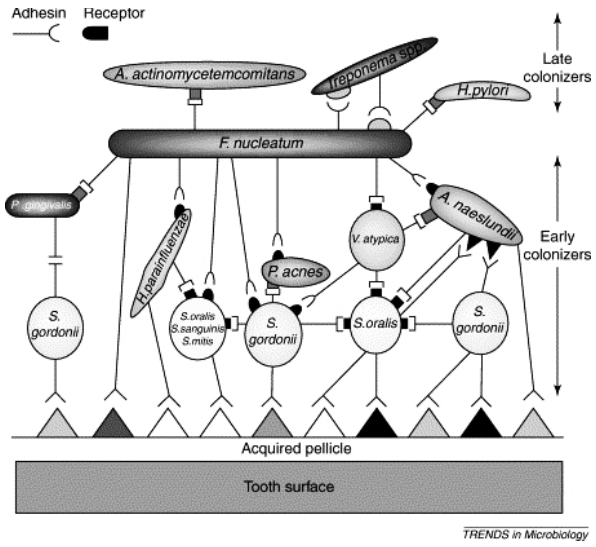

Fig. 4*: Diagrammatic representation of the proposed sequence of events in human oral biofilm formation on the tooth surface. Source: [100]*

Not all bacteria that enter the oral cavity adhere and colonize. Only a small fraction of the bacteria attach and remain in the oral cavity but the majority of them are removed. Bacterial clearance occurs by mechanical flushing, i.e. swallowing, chewing or spitting that is moreover facilitated by binding of salivary components to bacterial adhesins [101]. These interactions can result in clumping (saliva-mediated agglutination) or steric hindrance of adhesion [91].

Salivary Glycoproteins

Salivary glycoproteins provide receptor structures for microbial adherence and they exert a number of protective functions in the oral cavity. Chemically, salivary glycoproteins can be broadly divided into two groups: (1) Salivary glycoproteins of serous cell origin and (2) of mucous cell origin. Group (1) contains *N*-glycosidic units characterized by the presence of mannose and a glycopeptide linkage of *N*-acetylglucosamine-Asn. Group (2) is more heavily glycosylated, contains *O*-glycosidic carbohydrate units and has a *N*-acetylgalactosamine-Ser/Thr glycopeptide linkage. Some glycoproteins, however, such as S-IgA have both *N*- and *O*-linked carbohydrate units. Beside this classification, salivary glycoproteins can also be grouped into several families (e.g. salivary mucins, PRPs). Their members share common structural features with the exception of structural differences arising from transcriptional and post-translational modifications [102]. The following sections include salivary glycoproteins that are relevant for this thesis.

Salivary mucins

Salivary mucins are glycoproteins, that are characterized by a high number and diversity of oligosaccharide side chains, *O*-glycosidically linked to a polypeptide backbone of repeating amino acid sequences. The glycopeptide-linkage is formed between *N*-acetylgalactosamine and threonine or serine [103]. Sugar residues are galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose and sialic acid [104]. Because of their strong glycosylation the conformation of mucins is often compared with a bottlebrush. Unglycosylated regions of mucins are vulnerable to proteolysis [105]. Mucins form viscoelastic, hydrophilic gels, that stick to all kinds of mucous surfaces and function as physical barriers, in this way protecting the underlying tissue. As constituents of the pellicle they protect oral surfaces against chemical and mechanical damage as well as microbial invasion [106]. Among salivary mucins, there is a high molecular weight species, MUC5B (> 1000 kDa), and a lower molecular weight species, MUC7 (200-250 kDa) [107] (Fig. 5).

Only 15% of the total molecular mass of MUC5B stems from the protein core. The protein core is composed of disulfide-linked subunits. The carbohydrate content contributes 78% and sulfate 7% to the molecular of MUC5B. The major oligosaccharides range in size from 4 to 16 sugar residues [107]. In saliva of secretors, ABH, Lewis a, and Lewis b blood group antigens are mainly expressed on MUC5B [108].

MUC7 is composed of a single peptide chain, which account for 30% of the total molecular mass. The carbohydrate content is about 68% with oligosaccharides of 2 to 7

monosaccharide residues in length. Approximately 170 oligosaccharide side chains are distributed over the polypeptide backbone. It is known that MUC7 contain terminal α2,3-linked sialic acids, α1,2-linked fucose and β1,3-linked galactose [107].

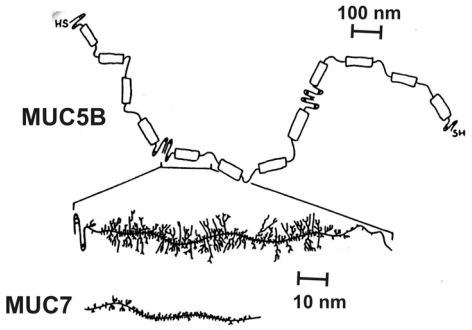

Fig. 5*: Schematic model of salivary mucins. MUC5B shows several subunits. MUC7 is composed of one subunit. Source: modified from [109]*

Salivary agglutinin (gp-340)

Salivary agglutinin is a 300 to 400 kDa glycoprotein that was originally identified as the protein responsible for its *Streptococcus mutans* aggregation properties [110]. Recently it has been shown that agglutinin is identical to the lung glycoprotein gp-340, a member of the scavenger receptor cysteine-rich (SRCR) superfamily [111, 112]. gp-340 is a product of an alternatively spliced form of the *DMBT1* gene. This gene codes for 3 distinct conserved proteins domains. One of these domains is the SCRC domain [113]. It has been reported that blood group antigens and Lewis antigens are present on agglutinin, dependent on the secretor status. In addition, the presence of α2,3-bound sialic acid-containing epitopes has been shown [114].

Salivary α-amylase

The calcium-requiring metalloenzyme α-amylase catalyzes the hydrolysis of internal α(1-4) glycosidic linkages in starch, glycogen, and glucose polymers [107]. Salivary amylases comprise two major families; family A amylases are glycosylated 62 kDa glycoproteins whereas family B amylases are nonglycosylated 55 kDa proteins [115, 116]. More than 25% of amylase secreted into saliva is assumed to be glycosylated [115, 117]. These contain four biantennary *N*-glycosidically linked oligosaccharide units. One of these units possesses a terminal sialic acid residue [107]. The role of salivary amylase in the digestion of carbohydrates is thought to be minimal [101]. In contrast it has been demonstrated in the past that amylase can play a role in the colonization and metabolism of oral bacteria. Amylases

have been identified as constituent of enamel pellicle [118], and shown to act as receptors for bacterial adhesion [119]. In addition, amylases have been shown to retain their enzymatic activity when bound to the bacterial surface [120].

Proline-rich glycoprotein

The proline-rich glycoprotein (PRG) is a member of the proline-rich family of salivary proteins (PRPs) [121]. These highly polymorphic proteins comprise about 70% of the total protein in human saliva and are encoded by six different genes that map to chromosome 12. Acidic PRPs (encoded by *PRH1*, *PRH2*) are needed to maintain calcium homeostasis in the mouth by binding calcium and inhibiting hydroxyapatite crystal growth. They are also capable of mediating the binding of microorganisms to tooth surfaces. Basic PRPs (encoded by *PRB1*, *PRB2, PRB3* and *PRB4*), that include also PRG, bind oral bacteria and exhibit masticatory-lubricating properties. For the non-glycosylated basic PRPs it was suggested, that they precipitate tannin and thereby prevent adsorption of this potential toxin from the alimentary tract [122-124]. PRG (encoded by *PRB3*) has a molecular mass of approximately 89 kDa. Concerning the glycosylation of PRG, there are different statements in the literature. Gillece-Castro *et al.* reported a carbohydrate content of 50% in the form of highly fucosylated *N*-linked saccharides. The major structure was a biantennary asialosaccharide that contained two fucose residues on one antenna [121]. A prior study of Reddy and co-workers stated six *N*-glyosidically linked triantennary oligosaccharide units with three neutral and three sialic acid containing units [125]. Analogous to the salivary mucins, PRG is responsible for lubricating food and interacting with oral bacteria [101].

Carbonic anhydrase VI

Carbonic anhydrase VI (salivary carbonic anhydrase) is analogous to gustin, a zinc-metalloprotein of 37 kDa, that was associated with taste sensation [126]. Carbonic anhydrases participate in the maintenance of pH homeostasis in various tissues and biological fluids by catalyzing the reversible reaction $CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$. Carbonic anhydrase VI is the only secretory isoenzyme of the carbonic anhydrase family. It is not only found in saliva but also in the upper alimentary tract and in the stomach [127].

Secretory immunglobulin A (S-IgA)

Secretory IgA is the predominant immunglobulin within all human mucosal secretions, including saliva. It protects the mucosal surface against invasion by pathogens. S-IgA occurs mainly as dimer in which the two IgA molecules are joined together *via* a small J chain (16

kDa) (Fig. 6). It is produced by plasma cells within the major and minor salivary glands. The epithelial cells express the polymeric immunglobulin receptor that binds one heavy chain of dimeric IgA. This complex is then translocated across epithelial cells. When reaching the mucosal surface the secretory component (50-90 kDa) is cleaved from the polymeric immunglobulin receptor and the IgA/J chain/secretory component-complex (S-IgA) is secreted. The heavy chain, the J chain and the secretory component are glycosylated. IgA occurs in two isotypic forms: IgA1 and IgA2. The glycosylation patterns differ between IgA1 and IgA2. The IgA1 heavy chain carries both, *N*-linked oligosaccharides and *O*-linked oligosaccharides whereas the heavy chain of IgA2 carries only *N*-linked oligosaccharides. The secretory components is mainly *N*-glycosylated and over 70% of these glycans are sialylated. It was suggested, that the secretory component carries different Lewis and sialylated Lewis epitopes. (reviewed by [107, 128])

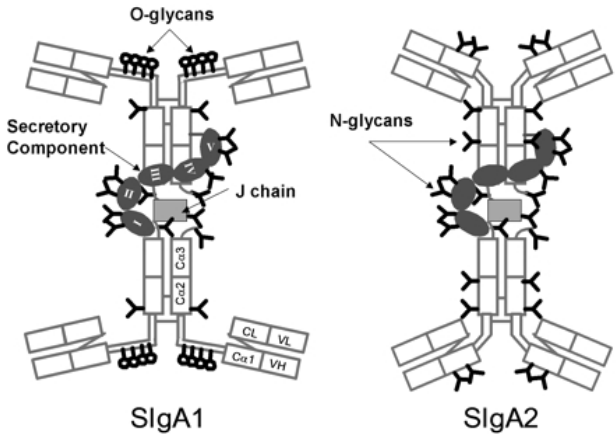

Fig. 6*: Schematic representation of human dimeric secretory IgA1 and IgA2. Source: [128]*

Zinc-α_2-glycoprotein

Zn-α_2-glycoprotein has a molecular weight of 38.5. Its name derives from its tendency to precipitate with zinc salts. Zn-α_2-glycoprotein is normally present in most body fluids such as serum, saliva, sweat or urine [129]. The carbohydrate content (three *N*-glycans) was reported to be 18% including sialic acid, galactose, mannose, fucose and *N*-acetylglucosamine. Zn-α_2-glycoprotein shares a high degree of amino acid sequence identity with the α chains of the class I major histocompatibility complex. Moreover this protein was shown to be a member of the immunglobulin gene superfamily [130].

Parotid secretory protein

The parotid secretory protein has been described for the first time in rodents, pigs and cattle. In a recent study it could be identified as a member of human oral and airway epithelial

proteins, that are similar to lipopolysaccharide-binding proteins. It is assumed, that the parotid secretory protein can serve as a template for the design of anti-inflammatory peptides [131]. So far, there is no published data on the glycosylation of the parotid secretory protein. However, a closely related protein, the palate-lung-nasal epithelium clone (PLUNC), is known to be sialylated [132]. In addition, the electrophoresis pattern of this molecule provides indication for glycosylation (Sven-Ulrik Gorr, personal communication).

Lactoferrin

Lactoferrin is an iron-binding glycoprotein and belongs to the transferrin family. It has a molecular mass of about 77 kDa with two *N*-linked biantennary oligosaccharide units per molecule. Lactoferrin contains approximately 7% carbohydrates including terminal sialic acids, fucose and galactose [133]. The two glycans are structurally heterogeneous and differ from those of other transferrins. Lactoferrin is synthesized by glandular epithelial cells, as well as neutrophils and occurs mostly in secretions which bathe human mucosal surfaces [107]. It is known that lactoferrin has antibacterial functions. It is effective against bacteria that require iron for their metabolic processes and it was shown to be antibacterial independent of iron deprivation [101]. The significance of glycosylation for lactoferrin is not completely understood, although protection against proteases such as the pancreatic enzyme trypsin has been suggested [134].

Fibronectin

Fibronectins occur on cell surfaces, within basement membranes, in the extracellular matrix and within several body fluids, such as saliva. They have molecular masses of 210 to 260 kDa and contain 5-12% carbohydrate [107]. The carbohydrate content is dependent on the source of fibronectin [135]. Fibronectin usually exists as a dimer composed of two nearly identical 250 kDa subunits. The subunits are linked by a pair of disulfide bonds. Each monomer consists of three types of repeating units. Glycosylation sites, that are either *N*-linked or *O*-linked, reside predominantly within type III repeats and a collagen-binding domain. Fibronectins play an important role in cell adhesion [136] and interaction with various microorganisms, such as *Staphylococcus aureus* [137-140]

Current Knowledge About the Interaction of *Helicobacter pylori* with Salivary Components

Several hypothetical scenarios can be envisioned to describe the passage of *H. pylori* through

the oral cavity on its way to the human stomach. This organism might be simply swallowed without showing any interactions with saliva components or it binds to or is bound by salivary components. In the latter case the coaggregation with oral bacteria or attachment to salivary proteins might be possible.

A coaggregation of *H. pylori* with the secondary colonizing strain *Fusobacterium* has been shown [141]. It was suggested, that an adhesin was present on the fusobacteria whereas the corresponding receptor was present on the helicobacters. Thus, with regard to the great number of bacterial species in the oral cavity, further coaggregation partners are very likely.

Studying adhesion of *H. pylori* to salivary glycoproteins is obviously important, because known receptor structures recognized by *H. pylori* adhesins, as mentioned above, are also expressed on salivary glycoproteins. In addition, an interaction of *H. pylori* with salivary mucin MUC5B (MG1) has been reported in several studies, whereby disagreement exists concerning the responsible receptor epitope. Namavar *et al.* attribute this adhesion to an interaction between the neutrophil-activating protein of *H. pylori* and sulfated oligosaccharide motifs on salivary mucin MUC5B [51, 142]. Prakobphol *et al.* suggested that *H. pylori* binds to carbohydrate structures, mainly Lewis blood group epitopes, of MUC5B [143]. In the same study, also attachment to oligosaccharide motifs of MUC7 and gp-340 could be revealed. Concerning the interaction of *H. pylori* with gp-340, Prakobphol *et al.* showed initially the reverse case, namely that gp-340 binds to *H. pylori* [112]. This observation was considered relevant. Either *H. pylori* shows the phenomenon of becoming part of the oral biofilm by coadhesion to oral bacteria or by adhesion to pellicle proteins as mentioned above. Or it is bound by salivary proteins or binds them in solution and is thus cleared from the oral cavity by mechanical flushing. In addition, *H. pylori* could somehow change its pathological properties by interacting with salivary proteins.

Beside these known interactions of *H. pylori* with salivary glycoproteins further binding properties have been described. These were noted for proteins that occur in saliva too, such as lactoferrin [57, 58], fibronectin, and S-IgA [33].

These first studies on the interactions between *H. pylori* and salivary components as well as the fact that the oral cavity is the first part of the intestinal tract and the entry point for the gastric pathogen *H. pylori*, led to the underlying question of this thesis, namely whether saliva might be bound by the adhesins of this bacterium.

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Chapter 2

The Aims of this Thesis

Because the oral cavity is the only possible entry point for gastric *Helicobacter pylori* (*H. pylori*) (**chapter 1**) it is important to know, whether *H. pylori* passes simply through the oral cavity, or whether this bacterium interacts with components of saliva analogous to bacteria of the indigenous oral microbial flora. Salivary proteins, primarily glycosylated salivary proteins, are known to promote microbial adhesion. Therefore, the immediate aim of this thesis was to search for possible interactions of *H. pylori* with glycosylated salivary proteins and to evaluate a method to determine the molecular basis of such interactions.
This work has three parts:

1. Proteome analysis of human saliva
2. Establishment of an eligible glycoconjugate array to generally characterize binding properties of *H. pylori* and to give a first indication for minimal receptor structures
3. Comprehensive adhesion studies with *H. pylori* on salivary proteins.

1. Proteome analysis of human saliva

The main part of this section displays a proteome map of human saliva (whole saliva (WS), parotid and submandibular/sublingual (SMSL) secretions) by means of two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in order to obtain an overview about protein composition of WS in comparison to glandular secretions, and to have a tool for adhesion studies with *H. pylori* on human saliva. Some 2-DE studies of human saliva were already published, but these studies considered only WS and some important protein families (proline-rich proteins, salivary mucins) were missing in the previously published saliva proteome maps. Therefore, the first step was to optimize the method of sample preparation for 2-DE of human saliva (**chapter 3**), in order to obtain more complete proteome maps for above mentioned objects. The second step was the comparative proteome analysis of WS, parotid and SMSL secretions by MALDI time-of-flight (TOF) MS and MALDI TOF/TOF MS (**chapter 4**). In this part of the thesis intra- and inter-individual variations in human saliva proteome maps were considered particularly with regard to further adhesion studies.

2. Glycoconjugate array

Because many oral microbes colonize the mouth by interaction with glycosylated salivary proteins, first adhesion studies were carried out by bacterial overlay with fluorescence-labeled bacteria on purified glycoproteins and synthetic neoglycoproteins, that were immobilized on nitrocellulose membranes as dots. The aim of this part of the thesis was to develop an array

suitable to characterize the *H. pylori* wild-type strain and its adhesin-deficient mutants (*babA* and *sabA* mutants) and to narrow down minimal receptor structures necessary for binding. The glycoconjugate array included natural occurring glycoproteins and structural related neoglycoproteins, mainly with focus on molecules that are known to be bacterial receptor structures of *H. pylori*. Binding patterns of *H. pylori* wild-type strain and its adhesin-deficient mutants to (neo)glycoproteins were compared with the binding patterns of lectins with similar specificities (*Lotus tetragonulobus* and *Ulex europeaus* lectin) and oral bacteria as control (**chapter 5**). In order to demonstrate that the bacterial overlay assay with fluoresecence-labeled bacteria on glycoconjugate arrays is an appropriate method for the display of binding specificities also of other bacterial strains it was additionally carried out with two species of oral bacteria (actinomyces and streptococci) as probes, that have well-defined binding properties (**chapter 7**).

3. Adhesion studies on human saliva

The analysis of the human saliva proteome and the investigation of binding specificities of *H. pylori* and its adhesin-deficient mutants were the basis for this section of the thesis. Interaction of *H. pylori* with the whole range of salivary proteins was studied comprehensively by bacterial overlay (**chapter 6**). Bacterial overlay was first carried out on blots with salivary proteins separated by 1-DE, and it was tried to identify putative receptors of *H. pylori* by MALDI-MS. In a next step bacterial overlay was adapted for transfer blots of 2-D gels and identification of *H. pylori* receptors was achieved by comparison of signal spots of *H. pylori* with protein spots of 2-D proteome maps of the preceding study. These experiments were also carried out with another bacterial strain (*Streptococcus gordonii*) in order to show the applicability of this method also for other species of bacteria (**chapter 7**).

Chapter 3

Establishment of Two-Dimensional Gel Electrophoresis of Human Saliva

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Abstract

A major aim of this thesis was to utilize two-dimensional gel electrophoresis (2-DE) and subsequent transfer of separated proteins onto nitrocellulose membrane (blotting) for a high resolution bacterial overlay. Identification of salivary proteins as possible substrates for bacterial binding should be facilitated by proteome analysis. Therefore, a protein map as complete as possible should be obtained by 2-DE to display the whole range of salivary proteins. The immediate aim of this study was to establish 2-DE of human whole saliva (WS) as a prerequisite for the aforementioned application. Different conditions for the first dimension were compared, convenient pH gradients were figured out, as well as appropriate sample preparation procedures. Additionally, a first proteome analysis of a resulting 2-D gel by mass spectrometry was performed. It turned out that in-gel rehydration in the first dimension with rehydration under voltage over a pH range of 3-10 was most suitable for obtaining a complete protein map of WS. Proteome analysis of WS, that was directly dialyzed and lyophilized showed a high keratin contamination and many other cellular components. By contrast, filtration of WS reduced the number of spots and showed a new, noticeable spot group in the anodic region of the gel. These spots were stained pink with coomassie blue and were supposed to be proline-rich proteins. Thus, filtration of whole saliva is an important methodological step of saliva preparation for 2-DE.

Introduction

Proteome analysis is used to separate, visualize and analyze complex protein mixtures in fluids, such as saliva [1, 2]. Up to now a rapidly growing number of proteins has been identified in human saliva by the use of various methods, e.g. two-dimensional gel electrophoresis-mass spectrometry (2-DE-MS) [3-9], 2-D liquid chromatography-MS [8, 10] or Fourier transform ion cyclotron resonance-MS [11]. However, 2-D polyacrylamide gel electrophoresis (PAGE) [12] is the overall accepted technology to separate complex protein mixtures because of its ability to separate thousands of proteins simultaneously, and to display, in a visual way, proteins with posttranslational modifications [1] or isoform variances [13].

Trends in salivary proteomics are not only approaching the cataloging of proteins but also functional and diagnostic studies [14]. In this regard 2-D PAGE of saliva might be suitable also for clinical investigations because of its diversity in potential applications. Sick and healthy status of subjects or other individual differences can be compared by 2-D gels of saliva because there are a number of diseases associated with a defined protein profile in human saliva including connective tissue disorders, cystic fibrosis and diabetes mellitus [15]. This task could be performed either by image analysis [7] or by using different staining methods in combination with glycosidases [16]. A further important and already performed application of 2-DE of humans saliva is, after electrophoresis, the protein transfer onto nitrocellulose membranes and immunoblotting [7, 16, 17]. Modifying the latter technique by probing 2-D transfers with labeled bacteria instead of antibodies, as already successfully employed with 1-D gels [18, 19], a powerful tool would be available to screen for bacterial receptors in saliva. Establishing a 2-D map of human saliva in which every spot is identified could moreover facilitate the identification of bacterial receptors. A prerequisite for all these applications is a 2-D map that reflects the complete range of salivary proteins by choosing convenient pH gradients and appropriate sample preparation procedures in order to produce a reproducible 2-D spot pattern.

The immediate aim of the present study was to investigate different sample preparation procedures and to compare comprehensively different pH ranges as well as different isoelectric focusing (IEF) methods in order to establish a 2-D map of human whole saliva (WS) suitable for a subsequent proteome analysis as well as for bacterial overlay applications.

Materials and Methods

Reagents

Glycerol (approximate 87%), sodium dodecyl sulfate (SDS), trichloroacetic acid (TCA), acetic acid, and glycine were purchased from Merck (Darmstadt, Germany). 3-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid (CHAPS), dithiothreitol (DTT), urea, Pharmalyte 3-10 for isoelectric focusing (IEF), dry strip cover fluid as well as agarose were obtained from GE Healthcare Bio-Sciences (München, Germany). Iodacetamide was from Sigma, thiourea was from Fluka (Buchs, Switzerland), tris(hydroxymethyl)aminomethane (Tris) was from USB Corporation (Cleveland, OH, USA) and ethanol was from J.B. Baker (Deventer, Netherlands). Coomassie brilliant blue R-250 was from Bio-Rad (Bio-Rad Laboratories GmbH, München, Germany).

Sample preparation

WS was obtained from a healthy female subject by expectoration after rinsing the mouth with water, according to general accepted guidelines [20]. Samples were kept on ice during the collection procedure and were then processed in four different ways.

***Table 1**: Saliva preparation procedures performed in this study*

Preparation step 1	Preparation step 2
x	dialyzed and lyophilized
filtrated	dialyzed and lyophilized
x	concentrated with Amicon Ultra®
filtrated	concentrated with Amicon Ultra®

WS was filtered twice with a disposable membrane filter unit (Minisart, Sartorius, Hannover, Germany). In a first filtration step a pore size of 5 µm was used in order to remove larger insoluble material. In the second filtration step a pore size of 0.2 µm was used in order to remove bacteria. Subsequent dialyzation (sample volume approximately 2 ml) against 2 l of double distilled water for 16-20 h at 4°C was performed using a Slide-A-Lyzer® Kit (Pierce, Rockford, IL, USA). Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce) with bovine serum albumin as a standard, yielding a protein concentration of 2-3 mg/ml. For lyophilizing saliva, samples were aliquoted (300 µg of protein), frozen in liquid nitrogen and transferred into the to lyophilizing apparatus (Christ

Alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Alternatively, samples were desalted and concentrated with Amicon® Ultra (Millipore, Bedford, MA, USA) centrifugal filter devices (molecular weight cut-off, 5,000) up to protein concentrations of 10-20 mg/ml. All samples were stored at -80°C.

Two-dimensional gel electrophoresis

IEF was carried out with the IPGphor system from GE Healthcare Bio-Sciences using precast IPG strips (pH 3-10 NL, pH 4-7, pH 6-11; 18 cm; Immobiline Dry Strip, GE Healthcare Bio-Sciences) as described by Görg *et al.* [21]. Samples were applied by cup-loading or by in-gel rehydration. For cup loading the IPG strips were rehydrated overnight in a reswelling solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT and 1% Pharmalyte 3-10. Lyophilized or concentrated saliva samples (300 µg of protein) were solubilized in 100 µl of sample buffer (same composition as reswelling solution). Sample solutions were applied to the reswelled gels in the Ettan IPGphor Cup Loading Strip Holder (GE Healthcare Bio-Sciences) according to the manufacturer's instruction. For in-gel rehydration the IPG strip was rehydrated directly with saliva (300 µg of protein solubilized in 350 µl of sample buffer). For 2-D separation of proline-rich protein 1 (PRP-1, kindly provided from Donald I. Hay, Forsyth Institute, Boston, MA, USA), 10 µg of purified PRP-1 were dissolved in 350 µl of sample buffer and applied by in-gel rehydration. Running conditions for the first dimension of 2-DE were used as recommended by the manufacturer (see table 2).

***Table 2**: Running conditions for isoelectric focusing used in this study*

Cup loading (pH 3-10 NL)	
Initial IEF	200 V (1h), 500 V (1h), 1000 V (1h)
Gradient	1000-8000 V, 30 min
IEF	8000 V, 3 h
Cup loading (pH 6-11 NL)	
Initial IEF	500 V (1h), 1000 V (1h)
IEF	8000 V, 3,5 h
In-gel rehydration loading (without voltage) (pH 3-10 NL)	
Reswelling	0 V, 12 h
Initial IEF	200 V (1h), 500 V (1h), 1000 V (1h)
Gradient	1000-8000 V, 30 min

IEF	8000 V, 3 h
In-gel rehydration loading (with voltage)	
Reswelling	30 V, 12 h
Initial IEF	200 V (1 h), 500 V (1 h), 1000 V (1 h)
Gradient	1000-8000 V, 30 min
IEF	8000 V, 3 h (pH 3-10 NL) or 4 h (pH 4-7)

The second dimension (SDS-PAGE) was performed on a vertical system (Ettan DALT six electrophoresis unit, GE Healthcare Bio-Sciences) with precast Ettan DALT gels (12,5%T, 3%C, 255 x 196 x 1mm, GE Healthcare Bio-Sciences). The IPG strip was applied on top of the gel, according to the manufacturer's instruction and gels were run overnight at 1W *per* gel and 25°C.

Staining

Gels were stained with Coomassie brilliant blue (R-250). In brief: Gels were first incubated for 1 h in 20% TCA and were then incubated in 0.1% Coomassie R-250 with 40% ethanol / 10% acetic acid for 2 h. Excess dye was removed with two changes of 40% ethanol / 10% acetic acid for 30 min respectively, before intensifying them with 1% acetic acid overnight. Gels were washed with deionized water and conserved in an aqueous solution of 30% glycerol. All staining and washing steps were performed under continuous gentle agitation.

Tryptic digestion

Sample preparation for MS and MS analysis was carried out by the company Protagen AG, Dortmund, Germany. 2-D gels were first analyzed using the automated line (Bruker Daltonik GmbH, Bremen, Germany) and then displayed with a robot control software (spControl 3.1). Spots were picked with the spot picking robot PROTEINEER sp and transported into pierced 96 well PCR plates as described by the manufacturer. Digestion was carried out using the PROTEINEER dp digestion robot with a reagent kit and digestion protocol provided by the manufacturer. Gel pieces were first washed and then dried using acetonitrile. Trypsin was added to the dried residues and the samples were incubated for 4-8 h at 37°C.

MALDI-analysis

The peptides obtained after tryptic digestion were extracted and spotted onto a target plate that was pre-coated with α-cyano-4-hydroxy-cinnamic acid (all reagents from Bruker Daltonik GmbH) and washed. Peptide mass spectra were obtained using a MALDI-time of

flight (TOF)-TOF mass spectrometer (Ultraflex TOF/TOF, Bruker Daltonik GmbH) equipped with a Scout^{TM} MTP MALDI target according to the manufacturer's instructions. Peptide mass fingerprint (PMF) spectra were obtained by measuring peptides in the mass range from 700-4,000 Da by summing up 200 shots per spot. For identifying the monoisotopic peak of each peptide signal, peaks were labeled using the Similarity-Neighborhood Approach (SNAP) algorithm provided by the manufacturer of the mass spectrometer. Protein spots that could not be identified by PMF were analyzed additionally by peptide fragmentation fingerprint (PFF) analysis. For this analysis the most prominent peaks were selected by an acquisition method that was triggered by an intelligent algorithm in the data base that can choose peptides suitable for fragmentation.

Database search

Software for searching PMF spectra were ProFound^{TM} (Genomic Solutions Ltd., Cambridgeshire, UK) and Mascot^{TM} (Matrix Science Ltd, London, UK) and for PFF spectra Mascot^{TM}, Sequest^{TM} (Thermo, San Jose, CA, USA) or PFFSolver (Protagen AG, Dortmund, Germany). Searches were performed against the NCBI nonredundant protein database without restriction by the taxonomy. Results from PMF spectra were collated into one table with a unified scoring system that allowed a comparison of the different algorithms on a scale from 0 to 100. Protein identifications with a unified score larger than 90 were considered to be correct. Results from individual PFF spectra were combined for each spot and protein identification was based on the combined score. Protein identifications with a combined score larger than 10 for Sequest and 100 for Mascot were considered to be correct.

Results and Discussion

Comparison of different running conditions and pH gradients

In order to produce high-quality 2-D maps of saliva for medical, functional or comparative studies, it is important to figure out appropriate pH ranges and running conditions that provide for a good pattern quality and a complete display of all spots.

The first step was therefore the comparison of different pH ranges in the IEF step in order to evaluate the optimal pH range. Therefore, zoom gels (pH 4-7 and pH 6-11) were made as well as a wide-pH-range gel (3-10NL) (Fig. 1). Dark traces at the right side of the pH 4-7 zoom gel and on the left side of the pH 6-11 zoom gel indicated that proteins accumulated at the end of the respective IPG strips. The reason for this phenomenon is evident in the middle gel (pH 3-10NL). It is obvious, that the pH range of salivary proteins extend at least

from pH 3-10. For our proteome analysis of WS this pH range proved to be most useful, as it was also used in several other proteome studies of WS [5-8]. On the other hand, proteomics trends towards functional studies, emphasizing finer distinctions between several subproteoms. Therefore zoom gels will be of greater importance in the future and could provide some additional information about certain proteins. When comparing zoom gels it is remarkable that the separation of protein spots in the pH 4-7 gel is better than in the pH 6-11 gel. This confirms the more difficult separation of alkaline proteins in 2-DE [1] and the requirement of sample cup-loading for focusing between pH 6-11.

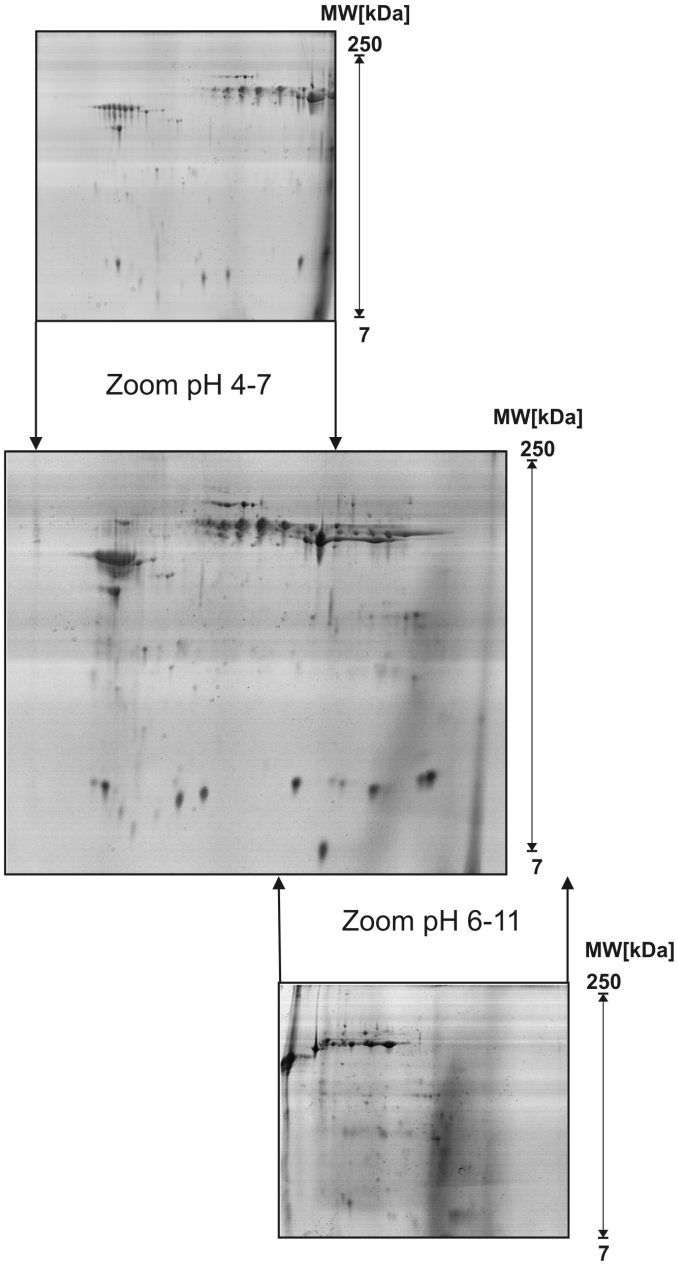

***Fig. 1**: Comparison of different pH ranges in the isoelectric focusing step (pH 4-7, 3-10NL, 6-11). For all procedures saliva was dialyzed/lyophilized and an amount of 300 µg of protein was loaded. IEF was carried out by cup-loading (pH 6-11) or by in-gel rehydration (pH 3-10NL, 4-7). Gels were stained with Coomassie blue R-250. Zoomed regions are marked with arrows on the middle gel (pH 3-10NL).*

Next, three different sample application methods, and thus, running conditions for IEF were compared (Fig. 2). It turned out that the overall spatial pattern of the protein spots was similar in all gels, independent of the electrophoresis settings. Although it is known that IPGs up to pH 10 are compatible with cup-loading as well as with in-gel rehydration [22], less spots and poorer resolution were seen in the anodic region of the 2-D gel with cup-loading IEF compared to in-gel rehydration IEF. This might be due to precipitation effects at the sample application point [23] or because of the higher protein concentration of the sample solution in the cup loading procedure (cup-loading: 3 mg/ml *versus* in-gel rehydration: 0.8 mg/ml). Differences between rehydration without voltage and under voltage were only marginal except that spots appeared more distinct in the former case.

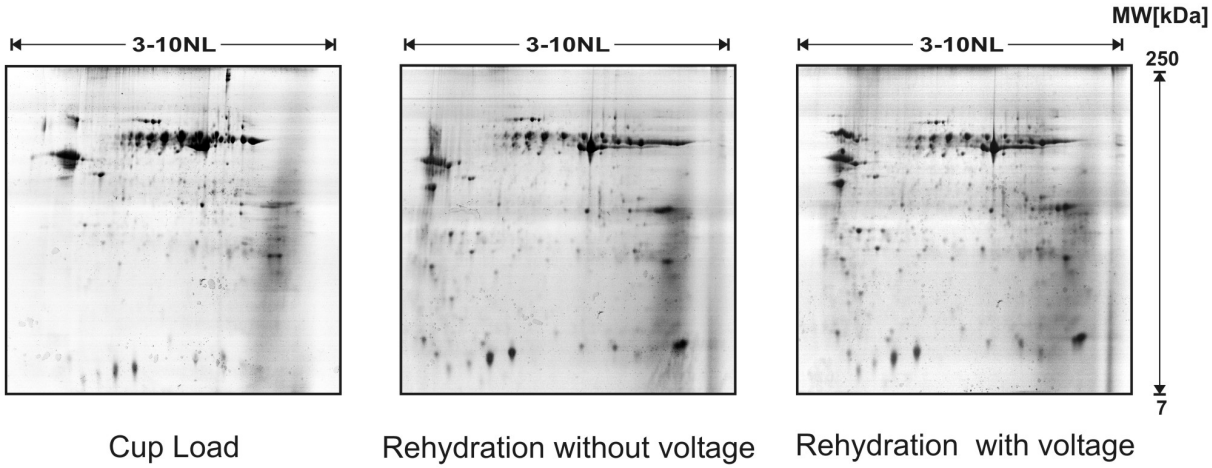

Fig. 2: *Comparison of different running conditions for isoelectric focusing (IEF). For all procedures saliva was dialyzed/lyophilized and an amount of 300 µg of protein was loaded. IEF was carried out over a pI range of 3-10. Gels were stained with Coomassie blue R-250. Running conditions for IEF are described in detail in Table 2 in the Material and Methods section.*

Identification of protein spots by mass spectrometry

In order to facilitate a later identification of bacterial receptors on 2-D transfers of human WS a first proteome analysis was carried out (Fig. 3). Because of above mentioned results the analysis was performed with WS rehydrated under voltage over a *pI* range of 3-10. Gels were stained with Coomassie blue and analyzed by the company Protagen AG. In this gel 351 protein spots were detected, picked and analyzed by MALDI-TOF and MALDI-TOF/TOF. Of these, 253 spots could be identified.

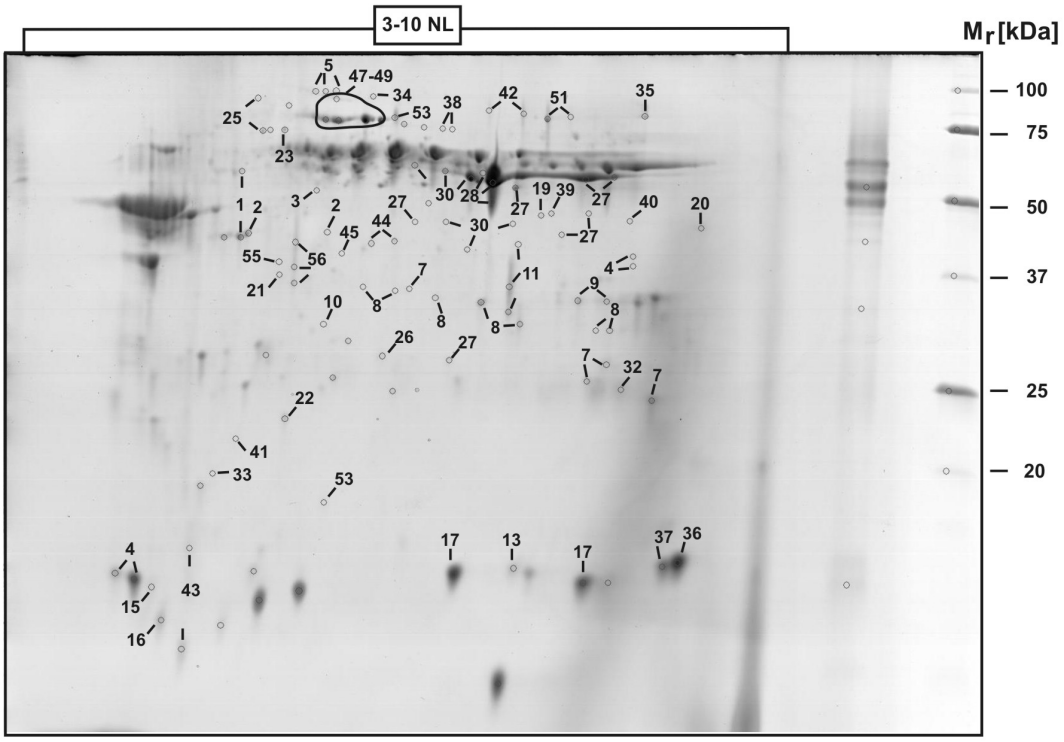

***Fig. 3**: 2-DE pattern of human whole saliva. Saliva was lyophilized/dialyzed and an amount of 300 µg was loaded. Separated proteins were detected by Coomassie blue staining. Circled spots are the spots identified without keratin. All spots that could be identified unambiguously are listed in Table 3.*

Among the 253 spots identified, 138 spots were identified as keratin (not circled in Fig. 3). All other protein spots identified (circled in Fig. 3) are listed in Table 3. Protein spots, that could not be identified unambiguously were disregarded in Table 3. Possibly, these proteins were of bacterial origin.

***Table 3**: List of proteins in unfiltrated human whole saliva identified by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Spots that were identified as keratin or that couldn't be identified unambiguously were disregarded.*

Spot No.	**Accession**	**Protein Name**	**Spot Count**
1	14250401	Actin, beta	2
2	728792	Actin 3	2
3	5031573	Actin-related protein	1
4	229674	Aldolase A	2
5	2804273	Alpha-actinin	1

Spot No.	Accession	Protein Name	Spot Count
6	12025678		1
7	442631	Annexin I (lipocortin I)	5
8	4502101		7
9	16306978	Annexin A2 (lipocortin II)	2
10	1703319	Annexin A4 (lipocortin IV)	1
11	14539767	Carbonic anhydrase VI	3
12	60822435	S100 calcium binding protein A9 (Calgranulin B)	3
13	539565	Cystatin D	1
14	4503109	Cystatin S (3)	3
15	359513	Cystatin SA	1
16	235948	Cystatin SA-III precursor of acquired enamel pellicle	1
17	118188	Cystatin SN	2
18	225541	Cystic fibrosis antigen	1
19	4503571	Enolase 1	1
20	4503471	Eukaryotic translation elongation factor 1 alpha 1	1
21	5453597	F-actin capping protein alpha-1 subunit	1
22	23200510	Glutathion-S-transferase	1
23	462325	Heat shock 70 kDa protein 1 (HSP70.1)	1
24	2495339	Heat shock 70 kDa protein 2 (HSP70.2)	1
25	5729877	Heat shock 70 kDa protein 8, isoform 1	1
26	4504517	Heat shock 27 kDa protein 1	1
27	15988376	Human salivary amylase	5
28	1633119		7
29	3212456		2
30	40254482		6
31	285975	Human rab GDI	1
32	223335	Ig κ light chain VLJ region	1

Spot No.	Accession	Protein Name	Spot Count
33	27894321	Interleukin 1 receptor antagonist isoform 4	1
34	33875446	JUP protein (junction plakoglobin)	1
35	494252	Lactoferrin	1
36	6729885	Lysozyme	1
37	307141		1
38	14603253	Phosphoglucomase 2	1
39	40068518	Phosphogluconate dehydrogenase	1
40	129902	Phosphogylcerate kinase 1	1
41	4507953	Phospholipase A2	1
42	514366	Poly-Ig-receptor	2
43	4505821	Prolactin-induced protein	2
44	13489087	Protease inhibitor 2	2
45	4505789	Protease inhibitor 5	1
46	352334	SAP-1	1
47	15988375	Serum Albumin	1
48	31615330		2
49	3212456		1
50	230777	Thioredoxin	1
51	4557871	Transferrin	3
52	4699632		1
53	13638501	Transglutaminase 3	1
54	21465999		1
55	422542	Tubulin alpha chain	1
56	4699583	Zinc-α_2-glycoprotein, chain B	3

The high abundance of keratin spots (~ 54 % of all spots identified) indicates a contamination of the saliva sample. Keratins are structural proteins that are expressed in epithelial cells. Two distinct classes of proteins are known that are always expressed as pairs: acidic type I and basic or neutral type II keratins [24]. Almost all keratin spots identified in WS were keratin 13 and its basic partner keratin 4. These keratins are expressed in suprabasal layers of non-

cornified, stratified, mucosal epithelia [24] including oral epithelium [25], providing evidence that this keratin contamination may originate from cellular debris within the oral cavity and not from external skin or hair contamination during the experimental procedure. Besides keratins, further spots of proteins of cellular origin were identified, e.g. actin or annexin. Both proteins have been detected in the oral epithelium in previous studies [26, 27]. The presence of cellular proteins, in particular the high abundance of keratin in human salivary 2-D map prevents the true display of human saliva proteome Therefore, the direct application of lyophilized / dialyzed saliva to 2-DE turned out to be useless, also, because it implies the risk to map bacterial proteins. Thus, an alternative way of saliva preparation was tested in this study.

Comparison of different sample preparation procedures

In previous 2-DE studies on human WS, saliva samples were always subjected to a centrifugation step, whereas no dialyzing step was performed [4-8]. Although centrifugation of saliva may remove cellular debris and keratin contamination, it was tried to find an alternative way of saliva treatment in the present study. Four different sample preparation procedures were compared (see also Table 1) including lyophilizing/dialyzing with or without a preceding filtration step as well as concentration with Amicon Ultra® with or without a preceding filtration step (Fig. 4). Filtration was divided into two steps: first, the removal of larger insoluble material by using a 5 µm pore size filter and subsequently a sterile filtration by using a 0.2 µm pore size filter as described previously [28].

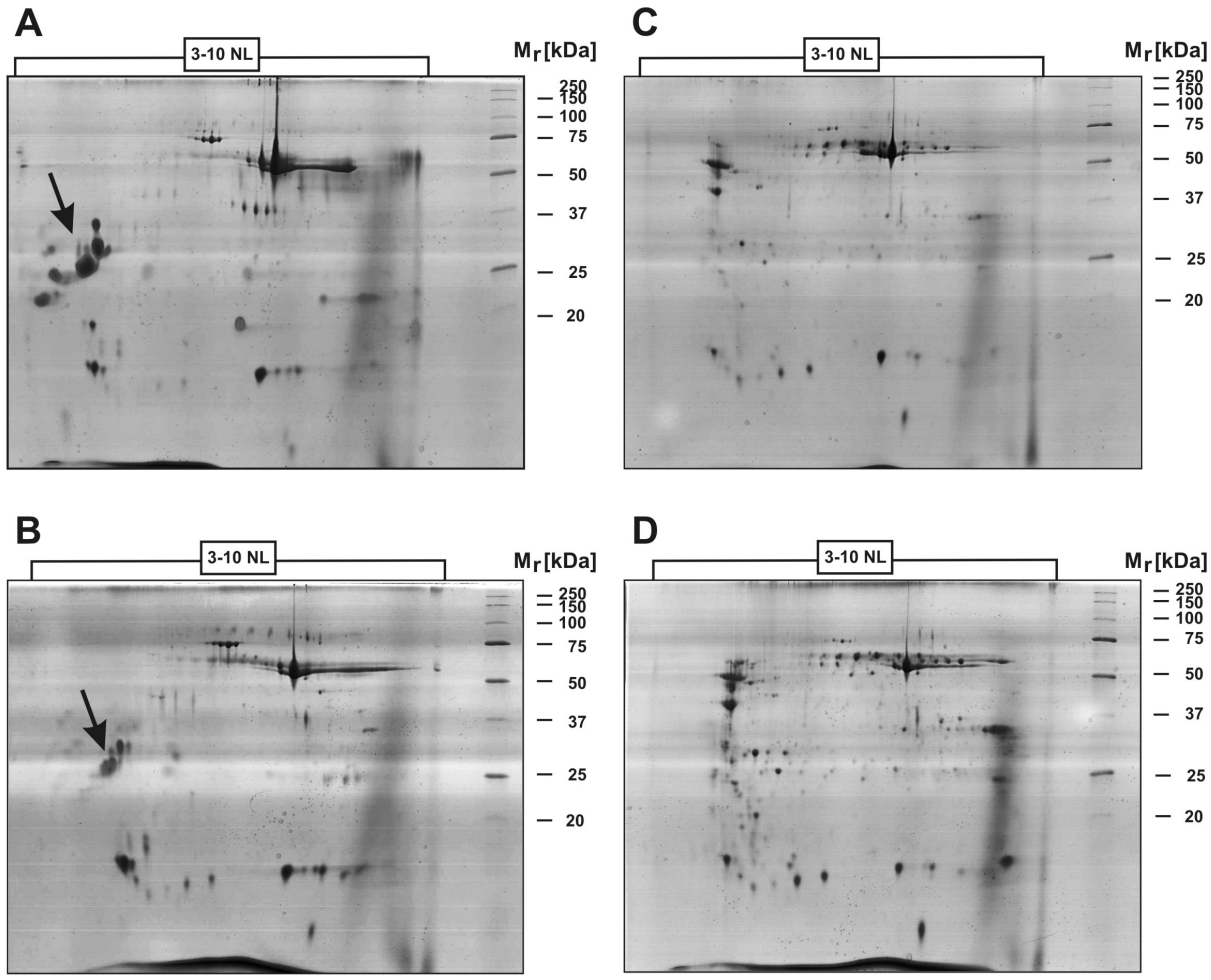

***Fig. 4**: Comparison of different ways of saliva sample preparation for two-dimensional gel electrophoresis. Human whole saliva was A. filtrated and concentrated with Amicon Ultra®, B. filtrated and dialyzed/lyophilized, C. only concentrated with Amicon Ultra® and D. only dialyzed/lyophilized. Isoelectric focusing was carried out by in-gel rehydration over a pI range of 3-10. Gels were stained with Coomassie blue. Spot groups that appeared pink with Coomassie blue staining are marked by arrows.*

Filtration of saliva samples (gels A, B) resulted in a noticeable effect on the 2-D spot pattern compared to saliva preparations without filtration (gels C, D). The number of spots was reduced, suggesting the absence of keratin and, perhaps, bacterial proteins from the samples (total spot count detected in gel A was 193). However, a new, pink spot group appeared in the anodic region of the gel (marked by arrows). It is known that PRPs, separated by gel electrophoresis, stain pink with Coomassie blue R-250 [29]. Thus, this spot group could be acidic PRPs. This notion was supported by applying purified PRP-1 to 2-DE (Fig. 5), resulting in two small, pink spots at the same location as the pink spots detected in 2-D gel of filtrated WS. The presence of PRPs in the 2-D map of the filtrated WS sample, that was absent in unfiltrated WS shows the advantage of the filtration steps, because PRPs are a

dominant group of proteins in human saliva [30]. A possible explanation for the appearance of PRP spots after sample filtration might be that these proteins are particularly sensitive to enzymatic cleavage by bacterial proteases [31]. It would be likely that this process could be considerably slowed down after removal of the naturally occurring oral bacteria in whole saliva by means of filtration.

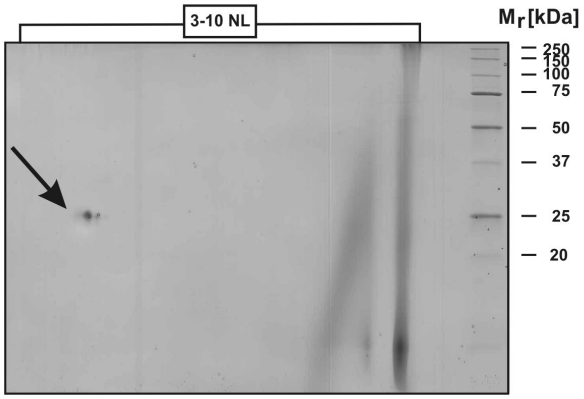

***Fig. 5**: 2-DE pattern of purified proline-rich protein 1 stained with Coomassie Blue R-250. The arrow shows the position of purified proline-rich protein 1 in the complete gel.*

In contrast to the advantageous effects of the filtration steps, only little differences were noticed between concentrating and lyophilizing saliva (A/B or C/ D respectively) demonstrating that each method leads to the same result. The need for desalting samples prior to electrophoresis had been examined earlier.

Concluding Remarks

When performing 2-DE studies of complex protein mixtures, the nature of the analyzed samples, the *pI* range of their proteins as well as the purpose of the study have to be considered. With regard to the aim of generating a proteome analysis of human saliva and of establishing a high-resolution bacterial overlay it turned out that focusing over a pH range of 3-10, by in-gel rehydration, is the best way to display the whole range of salivary proteins in a reproducible way. In terms of sample preparation, a compromise has to be found between a high number of protein spots and the true display of all typical salivary proteins, thus filtrating of WS prior to electrophoresis experiment was chosen as the method of choice for subsequent studies.

Acknowledgements

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Chapter 4

Proteome Analysis of Glandular Parotid and Submandibular-sublingual Saliva in Comparison to Whole Human Saliva by Two-dimensional Gel Electrophoresis

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Proteomics (2006); **6**: 1631-1639

Abstract

The secretions of the salivary parotid and submandibular-sublingual (SMSL) glands constitute the main part of whole human saliva (WS) in which proline-rich proteins (PRPs) and mucins represent dominant groups. Although proteome analysis had been performed on WS, no identification of PRPs or mucins by two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) was achieved in WS and no comprehensive analysis of both glandular secretions is available so far. The aim of this study was to compare the protein map of WS to parotid and SMSL secretions for the display of PRPs and mucins. WS and glandular secretions were subjected to 2-DE and spots were analyzed by matrix-assisted laser desorption/ionization-MS. New components identified in WS were cyclophilin-B and prolyl-4-hydroxylase. Also acidic and basic PRPs as well as the proline-rich glycoprotein (PRG) could now be mapped in WS. Acidic PRPs were found equally in parotid and SMSL secretions, whereas basic PRPs and PRG were found primarily in parotid secretion. Salivary mucin MUC7 was identified in SMSL secretion. Thus, the more abundant proteins of WS can be explained mainly by mixed contributions of parotid and SMSL secretions with only few components remaining that may be derived from local sources in the oral cavity.

Introduction

Proteins in saliva have important functions not only for mastication and digestion of food but also for mineralization of teeth, protection of oral mucosa and interactions with a complex oral microbiota [1-4]. Because saliva, in contrast to blood, is easy to collect and also contains many biologically active substances such as cytokines, growth factors and hormones it may have a great potential as a diagnostic fluid [5, 6]. Unfortunately, although the protein components of saliva and their functions are well known, sufficient qualitative and quantitative analytical methods suitable for diagnostics were not available in the past.

In comparison to other body fluids, whole human saliva (WS) is a complex mixture derived from not only different major and minor salivary glands but also from crevicular fluid as well as from contaminating bacteria and cellular debris [1]. 2-DE is a well suited method to display proteins in complex mixtures [7]. Several approaches had been undertaken in the past to separate saliva by 2-D PAGE [8-11] and more recently comprehensive proteome analyses have been completed for WS [12-16] and for glandular parotid secretion [17]. In a recent study, multidimensional protein identification technology (MudPIT) has been used for the analysis of the WS proteome [18]. The results show the applicability of 2-D chromatography to study the saliva proteome. More than 100 proteins were identified in WS, many of those not previously detected by an electrophoretic approach.

In contrast to MudPIT, the classical approach by a combination of 2-D PAGE and MS allows the separation of different isoforms and has the further advantage that probing with antibodies or other biologically reactive compounds can be performed on transfer-blots of 2-D gels [19]. Previous 2-DE analyses of WS do not provide complete information about two dominant and unique groups of salivary proteins, namely the proline-rich proteins (PRPs) and the salivary mucins in a comparative display with submandibular-sublingual (SMSL) and parotid glandular secretions. PRPs have important functions for calcium-binding, mineralization of dental hard tissues, and possibly for inactivating the effects of dietary tannins [20, 21]. Salivary mucins are important for lubrication and protection of oral epithelial surfaces as well as for food bolus formation and swallowing [22, 23]. Both PRPs and mucins adsorb to the tooth surface in form of the acquired pellicle [24] where they protect teeth from acid-induced demineralization [25] and provide adhesion sites for certain oral bacterial adhesins [4, 26].

In the present study, it was attempted to analyze WS as well as parotid and SMSL glandular secretions by state-of-the art proteomics approach applying high-resolution 2-DE

and MALDI-MS with the intention to comprehensively map PRPs and mucins in these different secretions.

Materials and Methods

Reagents

Urea, CHAPS, DTT, Pharmalyte 3-10 for IEF, dry strip cover fluid as well as agarose NA were purchased from Amersham Biosciences (Freiburg, Germany), Iodacetamide was from Sigma, thiourea was from Fluka (Buchs, Switzerland), Tris was from USB Corporation (Cleveland, OH, USA) and ethanol was from J.B. Baker (Deventer, Netherlands). Glycerol (approximate 87%), SDS, TCA, acetic acid, glycine, acetone and acetonitrile were obtained from Merck (Darmstadt, Germany). Coomassie brilliant blue R-250 was acquired from Bio-Rad (Bio-Rad Laboratories GmbH, München, Germany).

Sample preparation for 2-D PAGE

The study was approved by the Ethics Committee of the Medical Faculty of the University of Regensburg. Salivary samples were collected from four healthy (2 female, 2 male) donors. For spot identification, samples from one female individual out of this group were taken. Unstimulated WS was collected by expectoration and kept on ice. Immediately after collection, WS was filtrated twice with a single use filter unit (Minisart, Sartorius, Hannover, Germany) using a pore size of 5 µm to remove larger insoluble material and then using a pore size of 0.2 µm to remove bacteria from the sample. Parotid saliva was collected with Lashley cups (Stone Machine Company, Colton, CA, USA) after stimulation of the tongue with 2% citric acid solution [27]. While the orifice of the parotid duct was blocked by Lashley cups, the sublingual area was isolated with cotton wool rolls, and SMSL saliva was collected with a 50 ml sterile syringe fitted with a sterile silicon tube. As a quality control for purity of the separate glandular secretions, one-dimensional gels were run on aliquots of the samples and were stained by silver stain as well as by a carbohydrate stain and resulted in a clearly distinct pattern of bands. Samples were desalted and concentrated with Amicon® Ultra (Millipore, Bedford, MA, USA) centrifugal filter devices (molecular weight cut-off, 5,000) yielding final protein concentrations of 5-25 mg/ml as determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. The samples were stored at -80°C until analyzed.

2-D gel electrophoresis

The first dimension, IEF, was performed in the IPGphor from Amersham Biosciences by in-gel rehydration as described by Görg *et al.* [28]. The IPG strip, pH 3-10 NL, 18 cm (Immobiline Dry Strip, Amersham Biosciences) was rehydrated directly with saliva (300 µg of protein) solubilized in 350 µl of sample buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT and 1% Pharmalyte 3-10. For analysis of PRP-1 (kindly provided from Donald I. Hay, Forsyth Institute, Boston, MA, USA) as standard control, 10 µg of PRP-1 were dissolved in 350 µl of sample buffer. Low voltage (30 V, 12 h) was applied during the rehydration step, IEF was started with 200 V for one hour and increased to 500 V and 1,000 V for one hour, respectively, before continuously (gradient, 30 min) raising the voltage to 8,000 V for a final focusing time of 3 h. IPGs were stored at -80°C until analyzed. The second dimension (SDS-PAGE) was performed in a vertical apparatus (Ettan DALT six electrophorese unit, Amersham Biosciences). The IPG strip was applied on top of the Ettan DALT gel (12,5%T, 3%C, 255 x 196 x 1mm, Amersham Biosciences), according to the manufacturer's instruction and the gel was then run for 16 h (1 W per gel, 25°C).

Staining

2-D gels were stained with Coomassie brilliant blue (R-250) under gentle agitation. The proteins were first fixed with 20% TCA for 1 h and then stained with 0.1% Coomassie R-250 in 40% ethanol / 10% acetic acid for 2 h. Gels were destained twice in 40% ethanol / 10% acetic acid for 30 min before intensifying with 1% acetic acid overnight. After washing with deionized water, gels were conserved in an aqueous solution containing 30% glycerol and were stored at 4°C.

Sample preparation for MS

For MS-based protein identification, both a manual [29] and a robotic procedure were performed. For the robotic approach, 2-D gels were analyzed using the automated PROTEINEER® line (Bruker Daltonik GmbH, Bremen, Germany). 2-D gels were rinsed with water in order to remove excess stain from the surface. Then the gels were fitted between a glass plate and a metal frame in order to prevent gel movement during the picking process. This assembly was mounted onto the surface of a high-resolution scanner that is integrated into the spot picking robot PROTEINEER sp with enough water to cover the gel. Using the robot control software spControl 3.1, an image of the 2-D gel was acquired. The spot detection was performed automatically, using the integrated spot detection algorithm. Spots

were picked into pierced 96 well PCR plates that were coded with a transponder for sample tracking. For spot picking, the method for backed gels was provided by the manufacturer.

Digestion and MALDI target spotting

The samples were digested using the PROTEINEER dp digestion robot. For the manual procedure, the samples were prepared as described elsewhere [29]. Following the automatic procedure, the sample plates were transferred from the spot picker to the appropriate positions on the digesting robot. The robot can identify the samples on the deck by means of an integrated transponder reader. The samples were digested using the reagent kit and digestion protocol provided by the manufacturer. Briefly, the gel plugs were washed to remove the Coomassie stain and dried using acetonitrile. Trypsin was applied to the gel plugs and samples were incubated for 4-8 h at 37°C. The resulting peptides were extracted and spotted onto a AnchorChip® MALDI target that was pre-coated with α-cyano-4-hydroxy-cinnamic acid (all reagents from Bruker Daltonik GmbH) and washed.

MALDI-analysis

For the analysis of tryptic peptides, MALDI-TOF MS was applied using the UltraFlex™ (Ultraflex TOF/TOF, Bruker Daltonik GmbH) equipped with a Scout™ MTP MALDI target according to the manufacturer's instructions. In a first analysis (PMF analysis) the masses of the intact peptides generated in the digestion process were measured in the mass range from 700-4,000 Da. The target spots were analyzed by summing up 200 shots per spot while the laser attenuation was adjusted automatically to an optimal level by utilizing a fuzzy-logic feedback algorithm. For the manual measurement, adopting laser attenuation and summing MS spectra were performed individually by the user. The peaks in the spectrum were labeled using the Similarity-Neighborhood Approach (SNAP) algorithm provided by the vendor of the mass spectrometer in order to identify the monoisotopic peak of each peptide signal. Thereafter, the spectra were exported to the Proteinscape database (Bruker Daltonik GmbH) for protein identification. Protein spots that could not be identified by PMF were analyzed in a second round performing PFF analysis from the most prominent peaks in the PMF spectrum. The acquisition of PFF spectra was triggered by an intelligent algorithm in the data base that can choose peptides suitable for fragmentation based on different criteria such as intensity, selectivity from neighboring peptide masses and / or protein identification.

Protein identification

The MALDI spectra were automatically imported into the database and linked to the appropriate spots in the 2-DE gel image. In the database, the MALDI spectra were recalibrated using known contaminant peaks e.g. from keratin or trypsin in order to obtain precise peptide masses. The masses of contaminant peaks were removed from the spectra leaving only peaks with unknown masses for the database search. From PMFs, protein identification was achieved by searching the mass spectra against the NCBInr protein database without restriction by the taxonomy using several external search algorithms ProFound™ (Genomic Solutions Ltd., Cambridgeshire, UK) and Mascot™ (Matrix Science Ltd, London, UK). The results were collated into one table with a unified scoring system that allowed a comparison of the different algorithms on a scale from 0 to 100 [30]. The significance of the search result was then evaluated based on the unified score. Protein identifications with a unified score larger than 90 were considered to be correct.

For PFF spectra arising from the MALDI TOF/TOF mode, protein identification was achieved by searching a protein database using several algorithms including Mascot™, Sequest™ (Thermo, San Jose, CA, USA) or PFFSolver (Protagen AG, Dortmund, Germany). The results from individual PFF spectra were combined for each spot and protein identification was based on the combined score. For Mascot, this was achieved by the algorithm itself, for Sequest and PFFSolver, the algorithm provided by the Proteinscape database was used. Protein identifications with a combined score larger than 10 for Sequest and 100 for Mascot were considered to be correct. The significance level calculated by the Mascot software was a score of 50. The threshold for protein identification was set considerably higher in order to prevent false positive identifications.

Results

Separation of salivary proteins by 2-D PAGE and identification of protein spots

In Coomassie blue-stained WS (Fig. 1A), major protein groups were represented by different isoforms of salivary amylase (spots 15) and a group of PRPs. Most PRPs appeared as typical diffuse pink spots [31] grouped within one zone of the acidic region (p*I* 3.2 - 4.9, spots 23). Additional pink spots appeared between p*I* 5.3 – 6.2 (spot 23) in the same M_r position and in the basic region (spots 24 and 25). Interestingly, the pink spots could only be seen, when saliva was filtrated with a membrane filter of 0.2 µm pore size prior to 2-DE.

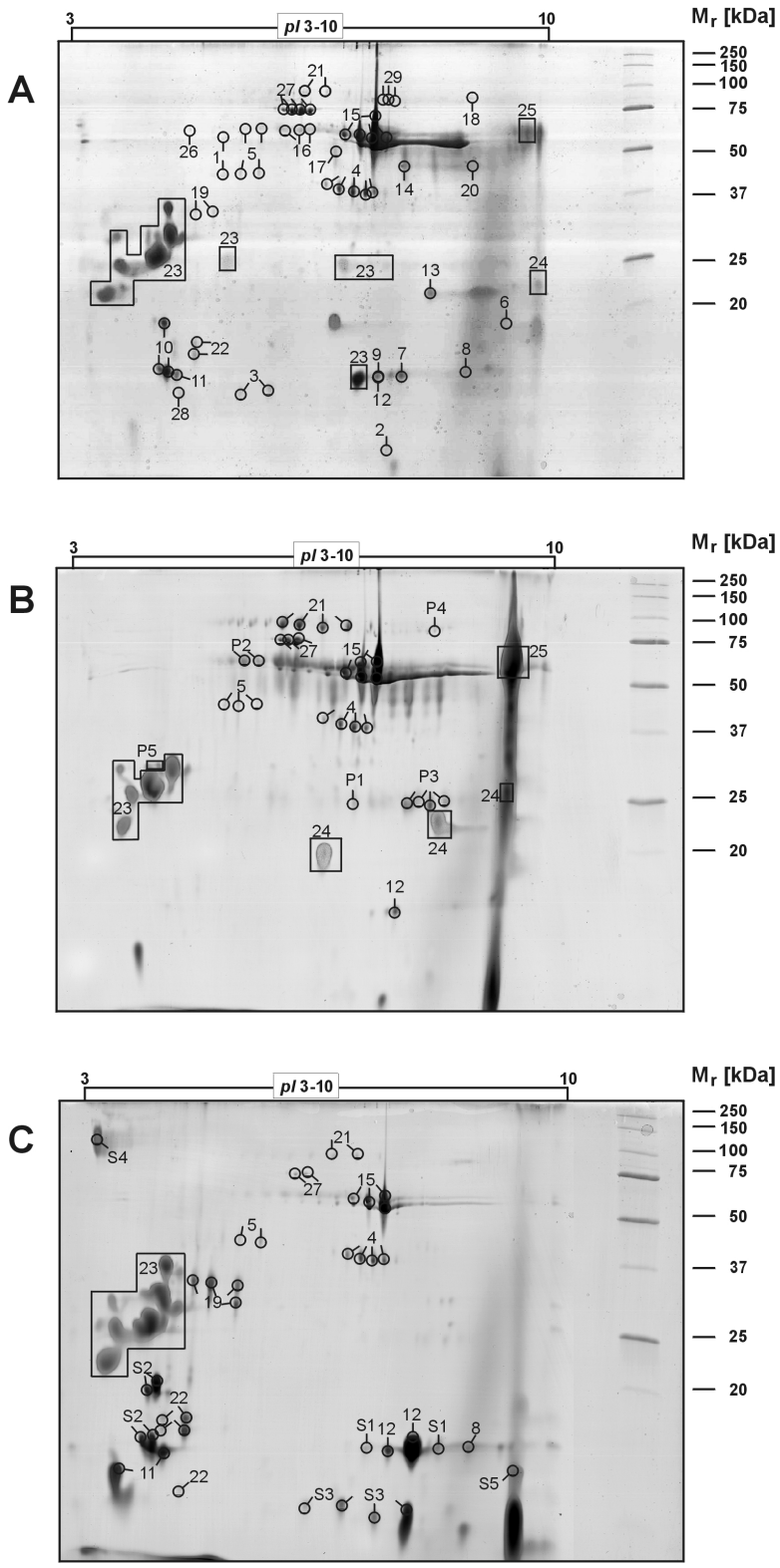


Fig. 1: *2-DE pattern of human whole saliva (**A**), parotid saliva (**B**) and submandibular/sublingual saliva (**C**). Separated proteins were stained with Coomassie Blue R-250. Spot numbers refer to proteins listed in Table 1 and the Table in the Supplementary Materials. The number of circles does not represent the number of total spots excised (for spot counts and for a more detailed description of mapped proteins see Table in the Supplementary Material).*

A total of 192 spots were picked from the WS-gel shown in Fig. 1A. The peptides resulting from tryptic digestion were analyzed and 131 spots could be identified by MALDI-MS (Table 1).

***Table 1**: List of proteins in whole saliva (WS) as well as in parotid and submandibular-sublingual (SMSL) secretions identified by two-dimension (2-D) polyacrylamide gel electrophoresis (PAGE) and mass spectrometry (MS) in comparison to previous proteome studies.*

Spot No.	Protein Name	Source of Protein			Previous Proteome Studies		
		WS	Parotid Saliva	SMSL Saliva	2-D PAGE	MudPIT [h]	LC-MS [i]
1	Actin	x			e, g	x	x
2	Calgranulin A	x			b, c, d, e	x	x
3	Calgranulin B	x			b, c, d	x	x
4	Carbonic Anhydrase VI	x	x	x	d, f, g	x	
5	Chain B, D Human Zn-α_2-Glycoprotein	x	x	x	c, d, e, f, g	x	x
6	Cyclophilin B	x					
7	Cystatin B	x			d, f	x	x
8	Cystatin C	x		x	d	x	x
9	Cystatin D	x			c, d, f	x	x
10	Cystatin SA-III precursor of acquired enamel pellicle	x			b, d, e	x	
11	Cystatin SA	x		x	b, c, d, e, f, g	x	x
12	Cystatin SN precursor	x	x	x	b, c, d, e, g	x	x
13	Cytokeratin 1	x					
14	Enolase 1	x			d, e, g	x	
15	Human salivary amylase	x	x	x	a, b, c, d, e, g	x	x
16	Ig alpha-1 chain C region	x			a, c, d, f, g	x	x
17	Keratin 10	x					
18	Lactoferrin	x			a, d	x	
19	Parotid secretory protein	x		x	d	x	
20	Phosphoglycerate kinase	x			g		
21	Poly-Ig-receptor	x	x	x	a, c, d, e, f, g	x	x

Spot No.	Protein Name	Source of Protein			Previous Proteome Studies		
		WS	Parotid Saliva	SMSL Saliva	2-D PAGE	MudPIT [h]	LC-MS [i]
22	Prolactin-inducible protein	x		x	c, d, e, g	x	x
23	Proline-rich phosphoprotein (gene *PRH1* Db allele)	x	x	x			
24	Proline-rich protein *Bst*NIn, subfamily 1, isoform 3	x	x				x
25	Proline-rich protein *Bst*NI, subfamily 3 (PRG)	x	x		f		x
26	Prolyl-4-hydroxylase	x					
27	Serum albumin	x	x	x	a, b, c, d, e, f, g	x	x
28	Thioredoxin	x			e	x	x
29	Transferrin	x			e, g		
P1	HSPC059		x				
P2	Ig alpha-2 heavy chain		x		g	x	x
P3	Ig κ light chain VLJ region		x		a, c, d, e, f, g	x	x
P4	Lactoperoxidase, Salivary peroxidase		x			x	x
P5	Salivary acidic proline-rich phosphoprotein 1/2 precursor		x		f	x	x
S1	CST1			x			
S2	Cystatin S			x	c, d, g	x	x
S3	Histatin 1			x	b, d, f	x	x
S4	Mucin 7			x			x
S5	0808206A peptide PA, proline-rich peptide			x			

a Beeley et al. (1991): 2-DE and immunoblotting (parotid saliva)

b Yao et al. (2002): 2-DE and MS (WS, acquired enamel pellicle)

c Ghafouri et al. (2003): 2-DE and MS (WS)

d Vitorino et al. (2004): 2-DE and MS (WS)

e Huang et al. (2004): 2-DE and MS (WS)

f Hardt et al. (2005): 2-DE and MS (parotid saliva)

g Hu et al. (2005): 2-DE and MS (WS)

h Wilmarth et al. (2004): MudPIT (WS)

i Hu et al. (2005): LC-MS (WS)

The proteins that were identified for the first time in WS by a combination of 2-DE and MS include cyclophilin B (spot 6), prolyl-4-hydroxylase (spot 26), acidic PRP (spots 23), basic PRP (spot 24), and proline-rich glycoprotein (PRG) (spot 25). In agreement with their predominant appearance in the 2-D gel, salivary amylase and PRPs also amount to the majority of spots (Table in the Supplementary Material).

In Coomassie-stained glandular secretions (Figs. 1B, C) a smaller number of spots as compared to WS were apparent. In parotid saliva, from 58 spots analyzed, 43 spots were identified and in SMSL secretion from 63 spots analyzed, 53 spots were identified. Most spots were found to be identical to the ones identified in WS. In parotid saliva (Fig. 1B), five proteins were found that could not be detected in WS, including HSPC059 (spot P1), Igα-2 heavy chain (spot P2), Igκ light chain VLJ region (spots P3) lactoperoxidase (spot P4), and salivary acidic proline-rich phosphoprotein 1/2 precursor (spot P5). In SMSL saliva (Fig. 1C), CST1 (spots S1), cystatin S (spots S2), histatin 1 (spots S3), MUC7 (spot S4), and 0508206A peptide PA (proline-rich peptide, spot S5) could be found as additional proteins that were not detectable in WS. Comparing SMSL spot intensities to WS and parotid secretion, lower amounts of salivary α-amylase were noted and PRG as well as basic PRPs could not be identified in the basic p*I*-region.

Identification of PRPs by comparison to purified PRP-1

Following 2-D PAGE of purified PRP-1, two pink spots within a p*I* range of about 4,5 and M_r of about 22,000 were revealed (gel not shown). PMF- and PFF-spectra of these spots (Fig. 2B, C) were in agreement with the database entry "salivary acidic proline-rich phosphoprotein 1/2 precursor" and, thus, substantiated the identity of the proteins in these spots as PRP-1. As seen in Fig. 2A, the PMFs of the pink spots in WS and glandular secretions (all: spot 23) differ from those of purified PRP-1 (Fig. 2B). An additional peptide peak with a mass of 2,136 Da was detected that was not present in purified PRP-1. The amino acid sequence of this peptide as revealed by PFF analysis (Fig. 2C), identified PRP type Db (gene *PRH1*) [32]. This PRP variant has an additional 21-amino acid repeat that is missing in the amino acid sequence of PRP-1-precursor [32, 33] (Fig. 2D). It is noteworthy that a horizontal scattering of Db protein spots within a p*I* range from 3.2 to 6.2 was observed.

In parotid saliva (Fig. 1B), an additional subclass of acidic PRPs was found (spots P5) that showed a mass spectrum comparable to that of purified PRP-1 (Fig. 2B). As evident from Fig. 2D, the N-terminal region of the PRPs was not detectable by MALDI mass spectrometry due to the paucity of tryptic cleavage sites and the limited mass range for PMF spectra. Due to

this limitation of analysis, it could not be distinguished which of the gene products of *PRH2* (PRP-1, PRP-2, PRP-4, PRP-4, PIF-f, PIF-s) is represented by spot P5. In WS from another individual (Fig. 3B, Individual 3), different PRP species were found that did not contain the peptide peak with a mass of 2,136 Da, confirming the existence of inter-individual variations in this diverse family of proteins [34] (data not shown).

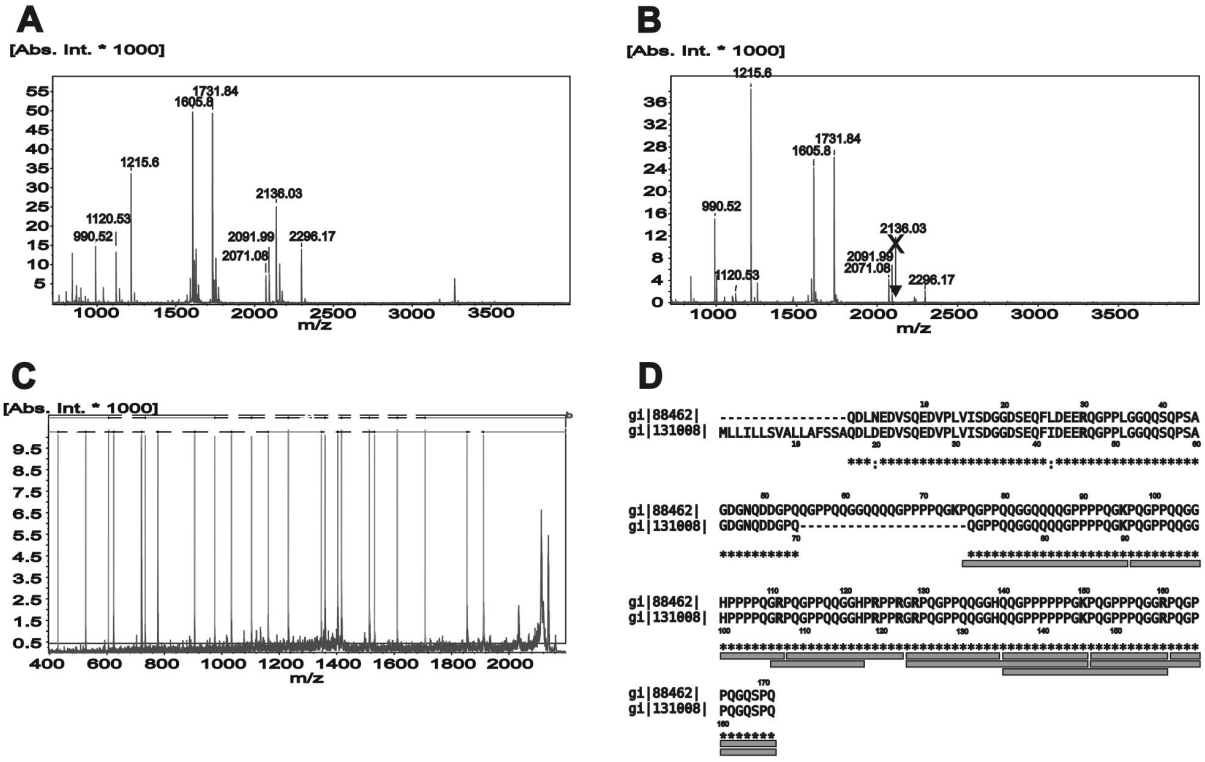


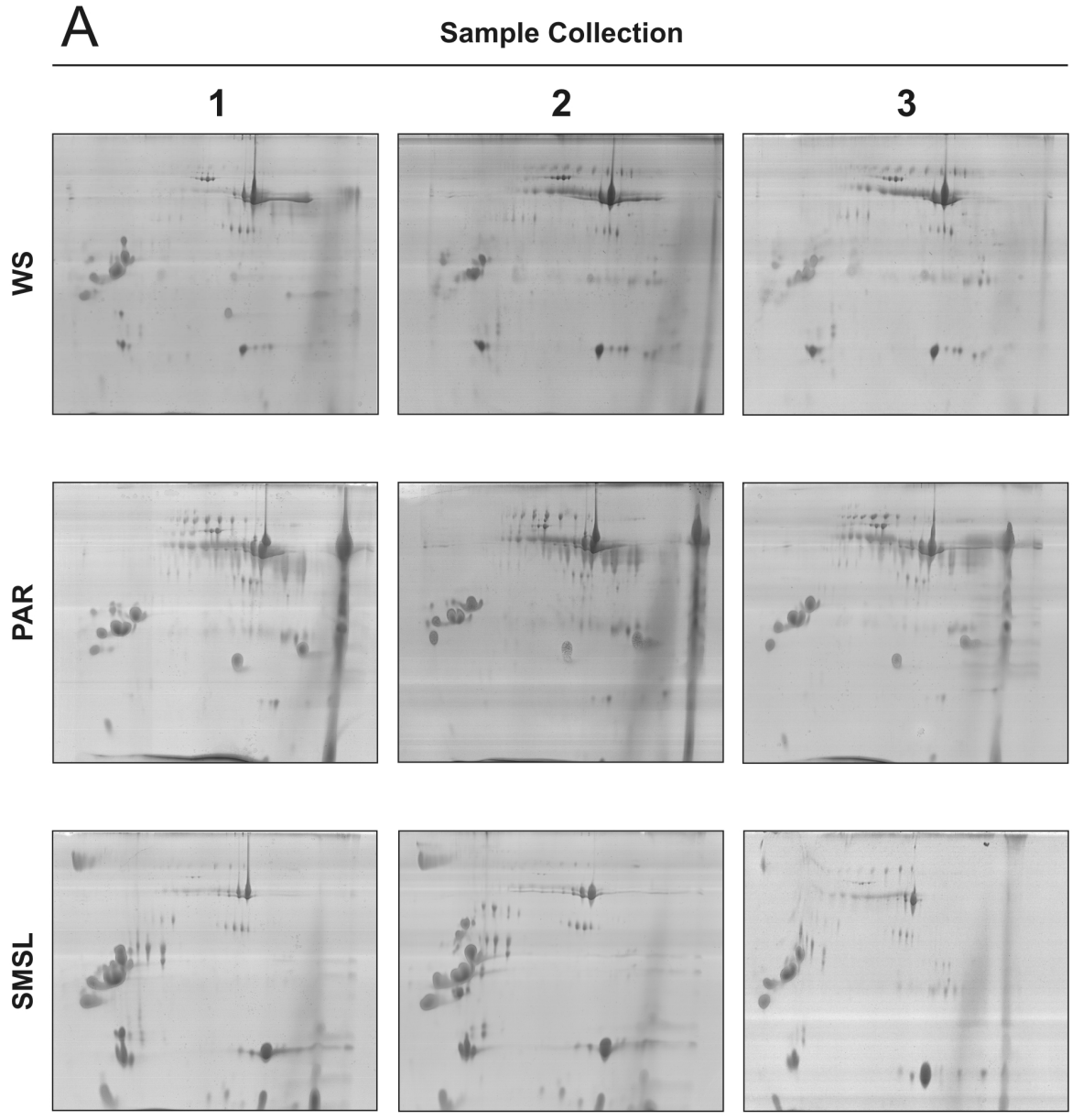
Fig. 2*: Identification of acidic proline-rich proteins by MS. A. PMF spectrum of proline-rich phosphoprotein identified in whole human saliva as well as in glandular secretions (gene* PRH1*, Db allele) B. PMF spectrum of purified PRP-1 (gene* PRH2*). C. PFF spectrum of the 2,136 Da peptide that is missing in PRP-1. D. Comparison of amino acid sequences of the two acidic PRPs identified. gi|131008|, Salivary acidic proline-rich phosphoprotein 1/2 precursor (gene* PRH2*); gi|88462|, proline-rich phoshoprotein (gene* PRH1*, Db allele).The peptide sequences identified by MALDI-PFF are highlighted in grey.*

Intra-individual and inter-individual comparisons of the 2-D protein patterns of WS and glandular secretions

For comparison of intra-individual variations, WS as well as glandular parotid and SMSL secretions were collected from one individual on three different days. A visual comparison of the Coomassie-stained 2-D gels derived from these samples (Fig. 3A) showed a good

reproducibility of the major spots in WS that was even better when comparing the glandular secretions.

For comparison of inter-individual differences, WS as well as glandular secretions were collected from three additional individuals. Although major spots appeared similarly in all individuals, quantitative differences were observed when comparing the spot patterns of WS (Fig. 3B). An example is albumin (spots 27) that was found in greater amounts in individual 3. These differences were considerably less pronounced when comparing the spot patterns of glandular secretions. Inter-individual variations were most notable in the region of the acidic PRPs (spots 23) in WS and glandular secretions.



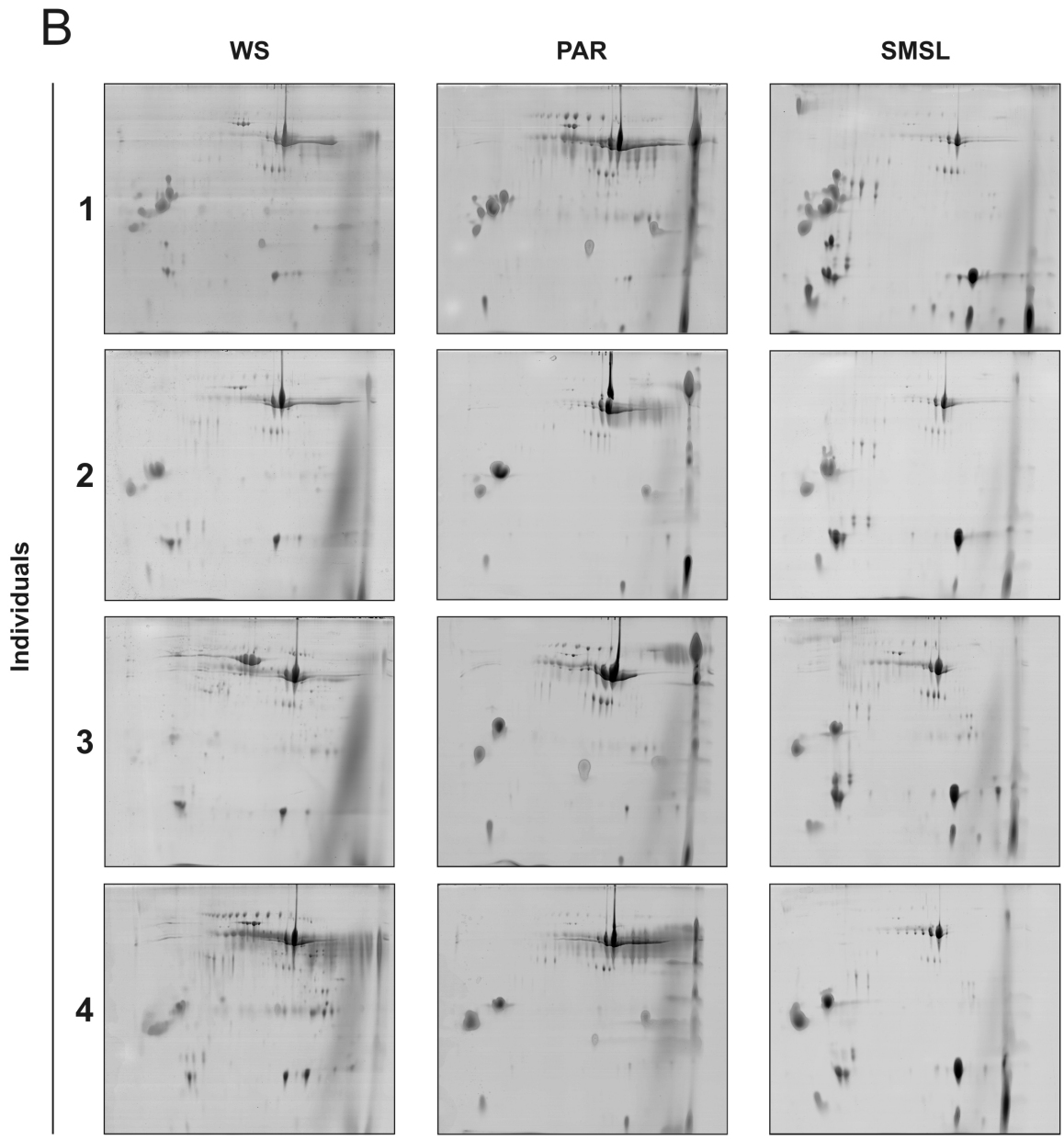


Fig. 3: *Intra- and interindividual comparisons of Coomassie-stained 2-DE patterns of whole saliva (WS), parotid (PAR) and submandibular-sublingual (SMSL) saliva. (**A**) Intraindividual comparison of samples collected at different days. Gels at collection time point 1 are the ones analyzed (identical to Fig. 1). (**B**) Interindividual comparison of samples collected from four different individuals. Individual 1 is the donor analyzed in Fig. 1.*

Discussion

A number of investigations using a combination of 2-DE and MS have been carried out for the study of human saliva [10-17]. Even though an impressive number of proteins could be identified, two major classes of proteins in human saliva, namely the PRPs and the salivary

mucins, had so far escaped identification in WS by a combination of 2-D PAGE and MALDI-MS. In the present study, it was now possible to enlarge the WS protein map and combine it with a comprehensive analysis of separated parotid and SMSL glandular secretions for the display of acidic PRPs, basic PRPs, PRG as well as salivary mucin MUC7. The results show that, with little intra- and inter-individual variations, most components in WS can be explained by mixed contributions of the two major salivary glands with only few components remaining that may be derived from local sources in the oral cavity.

Some spots were readily apparent in WS that could not be detected in glandular salivas, e.g. calgranulin A (spot 2), calgranulin B (spot 3) or transferrin (spots 29). These components may not be derived from the salivary glands but rather from local sources in the oral cavity such as crevicular fluid [35]. Furthermore, some spots were seen in glandular secretions, that could not be detected in WS, e.g. histatin 1 (spots S3), cystatin S (spots S2) or immunglobulins (spots P2, P3). This might in part be explained by the fact that both SMSL and parotid saliva become diluted once they mix with each other. However, for spots as strong as histatin 1 and cystatin S it is unlikely that the effect of dilution would be the only cause for disappearance of these spots in WS. An additional explanation could be that certain proteins selectively form macromolecular aggregates in WS, as has been described for MUC5B [36], and was suggested to lead to the formation of salivary micelles [37]. Such macromolecular aggregates could be lost during sample preparation due to precipitation or filtration, as in the case of WS. Such a loss would be even more likely for proteins if they would bind to or would be bound by bacteria retained in the filter.

The overall qualitative pattern of protein spots showed comparatively little intra-individual variation at different time points of sample collection. This holds particularly true for parotid and SMSL secretions. In WS greater variations were observed, that could be explained by changes in the relative amount of volume contributed by the major salivary glands to mixed saliva but might be also due to other changes in the local environment of the oral cavity. It has to be conceded, however, that in the present study the collection of saliva took place under controlled conditions. Under extreme conditions, such as strong physical activity, emotional stress, disease, or pharmacological influence, more pronounced changes are likely to be expected, as had been shown in past studies (reviewed by [38]). The inter-individual comparison showed a consistent similarity of most major spots. Differences among individuals, seen in WS only, may reflect the momentary status of the local environment in an individual's oral cavity. This was particularly evident for proteins that enter the oral cavity by way of diffusion through the epithelial barrier or through the gingival crevice, such as serum

albumin (spots 27), that was found in high amounts in the 2-D gel of individual 3 (Fig. 3B). Serum albumin is known to be a marker for leakage of plasma proteins into saliva [39] and is related to the level of gingival inflammation [40] which in turn can be related to the degree of oral hygiene [41]. In contrast to WS, the inter-individual comparison of pure glandular secretions showed a higher degree of similarity. Differences seen in the spot pattern of glandular secretions among individuals were most notable for acidic PRPs (spots 23) and in this case might be due to the known genetically determined heterogeneity for this particular family of proteins [34].

The identification of PRPs by MS is complicated by the fact that the arginine/lysine-proline bond is not commonly cleaved by trypsin [42]. Therefore, only one tryptic peptide within the mass range of 700-4,000 Da can be expected for the acidic PRPs. Thus, the PRPs were not identified using the usual database search parameters with trypsin specificity. When allowing the cleavage of the R-P and K-P bond in the database search [43], PRP-1 can theoretically be cleaved into 19 peptides. Advanced bioinformatics for spectra calibration and removal of contaminating peptides were applied prior to database searching in order to increase the significance of the protein identifications achieved. Choosing these parameters, in combination with a comparison to a sample of purified PRP-1, allowed the unambiguous identification of PRPs by MALDI-PMF and MALDI-PFF measurements. Thus, the presence of several acidic and basic PRPs, recently shown in WS by Wilmarth *et al.* using the MudPIT technique [18] was confirmed in the present study by a combination of 2-D PAGE and MALDI-MS and extended by including the analysis of parotid and SMSL glandular secretions.

In agreement with previous knowledge concerning the composition of proteins in WS, it should be expected that various subclasses of PRPs are to be revealed by 2-DE. Among the six PRP genes known, *PRH1* and *PRH2* code for acidic PRPs (*PRH1* for Pa, Db and PIF, *PRH2* for PRP1-4, PIF-f and PIF-s). *PRB1*, *PRB2 and PRB4* code for basic PRPs and *PRB3* codes for PRG. *PRB1 – PRB4* are of the *Bst*NI type which means that the restriction enzyme *Bst*NI cuts the repetitive region of these genes frequently [44]. As a consequence of this genetic heterogeneity and of posttranslational modifications, at least 20 different members of the PRP family should occur in human saliva that show different electrophoretic mobility [45]. In the present study, within the group of acidic PRPs, only the Db protein (gene *PRH1*) could be detected in WS as well as in both glandular secretions. Solely in parotid saliva, a gene product of *PRH2* (spot P5) could be detected but due to analytical limitations it could not be definitively determined to which of the various types (PRP-1, PRP-2, PRP-3, PRP-4,

PIF-s, PIF-f) this protein belongs. Basic PRPs could not be sufficiently separated due to the limitations of isoelectric focusing in the basic region. Nevertheless, a type of basic PRP (*Bst*NI-type, subfamily 1) and PRG (synonyms: *Bst*NI-type, subfamily 3-GI protein) were identified. Notably, in contrast to WS and parotid saliva, these latter proteins could not be identified in SMSL saliva.

Another group of well characterized salivary proteins, that had not been detected by 2-DE in previous reports are the mucins MUC5B and MUC7 [3]. In this study, it was possible for the first time to resolve MUC7 after 2-D PAGE of SMSL saliva. Because MUC7 is predominantly derived from SMSL glands and becomes diluted once it is mixed with parotid saliva it may not anymore be readily detectable after 2-D PAGE of WS. Another possible explanation for the failure to detect MUC7 in WS could be that salivary micelles in WS containing MUC7 [37] might be lost during sample preparation. The fact that MUC5B could not be detected might likely be due to its high molecular weight (> 40 x 10^6 Da), that may prevent it from entering either the IPG-strip or the second dimension gel.

Despite some limitations of 2-DE and, although, novel techniques such as MudPIT provide a considerably larger number of identified proteins, the possibility of a subsequent transfer of a 2-D gel onto a protein-binding membrane combined with immunological or biochemical blotting techniques may allow further insight into the biological functions of certain distinct proteins.

Acknowledgements

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Chapter 5

Identification and Characterization of Binding Properties of *Helicobacter pylori* by Glycoconjugate Arrays

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Abstract

The microaerophilic bacterium *Helicobacter pylori* (*H. pylori*) is well established for its role in development of different gastric diseases. Bacterial adhesins and corresponding binding sites on the epithelial surface allow *H. pylori* to colonize the gastric tissue. In the present investigation, the adhesion of *H. pylori* to dot blot arrays of natural glycoproteins and neoglycoproteins was studied. Adhesion was detected by overlay with fluorescence-labeled bacteria on immobilized (neo)glycoproteins. The results confirmed the interaction between the adhesin BabA and the H-1-, Lewis b-, and related fucose-containing antigens. In addition, *H. pylori* bound to terminal α2-3-linked sialic acids as previously described. The use of a *sabA* mutant and sialidase treatment of glycoconjugate arrays showed that the adherence of *H. pylori* to laminin is mediated by the sialic acid-binding adhesin, SabA. The adhesion to salivary mucin MUC5B is mainly associated with the BabA adhesin, and to a lesser extent with the SabA adhesin. This agrees with reports, that MUC5B carries both fucosylated blood group antigens and α2-3-linked sialic acids. The adhesion of *H. pylori* to fibronectin and lactoferrin persisted in the *babA/sabA* double mutant. Because binding to these molecules was abolished by denaturation rather than by deglycosylation, it was suggested to depend on the recognition of unknown receptor moieties by an additional unknown bacterial surface component. The results demonstrate that the bacterial overlay method on glycoconjugate arrays is a useful tool for exploration and characterization of unknown adhesin specificities of *H. pylori* and other bacteria.

Introduction

The spiral, gram-negative bacterium *Helicobacter pylori* (*H. pylori*) persistently colonizes the gastric mucosa of 50% of the human population and is associated with the induction of chronic type B gastritis, peptic ulceration, and the development of gastric carcinoma and MALT (mucosa-associated lymphoid tissue)-lymphoma (for a review see [1]). Close association of *H. pylori* with gastric epithelial cells [2] and the mucin layer covering the epithelial surface [3, 4] is believed to facilitate the permanent colonization of the stomach by this bacterium. An intimate association with the epithelial cells allows the bacteria to encroach upon signal transduction processes, in particular, by action of the so-called Cag type IV secretion system [5, 6], resulting in rearrangement of the cytoskeleton [7] and the induction of proinflammatory cytokines [8].

In the past, biochemical as well as genetic approaches have been chosen to identify bacterial factors involved in the adherence process of *H. pylori* to gastric cells. By using affinity purification and overlay assays, a subset of putative receptors could be identified, including fucosylated, sialylated, or sulphated oligosaccharides, glycolipids, glycoproteins, mucins, and lipid-like compounds [9-16]. Recently, two major receptor structures and their corresponding adhesins were characterized in more detail. One is a fucosylated oligosaccharide structure present both in the H-1 and Lewis b blood group antigens (blood group O phenotype) [10, 17] that was identified as a receptor motif for the *H. pylori* outer membrane protein (OMP) BabA, the blood group antigen binding adhesin [18]. Another is the sialyl-Lewis x antigen, that is recognized by the OMP SabA, the sialic acid-binding adhesin [19]. The fucosylated blood group antigens are highly expressed in gastrointestinal epithelium which favors colonization of the gastric mucosa. The sialyl-Lewis x antigen is described to be predominantly expressed in inflamed tissues and might promote the chronicity of the infection process once gastritis is established [19].

Beside these well-characterized interactions, other eucaryotic receptors and bacterial adhesin candidates have been described, suggesting the enrolment of additional interactions that are not fully explored yet. In this regard, extracellular matrix (ECM) proteins, such as laminin and collagen type IV, have been proposed as receptors for *H. pylori* [20, 21]. In addition, surface-exposed components of *H. pylori* such as the OMPs AlpAB [22, 23] and HopZ [24] as well as Lewis x structures in the O-antigen side chain of the lipopolysaccharide [25] have been shown to be involved in adherence to gastric cells.

Recently, arrays of immobilized purified glycoproteins and neoglycoproteins have been successfully used to explore the adhesin specificities of oral actinomyces and viridans streptococci [26, 27]. These arrays are well suited to assess bacterial adhesin specificities because both the naturally occurring glycoproteins and related oligosaccharide structures, presented on neoglycoproteins, can be compared for receptor activity and for exploration of the minimal oligosaccharide motifs necessary for binding. This method has now been adapted using a set of *sabA*- and *babA*-deficient fluorescence-labeled mutants of *H. pylori* to investigate a wider range of natural and synthetic glycoproteins for receptor activity.

Material and Methods

Bacteria and growth conditions

The wild-type *H. pylori* strain J99 [28] and its isogenic mutants in the *sabA* and/or *babA* genes [19] were grown for 48-72 h at 37°C in a microaerophilic atmosphere on Wilkins-Chalgren agar (Oxoid, Wesel, Germany) containing 10% horse blood, Dent supplement (Oxoid, Wesel, Germany) and 0.4 g KNO_3 per liter. *Streptococcus gordonii* strain DL1 (Challis) and the sialic acid binding deficient mutant strain D102, kindly provided by Yukihiro Takahashi (The Nippon Dental University School of Dentistry at Tokyo, Japan) were grown in complex medium as previously described [29].

SDS-polyacrylamide gel electrophoresis and immunoblot

For immunoblot analysis the bacteria were collected from agar plates and suspended in 300 µl sample solution [30]. Boiled aliquots were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% acrylamide gel using a mini-gel apparatus (Biorad, München, Germany) and blotted onto nitrocellulose membranes at 1 mA/cm^2 using a semi-dry blot system (Biotec Fischer, Reiskirchen, Germany). The membranes were blocked with 3% BSA in Tris-buffered saline (TBS) (50mM Tris-HCl, pH 7.5; 150 mM NaCl) and incubated with antisera AK277 (anti-BabA) [31] or AK278 (anti-SabA) for at least 2 h (1:10,000 dilution). Alkaline phosphatase-coupled protein A was used to visualize the bound antibody by decomposition of nitroblue tetrazolium.

Glycoproteins and neoglycoproteins

Dry nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA, pore size 0.4 µm) were spotted with 1 µl volumes containing 1 µg of glycoproteins or neoglycoproteins.

***Table 1**: Neoglycoproteins used in this study*

Sugar chains	Neoglycoprotein conjugates
Lacto-*N*-tetraose	Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[a]
Lewis a	Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[a]
H-1	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[a]
Lewis b	Fucα1-2Galβ1-3[Fucα1-4]GlcNACβ1-3Galβ1-4(Glc)-APD-HSA[a]
A-Trisaccharide	GalNAcα1-3[Fucα1-2]Galβ1-O-APE-HSA[a]
sialyl-Lewis a	Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[a]
Lacto-*N*-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[a]
Lewis x	Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[a]
H-2	Fucα1-2Galβ1-4GlcNAcβ-O-APE-HSA[a]
Lewis y	Fucα1-2Galβ1-4[Fucα1-3]GlcNAcβ1-O-APE-HSA[a]
B-Trisaccharide	Galα1-3[Fucα1-2]Galβ1-O-APE-HSA[a]
sialyl-Lewis x	Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[a]
Lactose	Galβ1-4Glcβ-O-10 Å spacer-BSA[b]
6'-Sialyllactose	Neu5Acα2-6Galβ1-4(Glc)-APD-HSA[a]
3'-Sialyllactose	Neu5Acα2-3Galβ1-4(Glc)-APD-HSA[a]
2'-Fucosyllactose	Fucα1-2Galβ1-4(Glc)-APD-BSA[a]
3'-Sialyl-3-fucosyllactose	Neu5Acα2-3Galβ1-4[Fucα1-3]Glc-3 atom spacer -BSA[c]
N-Acetyllactosamine	Galβ1-4GlcNAc-3 atom spacer - BSA[c]
3'-Sialyl-*N*-acetyllactosamine	Neu5Acα2-3Galβ1-4GlcNAc-3 atom spacer-BSA[c]

a IsoSep AB, Tullinge, Sweden (10-20 mol oligosaccharides/mol HSA/BSA)

b Glycorex AB, Lund, Sweden (20 mol oligosaccharides/mol BSA)

c Dextra-Laboratories Ltd, Reading, UK (7-11 mole oligosaccharides/mol BSA)

The glycoproteins used were: fetuin (Calbiochem, Bad Soden, Germany), asialofetuin (Sigma), glycophorin A (Sigma), asialoglycophorin (Sigma), laminin (from human placenta, Sigma), MUC5B (kindly provided by M. J. Levine, Department of Oral Biology, SUNY, Buffalo, NY), transferrin (Sigma), fibronectin (from human plasma, Sigma), and lactoferrin (from human milk, Sigma). The neoglycoproteins used are listed in Table 1. Human serum albumin (HSA, fraction V, Sigma) and bovine serum albumin (BSA, fraction V, immunoglobulin-free, protease-free, Sigma) were included as negative (non-glycosylated) controls.

Pretreatment of dot blot arrays

For sialidase treatment, membranes were incubated with 0.1 U/ml of sialidase (from *Clostridium perfringens* type X, Sigma) in TBS containing 5% BSA (fraction V, Sigma), 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1% sodium azide at 37°C before overlay. For denaturation of spotted proteins, membranes were treated with 0.1% SDS (Merck, Darmstadt, Germany) in 20 mM sodium phosphate buffer (pH 7.2) containing 50 mM β-mercaptoethanol (Merck) at 100°C for 5 min. For *N*-glycosidase F digestion, 0.05 U/ml of recombinant Glyko® *N*-glycanase from *Chryseobacterium meningosepticum* (PROzyme, San Leandro, CA, USA) and 0.75% NP-40 (PROzyme, San Leandro, CA, USA) were added after denaturation and further incubation was carried out overnight at 37°C. All enzymatic pre-treatments of membranes were performed in sealed plastic bags.

Bacterial overlay

The method was performed as previously described [32] except that fluorescence labeled bacteria were used as probes. Bacteria at 1 x 10^8/ml in PBS were fluorescein labeled by incubation with fluorescein-5-isothiocyanate (Molecular Probes, Oregon, USA) at 100 µg/ml for 30 min at room temperature. Untreated and pretreated membranes were blocked in TBS containing 5% BSA (fraction V, Sigma), 1 mM $CaCl_2$, 1 mM $MgCl_2$ for 2 h at room temperature. Labeled bacteria were recovered by centrifugation at 900 x g for 7 min, resuspended in 10 ml blocking buffer and added to a final concentration of 2,5 x 10^7 bacteria in a total volume of 40 ml (about 1 ml of bacterial suspension per cm^2 of nitrocellulose membrane). The overlays were incubated for 30 min at 4°C in the dark without mixing and washed three times at room temperature for 5 min on a rotary shaker in TBS containing 0,05% Tween-20, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The fluorescence of adherent bacteria was detected by a Typhoon imaging system (Typhoon 9200, Amersham Biosciences, Freiburg, Germany).

Chemical labeling of glycoconjugates

For the oxidation of carbohydrates, nitrocellulose membranes with (neo)glycoproteins immobilized were incubated for 30 min in acetate buffer (0.1 M, pH 5.5) with 10 mM sodium periodate (ICN Biomedicals, Aurora, OH) at room temperature in the dark. After washing in PBS, membranes were incubated for 1 h at room temperature in acetate buffer containing 100 µg/ml biotin-LC-hydrazide (Pierce). The membranes were washed three times with TBS and were then blocked in TBS containing 3% BSA (Sigma) for 1 h at room temperature. The

membranes were subsequently incubated for 30 min in the dark with 5 mg fluorescein avidin-D (Vector Laboratories, Inc., Burlingame, CA) per ml in blocking buffer. Membranes were washed three times in TBS containing 0.1% Tween-20 and once in TBS. Fluorescent signals were detected by a Typhoon imaging system (Typhoon 9200, Amersham Biosciences).

Lectin blotting

Membranes were blocked for 1 h at room temperature with TBS containing 2% polyvinyl alcohol (average molecular weight 30,000 - 70,000, Sigma), 0.1% Tween 20, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Membranes were subsequently incubated for 1 h at room temperature in the dark with fluorescein-labeled UEA I (Vector Laboratories, Inc., Burlingame, CA) and fluorescein-labeled LTA (Sigma) at concentrations of 5 µg per ml in blocking buffer [32] The blots were washed three times in TBS containing 0.1% Tween-20 and the fluorescence of bound lectins was detected by a Typhoon imaging system (Typhoon 9200, Amersham Biosciences).

Aminogroup detection

Untreated and pre-treated membranes were washed three times in borat buffer pH 9.7 containing 0.05 M $Na_2B_4O_7$ x 10 H_2O (Merck) and 0.2% Tween 20 and then incubated for 1 h with 100 µg/ml of sulfo-NHS-LC-biotin (Pierce, Rodeford, IL). After washing two times in borat buffer and two times in TBS containing 0.1% Tween-20, the membranes were incubated for 30 min in the dark with 5 mg/ml fluorescein avidin-D (Vector Laboratories) in TBS containing 0.1% Tween-20. Membranes were washed again three times in TBS containing 0.1% Tween-20 and fluorescent signals were detected by a Typhoon imaging system (Typhoon 9200, Amersham Biosciences).

Results

Characterization of adhesin expression in H. pylori *mutants*

Using specific antisera directed against BabA and SabA, respectively, the expression of these OMPs was verified on *H. pylori* J99 wild-type (wt) (Fig. 1). The BabA and SabA proteins could be detected with apparent molecular weights of ~80 kDa and 70 kDa, respectively. The BabA protein was expressed in the J99 *sabA* mutant but not in the J99 *babA* and J99 *babA/sabA* mutants. The expression of the SabA protein could be detected in the J99 *babA* but not in J99 *sabA* or J99 *babA/sabA* mutants. These data confirm the correct mutagenesis of the desired genes in each mutant strain.

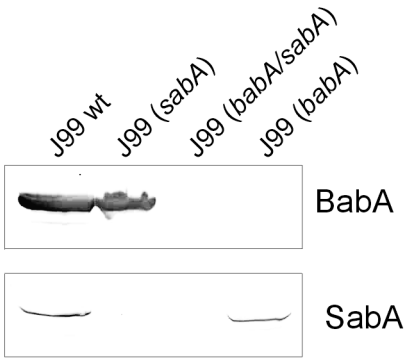

Fig. 1*: The characterization of adhesin expression in* Helicobacter pylori *J99 wild-type (wt) and adhesin-deficient isogenetic mutants. Protein lysates from* H. pylori *J99 wt, J99* sabA *mutant, J99* babA *mutant, and J99* babA/sabA *double mutant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and western transfers were incubated with anti-BabA and anti-SabA antisera. Bound antibodies were detected with alkaline phosphatase-coupled protein A and nitroblue tetrazolium color reaction.*

Validation of glycoconjugate array

To verify that proteins were properly immobilized on the nitrocellulose membrane and are glycosylated, the incubation of blots with 10 mM sodium periodate for oxidation of carbohydrates and subsequent incubation with biotin-LC-hydrazide was performed. The result confirmed that each protein spot on the array carries sugar chains with the exception of BSA and HSA, that were included as internal negative controls (Fig. 2A).

A further characterization of carbohydrates was performed by lectin blotting with *Ulex europaeus* agglutinin (UEA-I) and *Lotus tetragonolobus* agglutinin (LTA) that both bind to α-L-fucose and recognize the H-2 trisaccharide epitope [33, 34] (Fig. 2B, C).

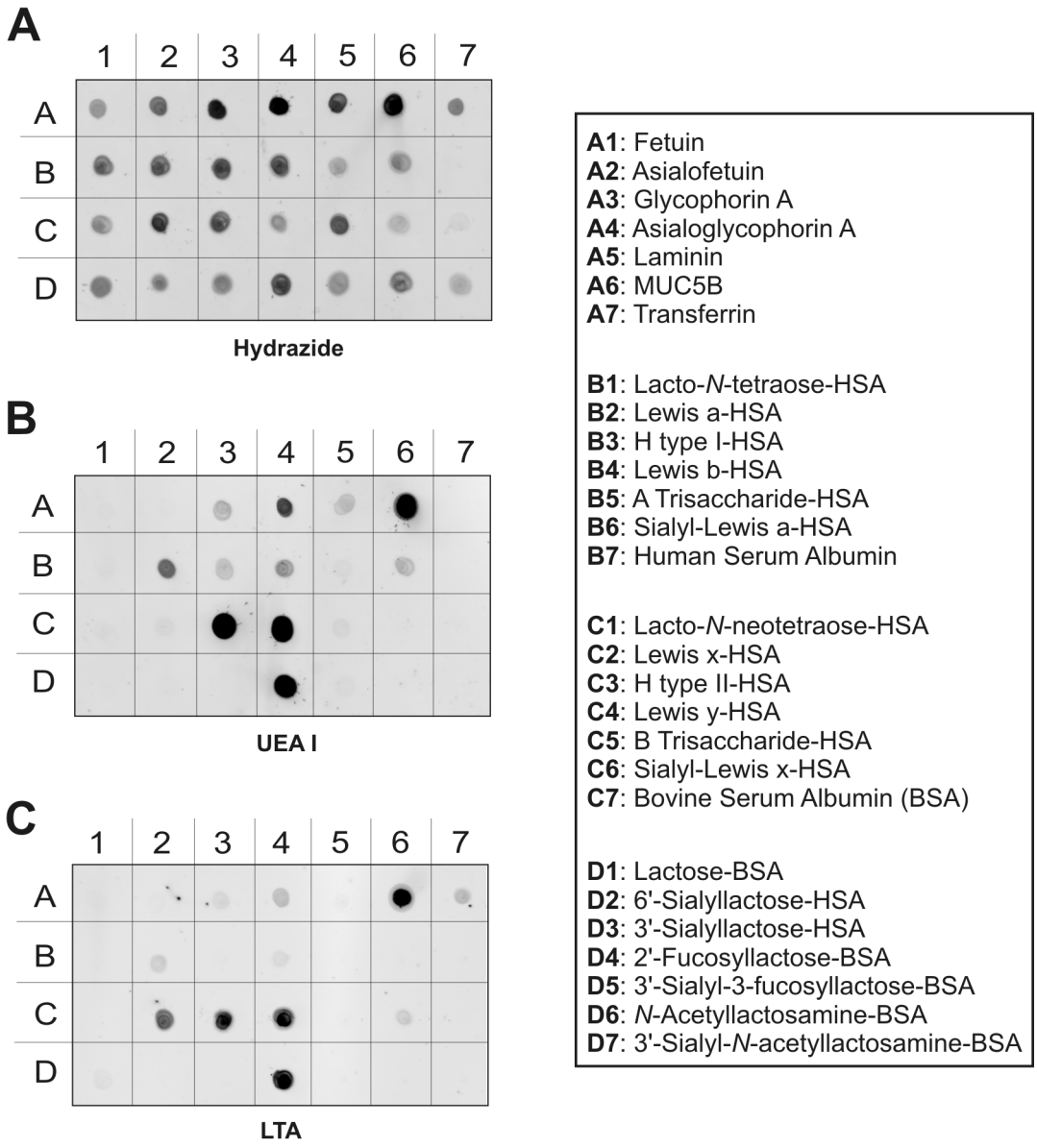

***Fig. 2**: Chemical labeling of carbohydrates on glycoconjugate arrays and recognition by fucose-specific plant lectins. Carbohydrates on (neo)glycoproteins spotted on nitrocellulose (1 µg per spot) were (A) oxidized by sodium periodate before labeling with biotin-LC-hydrazide and the detection by avidin-D fluorescein-5-isothiocyanate (FITC) or incubated with (B) FITC-conjugated UEA-I and (C) FITC-conjugated LTA. Bound lectins were detected by a fluorescence scanner. The locations of spotted (neo)glycoproteins are indicated below.*

In previous studies UEA-I was used for detection of receptor motifs recognized by *H. pylori* [35]. In the present study, both lectins bound strongly to H-2-, Lewis y-, and 2'-fucosyllactose-carrying neoglycoproteins. LTA but not UEA-I recognized also the Lewis x-antigen (dot C2). Weaker binding was noted to fucosylated blood group type 1 chains that were recognized by UEA-I and LTA to different extents. Strong binding of both lectins was

found to MUC5B (dot A6), indicating the presence of α-L-fucose on this glycoprotein as expected from the presence of type 2 human blood group determinants on this molecule [36].
To further validate the glycoconjugate overlay method, *Streptococcus gordonii* DL1, expressing an adhesin (Hsa) specific for α2-3-linked sialic acids [29, 37], was used as a probe. This strain bound strongly to fetuin, glycophorin A, laminin, and 3'-sialyllactose (Fig. 3A). Strain D102, which lacks the sialic acid binding adhesin, failed to bind any component on the glycoconjugate array (Fig. 3B).

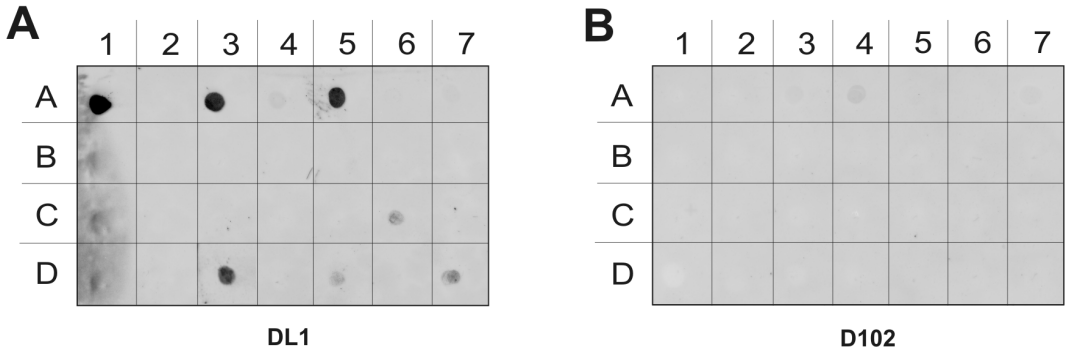

Fig. 3*: The adhesion of fluorescein-5-isothiocyanate (FITC)-labeled* Streptococcus gordonii *DL1 (A) and mutant strain D102 (B) to glycoconjugate arrays. (Neo)glycoproteins spotted on nitrocellulose (1 μg per spot) were overlaid with FITC-labeled bacteria and adherent bacteria were detected by a fluorescence scanner. The locations of spotted (neo)glycoproteins are the same as outlined in legend to Fig. 2.*

Lectin-dependent H. pylori *binding to natural and synthetic glycoproteins*

Binding characteristics of *H. pylori* adhesins were determined by comparing the binding of wt strain J99 with *babA*- and *sabA*-deficient mutant strains (Fig. 4). J99 wt bound to fetuin, glycophorin A, laminin, MUC5B, sialyl-Lewis a, sialyl-Lewis x, 3'-sialyllactose, 3'-sialyl-3-fucosyllactose, and 3'-sialyl-*N*-acetyllactosamine (Fig. 4A). These glycoproteins were recognized also by the J99 *babA* mutant strain (Fig. 4B). No binding of any *H. pylori* strain to 6'-sialyllactose (dot D2) could be detected. Strong binding of J99 wt was noted to H-1- and Lewis b-containing neoglycoproteins (Fig. 4A). These two determinants were also recognized by the J99 *sabA* mutant (Fig. 4C). Binding to MUC5B (dot A6) was still detected with both, the *babA* and the *sabA* mutant, the latter, however, showing a stronger signal. The J99 *babA/sabA* double mutant failed to bind to any component on the glycoconjugate array (Fig. 4D).

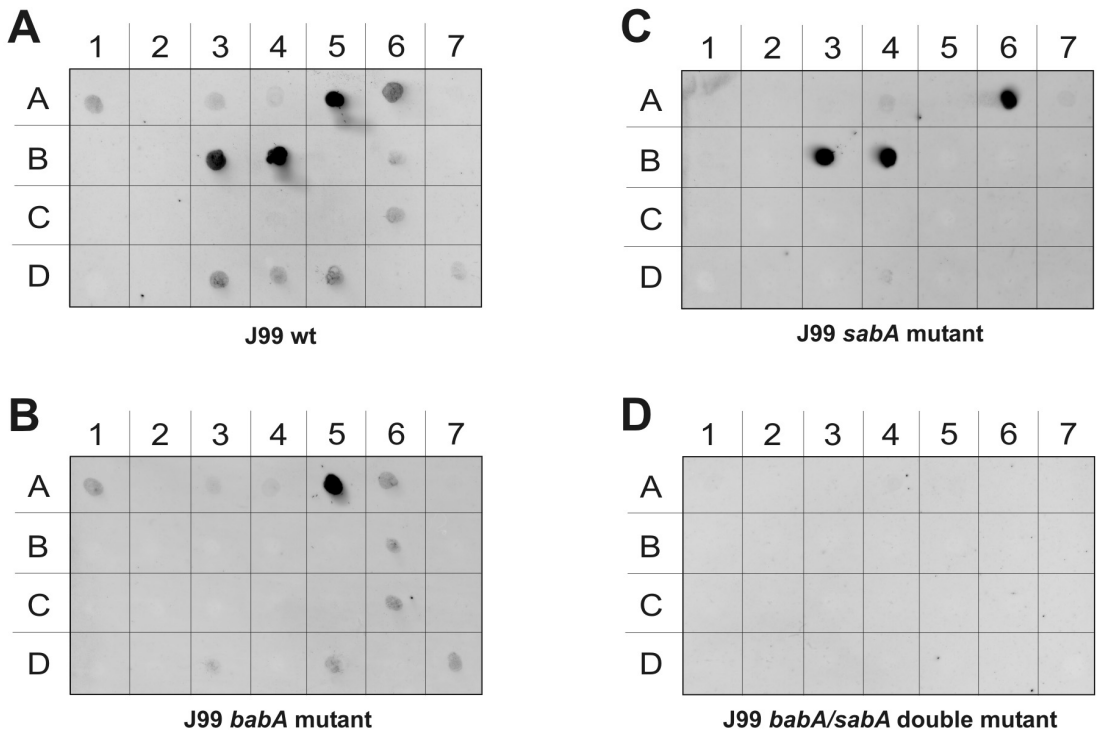

***Fig. 4**: The adhesion of fluorescein-5-isothiocyanate (FITC)-labeled* Helicobacter pylori *wild-type (wt) strain J99 (A),* babA *mutant strain (B),* sabA *mutant strain (C), and* sabA/babA *double mutant strain (D) to glycoconjugate arrays. (Neo)glycoproteins spotted on nitrocellulose (1 µg per spot) were overlaid with FITC-labeled bacteria and adherent bacteria were detected by a fluorescence scanner. The locations of spotted (neo)glycoproteins are the same as outlined in the legend to Fig. 2.*

To further confirm the sialic acid-dependency of SabA-mediated binding, blots were incubated with sialidase prior to overlay with bacteria (Fig. 5). Sialidase treatment abolished binding of J99 wt and the *babA* mutant to fetuin, laminin and sialic acid-containing neoglycoproteins (Fig. 5A, B), resulting in a binding pattern analogous to the *sabA* mutant (Fig. 4C). Residual binding of the *babA* mutant to MUC5B (Fig. 5C) was completely abolished by pre-treatment with sialidase (Fig. 5D).

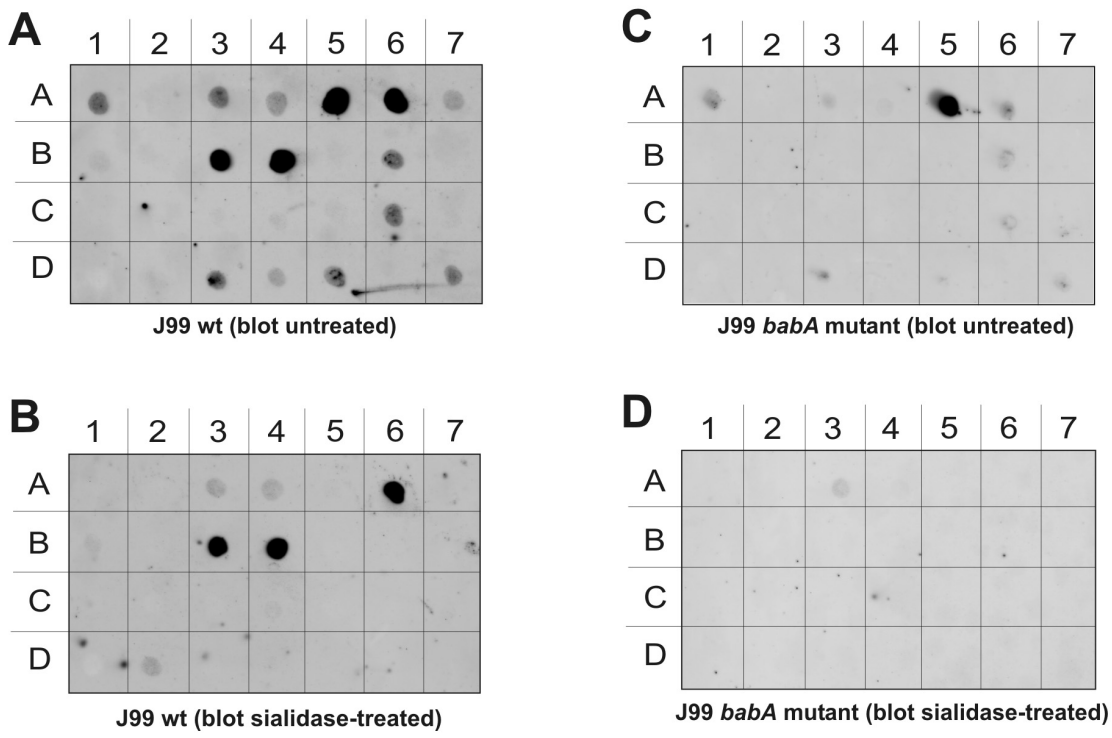

Fig. 5*: The adhesion of fluorescein-5-isothiocyanate (FITC)-labeled* Helicobacter pylori *wild-type (wt) strain J99 (A, B) and J99* babA *mutant strain (C, D) to glycoconjugate arrays. (Neo)glycoproteins spotted on nitrocellulose (1 µg per spot) were overlaid with FITC-labeled bacteria and adherent bacteria were detected by a fluorescence scanner. Blots remained untreated (A, C) or were sialidase treated (B, D) before the addition of bacteria. The locations of spotted (neo)glycoproteins are the same as outlined in the legend to Fig. 2.*

BabA- and SabA-independent binding of H. pylori *to fibronectin and lactoferrin*

In the course of testing additional glycoproteins as putative receptor candidates for *H. pylori*, adhesion to fibronectin and lactoferrin was noticed (Fig. 6). Remarkably, this was observed not only with the wt strain but also with the *babA/sabA* double mutant, suggesting that a different unknown bacterial surface component on *H. pylori* might be involved in this interaction. To map the receptor motif in these glycosylated proteins, the protein structure was denatured by sodium dodecyl sulfate (SDS) and heat treatment or the sugar residues were removed by treatment with *N*-glycosidase F. Following only denaturation, the adhesion to fibronectin and lactoferrin disappeared in both J99 wt and the *babA/sabA* double mutant. However, denaturation did not influence the SabA-dependent binding of J99 wt to laminin. Binding of J99 wt to laminin only disappeared, when denatured membranes were treated with

N-glycosidase F, confirming lectin-like interaction in this recognition process. Aminogroup detection was used to control that denaturation did not result in loss of immobilized proteins.

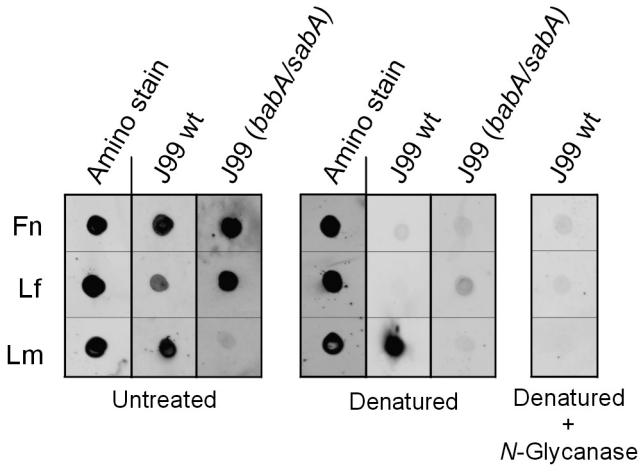

***Fig. 6**: SabA and BabA-independent binding of* Helicobacter pylori *to lactoferrin and fibronectin. Fibronectin (Fn), lactoferrin (Lf), and laminin (Lm) were spotted on nitrocellulose membranes (1 µg per spot) and were stained for amino groups by labeling with NHS-LC-biotin and detection by avidin-D FITC as well as for bacterial binding of FITC-labeled* H. pylori *wild-type strain (wt) J99 or* babA/sabA-*double mutant strain. Fluorescent signals were detected by a fluorescence scanner.*

Discussion

Numerous adhesive properties of *H. pylori* have been described, including hemagglutination, attachment to epithelial cells and binding to distinct receptors, such as oligosaccarides or proteins of the basement membrane [20, 38]. The aim of this study was to establish a screening system for exploration of novel receptor-adhesin interactions in *H. pylori*. The results of this study confirmed the binding specificity of *H. pylori* adhesin BabA to H-1-, Lewis b-, and Lewis b-related oligosaccharide determinants as well as the specificity of adhesin SabA to sialyl-Lewis x and sialyl-Lewis a containing oligosaccharides [10, 19]. Preferential binding of *H. pylori* to α2-3-linked sialic acid [13] was attributed to the SabA adhesin. In addition, the SabA adhesin was found sufficient to explain binding to laminin that had been previously observed [20]. Binding of *H. pylori* to salivary mucin MUC5B that also had been described [16] was now shown to be predominantly mediated by the BabA adhesin and to a lesser degree also by the SabA adhesin. Only the binding to fibronectin and lactoferrin could not be explained by the activities of the SabA or BabA adhesins. Because binding of *H. pylori* to these glycoproteins was abolished by denaturation rather than by

deglycosylation, it is proposed to depend on recognition of unknown protein moieties by an additional adhesive surface structure on *H. pylori*.

For the validation of the glycoconjugate array, lectins (UEA-I and LTA) were chosen that exhibit binding specificity for α-L-fucose [35] and in this respect are similar to the *H. pylori* BabA adhesin that is known to bind to the Lewis b antigen, an oligosaccharide structure containing terminal α1-2-linked-fucose [18]. The minimal structure in the glycoconjugate array for recognition by UEA-I was shown to be Fucα1-2Gal, present in both type 1 and type 2 chains of blood groups, but a preference for the H type 2 trisaccharide epitope (Fucα1-2Galβ1-4GlcNAc) was noticed that is in agreement with earlier reports [33, 34]. Strong binding to 2'-fucosyllactose confirmed that the *N*-acetyl group of type 2 chains is not necessary for binding [34]. Analogous to UEA-I, the binding specificity of LTA for H-2 antigen [34] could be confirmed. A significant difference between UEA-I and LTA was the additional recognition of the Lewis x antigen (Galβ1-4[Fucα1-3]GlcNAc) by LTA but not by UEA-I, that is also in agreement with previous reports [39]. So far, UEA-I had been used as a probe to identify potential receptors for *H. pylori* [35]. However, the results of this investigation demonstrate, that UEA-I recognizes a broader range of fucose-containing receptors than the *H. pylori* BabA adhesin. This becomes particularly evident from the finding that, in contrast to UEA-I which strongly bound 2'-fucosyllactose, *H. pylori* J99 wt and the *sabA* mutant showed only weak binding. Thus, although terminal Fucα1-2Gal might be sufficient for binding of *H. pylori*, significantly stronger binding occurs to the Fucα1-2Galβ1-3GlcNAc motif found in H-1 and Lewis b antigens.

In the past, two possible explanations for the binding of *H. pylori* to laminin were proposed. First, a lectin-like interaction of the bacterium with terminal sialic acids on laminin [40] and second, an interaction of *H. pylori* lipopolysaccharides with laminin [41]. Our results clearly show that a lectin-like interaction of the SabA adhesin with terminal sialic acid is responsible for binding to laminin. Thus, strong binding to laminin that was detectable with the J99 wt strain disappeared in the *sabA*-deficient mutant (Fig. 4) as well as after preincubation of the membranes with sialidase (Fig. 5). These findings expand the binding activities of the SabA adhesin beyond the previously reported recognition of sialyl-Lewis blood group antigens [19]. A broader recognition of terminal sialic acid-containing oligosaccharides is supported by the finding that the *sabA* mutant failed to bind to fetuin, glycophorin, 3'-sialyllactose-HSA, 3'-sialyl-3-fucosyllactose-BSA, and 3'-sialyl-*N*-acetyllactosamine-BSA. Because 6'-sialyllactose-HSA was not recognized as a receptor, it is

proposed that the previously found binding activity of *H. pylori* to terminal α2-3-linked sialic acid [13] can be attributed to the SabA-adhesin. In this respect, the binding specificity of the SabA adhesin to laminin appears similar to the sialic acid-binding adhesin of *Streptococcus gordonii* DL1 that also exhibits a preference for α2-3-linked sialic acids [29]. Interestingly, the elucidation of the oligosaccharides on laminin had revealed only the presence of terminal α2-3- but not α2-6-linked *N*-acetyl neuraminic acid [42]. This might explain stronger binding of *H. pylori* to laminin than to fetuin, the latter carrying both α2-3- and α2-6-linked *N*-acetyl neuraminic acids [43, 44].

Binding of *H. pylori* to salivary mucin MUC5B, that had been previously reported [16], could now be confirmed by binding of wt strain J99. Notably, both the *sabA* and the *babA* mutants still bound to MUC5B whereas the *sabA/babA* double mutant failed to bind. Strong binding of the *sabA*-deficient mutant to MUC5B indicates the importance of H-1-, Lewis b-, and Lewis b-related oligosaccharide epitopes for the recognition of this mucin by the BabA adhesin. Analysis of glycosylation had shown the presence of these oligosaccharide determinants on MUC5B [36]. Binding of the *babA*-deficient mutant to MUC5B was weaker and suggests an additional involvement of the SabA adhesin with corresponding terminal sialic acids on this molecule. This was further confirmed by pretreatment of the membranes with sialidase that removed the residual binding of the *babA*-deficient mutant but not of the wt strain J99 to MUC5B (Fig. 5). These data suggest that binding to MUC5B can solely be explained on the basis of both the BabA and the SabA adhesin. The requirement of an additional adhesin recognizing sulfated oligosaccharide structures, as previously proposed [16], could not be supported based on the present data. Because the recognition of various sialic acid-containing oligosaccharides differs between the SabA adhesin of *H. pylori* and the Hsa adhesin of *S. gordonii* DL1 (Figs. 3 and 4), sub-terminal sugars to α2-3-linked sialic acid seem to be involved in recognition. This becomes particularly apparent for MUC5B that is bound by *H. pylori* but not by *S. gordonii* DL1. Binding of *H. pylori* to MUC5B, a human salivary mucin, may enable *H. pylori* to colonize the oral cavity [16] which may in turn have implications for oral transmission of this pathogen [45].

Binding of *H. pylori* to lactoferrin had been previously described and was attributed to either a 60 kDa heat shock protein [46] or a 70 kDa lactoferrin-binding OMP of *H. pylori* [47]. For the 60 kDa heat shock protein, it was suggested that carbohydrate moieties of lactoferrin were involved in binding [46]. In this investigation, however, it could be demonstrated that binding of *H. pylori* to lactoferrin is not dependent on BabA or SabA activities because the *babA/sabA* double mutant still bound to this protein. Thus, the presence

of an additional binding activity on *H. pylori* has to be hypothesized. Analogous to lactoferrin, binding of *H. pylori* to fibronectin, an ECM component, was also independent of BabA or SabA activities, clearly distinguishing it from the SabA-dependent binding to laminin, another protein of the ECM. The fact that denaturation rather than deglycosylation of both lactoferrin and fibronectin abolished binding of *H. pylori* to these components, suggested that protein moieties rather than carbohydrates might play a role in receptor recognition. ECM proteins such as fibronectin, laminin or vitronectin are involved in integrin-mediated signal transduction pathways that regulate cellular processes including actin rearrangements, cell cycle regulation or survival of cells [48]. Several pathogenic bacteria such as *Staphylococcus aureus* or *Neisseria gonorrhoeae*, have learned to exploit this signaling network to invade epithelial cells by bridging fibronectin-binding proteins to β1-integrins on the epithelial surface [49, 50]. Because evidence for *H. pylori* invasiveness has been described [51, 52] but the mechanism of entry is not known, it will be interesting to identify the fibronectin-binding component on *H. pylori*.

The high specificity and reliability of the current overlay method as well as the simple handling, in combination with well defined bacterial mutants, may allow future analysis of complex mixtures of, for example, gastric epithelial cell membranes or salivary proteins for identification of natural receptors for *H. pylori* adhesion.

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Chapter 6

A Novel Approach for Identification of Receptors in Human Saliva for the BabA and SabA Adhesins of *Helicobacter pylori* by Bacterial Overlay in Combination with Proteomics Techniques

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Abstract

Because gastric infection by *Helicobacter pylori* (*H. pylori*) takes place via the oral route, possible interactions of this bacterium with salivary proteins could be of pathogenic importance. By using a combination of bacterial overlay and proteomics techniques, the interactions of *H. pylori* adhesins BabA and SabA with the whole range of salivary proteins were explored. Proteins of human whole saliva and glandular secretions were separated by one-dimensional and two-dimensional gel electrophoresis and transferred onto nitrocellulose. Adhesion of *H. pylori* strain J99 to transferred salivary proteins was detected by bacterial overlay with fluorescence-labeled organisms. Receptor molecules were identified by either matrix-assisted laser desorption/ionization mass spectrometry or by comparison with the proteome maps of a preceding study. Binding of *H. pylori* to MUC5B, MUC7 and gp-340 was confirmed and by use of isogenic adhesin-deficient mutants the adhesins responsible for binding could be revealed. Adhesion to MUC7 and gp-340 could be associated with the SabA and BabA adhesins, respectively, whereas binding to MUC5B was associated with both adhesins. Binding of *H. pylori* to the proline-rich glycoprotein was newly detected and assigned to the activity of the BabA adhesin whereas the SabA adhesin was found responsible for binding to other newly detected receptor molecules, including carbonic anhydrase VI, secretory component, parotid secretory protein and zinc-α_2-glycoprotein. Interactions between *H. pylori* surface adhesins and salivary receptors may modify the pathogenic properties of this organism. The establishment of the two-dimensional overlay technique represents an useful supplement in the adhesion studies of bacteria with complex protein mixtures.

Introduction

Adhesins of pathogenic bacteria are considered to be virulence factors important for initial binding and subsequent colonization of host tissues as well as for triggering a host response [1]. There is a need for techniques that allow to identify the corresponding host receptors for such bacterial adhesins both on the tissue surface and in the complex biological fluids coating most internal surfaces. A bacterium for which the importance of adhesin-receptor interactions has been well documented is the gastric pathogen *Helicobacter pylori* (*H. pylori*). More than 50% of all people worldwide are infected by this organism [2], that causes type B gastritis, gastric and duodenal ulcers and is involved in the development of gastric adenocarcinoma [3, 4].

Because the infection is acquired by oral ingestion of the bacterium [4], it is likely that, while being ingested, *H. pylori* may interact with human saliva, a predominant component in the oral cavity. Between 0.5 and 1.5 liters of saliva are produced per day [5] most of it being swallowed and ending up in the stomach [6, 7] and a smaller part being expectorated. Thus, it is not unlikely that saliva and its constituents could potentially influence *H. pylori* infectivity in the stomach or might function as a vehicle for a possible oral-to-oral transmission. In this regard, it is noteworthy that several studies suggest a transient or even permanent colonization of *H. pylori* in the oral cavity (reviewed by [8]).

A putative relevance of saliva for *H. pylori* pathogenesis is also supported by recent investigations that have shown adhesive interactions of *H. pylori* with salivary proteins. The inhibitory action of crude bovine and human salivary mucins on *H. pylori* haemagglutination has been known for a long time [9, 10]. More recently, adhesive interaction of *H. pylori* with human salivary mucin MUC5B (MG1) could be demonstrated by ELISA and by immunoblotting adsorbed salivary proteins eluted from the *H. pylori* surface [11, 12]. This interaction was confirmed by a study of Prakobphol *et al*. in which direct binding of *H. pylori* to MUC5B and also to the other major salivary mucin MUC7 (MG2) was shown by bacterial overlay and was suggested to be associated with the expression of Lewis blood group antigens on these glycoproteins [13]. In addition, the same group reported that the salivary agglutinin which is identical to the lung scavenger receptor cysteine-rich protein (gp-340) caused aggregation of *H. pylori*, could be eluted from the surface of this organism [14], and mediated binding of *H. pylor*i to nitrocellulose transfers of salivary proteins separated by SDS-PAGE [13].

It is known that carbohydrate motifs are important receptors for adhesin-mediated colonization of gastric tissues by *H. pylori* organisms [15]. Best characterized among many reported adhesins are the blood group antigen-binding adhesin BabA [16, 17] and the sialic acid-binding adhesin SabA [18] that belong to the family of outer membrane proteins (OMP). Carbohydrate receptor motifs recognized by the BabA adhesin include H-1-, Lewis b- and related fucose-containing blood group antigens whereas the SabA adhesin binds preferentially to sialyl-Lewis x antigen and more general to α2-3-linked sialic acid termini [18-20]. The assumption that these adhesins could also be important for binding salivary glycoproteins, was proven recently in a glycoconjugate array that showed dependency of *H. pylori* binding to salivary mucin MUC5B on both expression of the BabA and the SabA adhesin, respectively [20]. To further explore the whole range of human salivary glycoproteins specifically recognized by *H. pylori* adhesins, *H. pylori* wild-type (wt) strain J99 together with a set of well defined isogenic *sabA*- and *babA*-deficient mutants were used as probes in a bacterial overlay on nitrocellulose transfers of whole and glandular salivary secretions separated by SDS-PAGE. Taking advantage of a previous proteome analysis of human whole and glandular salivas [21], the bacterial overlay technique was now newly adapted for usage on transfers of two-dimensional (2-D) gels with the aim of a better resolution and possible identification of salivary receptor proteins that may be of pathogenic relevance during the passage of *H. pylori* through the upper alimentary tract.

Materials and Methods

Bacteria and growth conditions

The wt *H. pylori* strain J99 [22] and its isogenic mutants in the *sabA* and/or *babA* genes (J99*babA*, M917B, M61B) were grown for 48-72 h at 37°C in a microaerophilic atmosphere on Wilkins-Chalgren agar (Oxoid, Wesel, Germany) containing 10% horse blood, Dent supplement (Oxoid, Wesel, Germany) and 0.4 g KNO_3 per liter. Bacteria were harvested from plates by wiping off the colonies with a sterile cotton swab and adjusting the bacterial concentration to an optical density of 0.4 in phosphate-buffered saline, pH 7.2 (PBS).

Saliva collection

Unstimulated whole saliva (WS) was collected by expectoration into a polypropylene vial, kept immediately on ice, and then filtrated sequentially with single use filter units of a pore size of 5 µm and 0.2 µm (Minisart, Sartorius, Hannover, Germany). Parotid salivary secretion was collected with Lashley cups (Stone Machine Company, Colton, CA, USA) by

intermittent stimulation of the tongue with 2% citric acid solution. Submandibular/sublingual (SMSL) secretion was collected utilizing a 50 ml sterile syringe fitted with a sterile silicon tube as previously described [21]. Samples (4.5 ml of saliva) were concentrated with the help of Amicon Ultra centrifugal filter devices (Molecular weight cut-off, 5,000, Millipore, Bedford, MA, USA) by centrifugation at 3,000 x g for 30 minutes at 4°C. Salt concentration was lowered by addition of an equal part of sterile double-distilled water, containing 5 mM EDTA, to the concentrate followed by a second centrifugation step. The resulting protein concentrations were determined utilizing the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard.

1-D gelelectrophoresis and staining of proteins and carbohydrates

Salivary samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were denatured under reducing conditions and applied to 4-20 % gradient gels (Novex, Invitrogen, Karlsruhe, Germany) as previously described [23]. The amounts of proteins loaded were 0.75 µg per lane for silver stain, 40 µg per lane for Pro-Q Emerald® stain, 0.2 µg per lane for aminogroup detection, and 20 µg per lane for hydrazide stain, lectin blotting as well as bacterial overlay. Proteins in gels either were stained with silver (SilverXpress®, silver staining kit, Invitrogen) or with Pro-Q Emerald® 488 glycoprotein stain [24] (Invitrogen) according to the manufacturer's instructions. Alternatively, salivary proteins were transferred to nitrocellulose membranes (0.45 µm pore size, Invitrogen) in a semi-dry transfer unit (Invitrogen) under a constant voltage of 0.4 V/cm for 18 h at 4°C in 25 mM Tris (USB Corporation, Cleveland, OH, USA), 192 mM glycine (Merck, Darmstadt, Germany) in 20% v/v methanol (Merck) as transfer buffer. Proteins transferred and bound to the nitrocellulose membrane were detected by labeling aminogroups with sulfosuccinimidyl-6-(biotinamido)hexanoate (sNHS-LC-Biotin, Pierce) and transferred carbohydrates were oxidized with 10 mM sodium periodate (ICN Biomedicals, Aurora, OH) followed by labeling with biotin-LC-hydrazide (Pierce) as previously described [20]. Bound biotin was stained with fluorescein avidin-D (Vector Laboratories, Burlingame, CA, USA). Alpha-L-fucose-containing carbohydrates were detected by binding of fluorescein-labeled *Lotus tetragonolobus* agglutinin (LTA, Sigma-Aldrich, Taufkirchen, Germany) (5 µg/ml in blocking buffer) after blocking the membranes for 1 h at room temperature with 20 mM Tris-buffered saline, pH 7.6 (TBS) containing 2% polyvinyl alcohol (average molecular weight 30,000–70,000, Sigma), 0.1% Tween-20, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ as described previously

[20]. Fluorescent signals were recorded using a fluorescence scanner with a green laser excitation wavelength of 532 nm (Typhoon 9200, GE Healthcare, Freiburg, Germany).

2-D gel electrophoresis

Isoelectric focusing (IEF) was performed in the IPGphor from GE Healthcare Bio-Sciences by in-gel rehydration as described by Görg *et al*. [25]. The IPG strip, pH 3-11 NL, 18 cm (Immobiline Dry Strip, GE Healthcare Bio-Sciences) was rehydrated directly with saliva (300 µg of protein), solubilized in 350 µl of sample buffer containing 7 M urea (GE Healthcare Bio-Sciences), 2 M thiourea (Fluka, Buchs, Switzerland), 2% CHAPS (GE Healthcare Bio-Sciences), 1% DTT (GE Healthcare Bio-Sciences) and 1% Pharmalyte 3-10 (GE Healthcare Bio-Sciences). Low voltage (30 V, 12 h) was applied during the rehydration step, IEF was started with 200 V for one hour and increased to 500 V and 1,000 V for one hour, respectively, before continuously (gradient, 30 min) raising the voltage to 8,000 V for a final focusing time of 4 h. SDS-PAGE was performed as previously described [21] in a vertical apparatus (Ettan DALT six electrophoresis unit, GE Healthcare Bio-Sciences) with Ettan DALT gels (12,5%T, 3%C, 255 x 196 x 1 mm, GE Healthcare Bio-Sciences), according to the manufacturer's instruction and the gels were run for 16 h (1 W per gel, 20°C). A sample (100 µg of protein) of the respective salivary secretion for 1-D separation was included on all gels as a control. After lifting off the gel from the plastic support film by use of a Film Remover (GE Healthcare Bio-Sciences), salivary proteins were subsequently transferred onto nitrocellulose membranes (0.45 µm pore size, Schleicher & Schuell, Dassel, Germany) by electroblotting in a semi-dry transfer unit (Multiphor II with NovaBlot unit, GE Healthcare Bio-Sciences) for 2 h at a constant current of 0.8 mA/cm^2 using 25 mM Tris (USB), 192 mM glycine (Merck) in 20% v/v methanol (Merck) as transfer buffer. Proteins transferred from 2-D gels were detected on the nitrocellulose membranes by Sypro Ruby® blot stain (Invitrogen) according to the manufacturer's instruction but at 4°C and visualized by a Typhoon Imaging System (green laser, 532 nm) (GE Healthcare Bio-Sciences).

Bacterial overlay

All steps of the bacterial overlay procedure were performed in the cold. Sypro Ruby®-stained membranes were washed in TBS for 10 min to remove excess stain and transfers were then blocked in TBS containing 5% BSA (fraction V, Biomol, Hamburg, Germany), 1 mM $CaCl_2$, 1 mM $MgCl_2$ for 2 h. *H. pylori* organisms at a concentration of 1 x 10^8 bacteria/ml in PBS were labeled as previously described [20] by incubation with fluorescein-5-isothiocyanate (Molecular Probes, Oregon, USA) at 100µg/ml for 30 min. Labeled bacteria were washed

three times with PBS and recovered by centrifugation at 900 x g for 7 min, resuspended in 10 ml blocking buffer and added to the membranes a final concentration of 2.5 x 10^7 bacteria *per* ml in a total volume of 40 ml for 1-D transfers or 250 ml for 2-D transfers (equivalent to about 0.6 ml of bacterial suspension *per* cm^2 of nitrocellulose membrane). The overlaid membranes were incubated stationary for 30 min in the dark to allow bacterial binding and subsequently washed three times for 5 min on an orbital shaker (KM-2 Swip, Bühler, Johanna Otto GmbH, Hechingen, Germany) with TBS containing 0,05% Tween-20, 1mM $CaCl_2$ and 1mM $MgCl_2$ to remove unbound bacteria. Fluorescent signals of bound bacteria were recorded by use of a Typhoon imaging system.

Mass spectrometry and protein identification

Bands in the Pro-Q Emerald®-stained 1-D gels corresponding to bands on transfers to which *H. pylori* had bound, were cut out and analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) by the company Protagen (Dortmund, Germany) using the UltraFlex™ mass spectrometer (Ultraflex TOF/TOF, Bruker Daltonik GmbH, Bremen, Germany). Gel pieces were washed three times alternately with 10 mM NH_4HCO_3 and 5 mM NH_4HCO_3/50% acetonitrile. After drying the gel pieces in a vacuum centrifuge (Univapo, UniEquip, Martinsried, Germany) trypsin (Promega, Mannheim, Germany) was added to digest the protein overnight at 37°C. The peptides were extracted from the gel pieces and purified using C_{18} material (ZipTipTM, Millipore) before spotting onto the MALDI target. Peptide mass fingerprint (PMF) spectra and peptide fragmentation fingerprint (PFF) spectra were acquired and database searches were performed against the NCBI database. Proteins corresponding to spots on 2-D transfers to which *H. pylori* had bound, were identified with the help of previously established 2-D maps [21].

Results

Staining of salivary proteins and glycoproteins after separation by 1-D-PAGE

To obtain a comprehensive overview of protein and glycoprotein components in WS and glandular secretions, a series of different gel and blot stains was performed. As seen in Fig. 1, silver staining of gels (Fig. 1A) alone is not sufficient to detect the whole range of salivary proteins. In particular, the highly glycosylated components become visible only after staining for glycoproteins with Pro-Q-Emerald® 488 (Fig. 1B). From both, silver stain and Pro-Q-Emerald® glycoprotein stain, it becomes evident, that WS and the two different glandular secretions show a distinct banding pattern typical to what is known from previous studies

[23]. This also ensures, that the two separate glandular secretions could be successfully collected without contamination from each other. Both, proteins and glycoproteins, including the high-molecular weight components, were transferred to nitrocellulose membranes as revealed by labeling aminogroups (Fig. 1C) or by hydrazide stain of carbohydrates (Fig. 1D). Fucose-containing glycoproteins as putative receptors for the *H. pylori* BabA adhesin were detected by lectin blotting with FITC-labeled *Lotus tetragonolobus* agglutinin (Fig. 1E). As shown in a previous investigation [20] however, the specificity of *Lotus tetragonolobus* agglutinin for α-L-fucose does only partially overlap with the binding specificity of the *H. pylori* BabA adhesin.

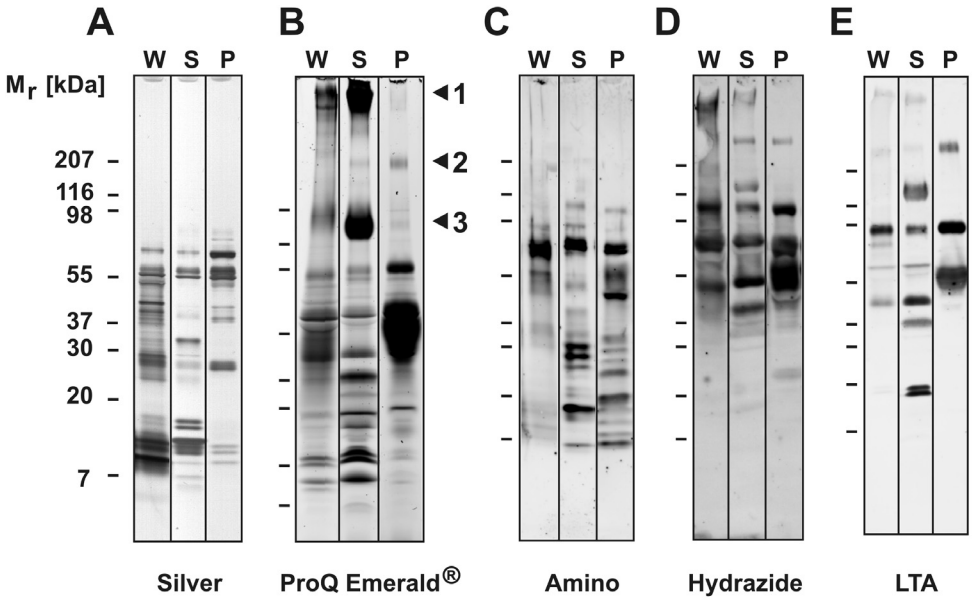


Fig. 1*: Staining of proteins and glycoproteins in whole saliva and glandular secretions. Whole saliva (W), submandibular-sublingual (S), and parotid (P) secretions were separated by SDS-PAGE and gels were stained with A, silver stain, and B, Pro-Q-Emeraldâ 488 glycoprotein stain. Nitrocellulose transfers of these gels were stained by C, aminogroup labeling with sNHS-LC-Biotin, D, glycoprotein stain with biotin-LC-hydrazide after oxidation of sugars with 10 mM sodium periodate, or E, lectin blotting with FITC-labeled* Lotus tetragonolobus *agglutinin (LTA). Bound biotinylated compounds were detected with FITC avidin-D and fluorescent signals were recorded using a fluorescence scanner. Glycoprotein bands in the Pro-Q-Emerald®-stained gels that had been cut out and identified by MALDI-MS and MALDI-MS/MS are indicated by numbered arrows.*

BabA and SabA-dependent recognition of salivary glycoproteins on 1-D transfers

To search for putative receptors among the range of salivary glycoproteins, bacterial overlays with FITC-labeled *H. pylori* were performed on nitrocellulose transfers of WS as well as SMSL- and parotid glandular secretions separated by 1-D PAGE (Fig. 2).

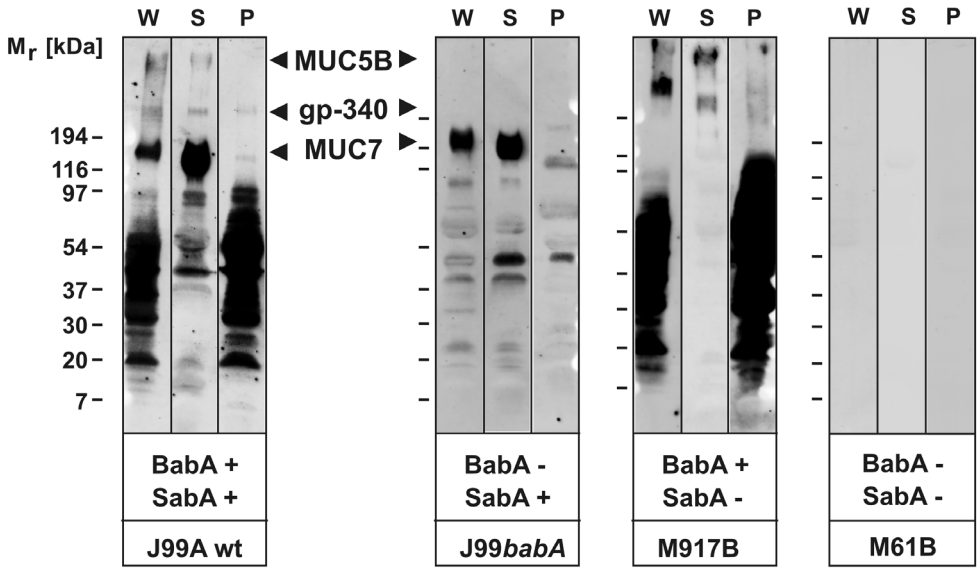


***Fig. 2**: Adhesion of FITC-labeled* H. pylori *J99A wild-type and adhesin-deficient mutants J99*babA*, M917B, and M61B to blots of whole saliva (W), submandibular-sublingual (S), and parotid (P) secretions separated by SDS-PAGE. The presence or absence of the adhesins BabA or SabA are indicated above the strain designation. Blots were overlaid with FITC-labeled bacteria. Following removal of non-adherent organisms, bound bacteria were detected by a fluorescence scanner. The locations of MUC5B, gp-340, and MUC7 that had been identified by MALDI-MS and MALDI-MS/MS in Pro-Q Emerald®-stained gels (see Fig. 1) are indicated.*

Binding of *H. pylori* wt strain J99A was observed to a diffuse band at the origin of the gel in WS and SMSL-secretions that was also strongly bound by the *sabA*-deficient mutant M917B but not by the *babA*-deficient mutant J99babA. The band was cut out at the corresponding place in the Pro-Q-Emerald®-stained gel (see arrow 1 in Fig. 1) and analysis by MALDI-TOF and MALDI-TOF/TOF identified this component as the high molecular weight mucin MUC5B (gi|51470790) (Fig. 3). *H. pylori* wt J99A bound also to another major band in the molecular range of about 200 kDa in WS and SMSL glandular secretion. This band was also strongly bound by the *babA* -deficient mutant J99*babA* but no binding was found with the *sabA* -deficient mutant M917B. Analysis by MALDI-TOF and MALDI-TOF/TOF identified

this band (see arrow 3 in Fig. 1) as the low molecular weight mucin MUC7 (MG2, gi|22748665) (Fig. 3).

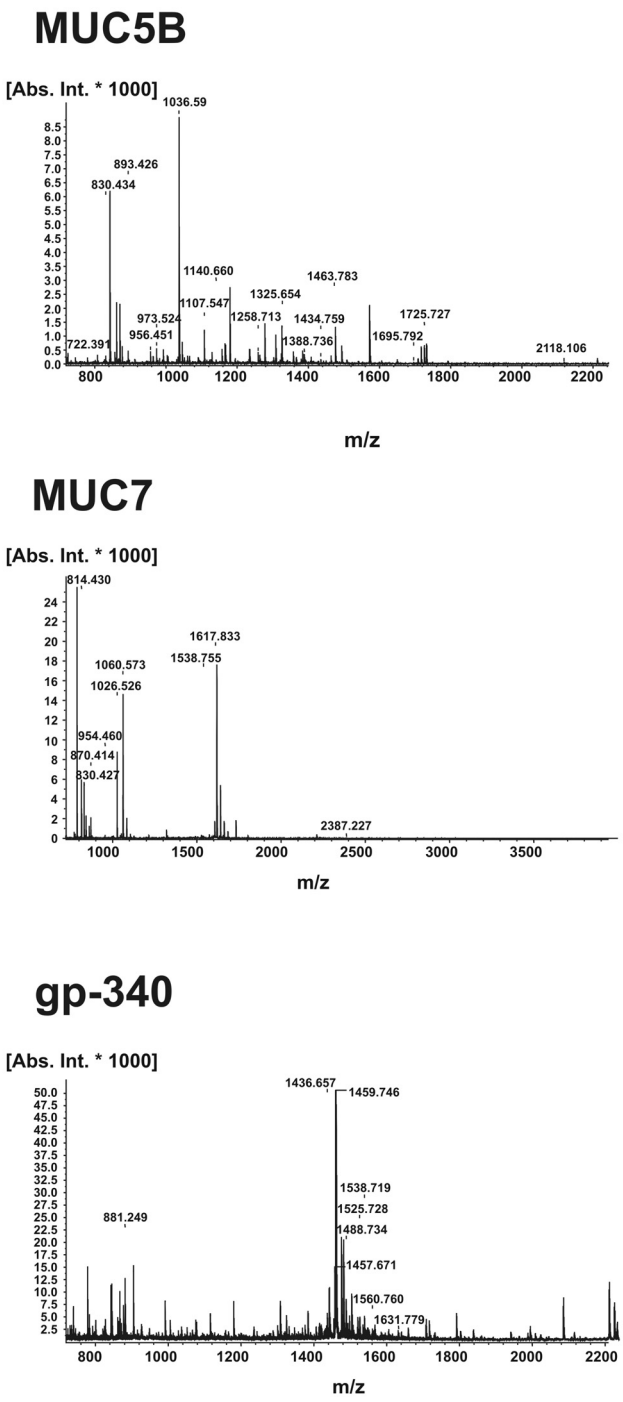


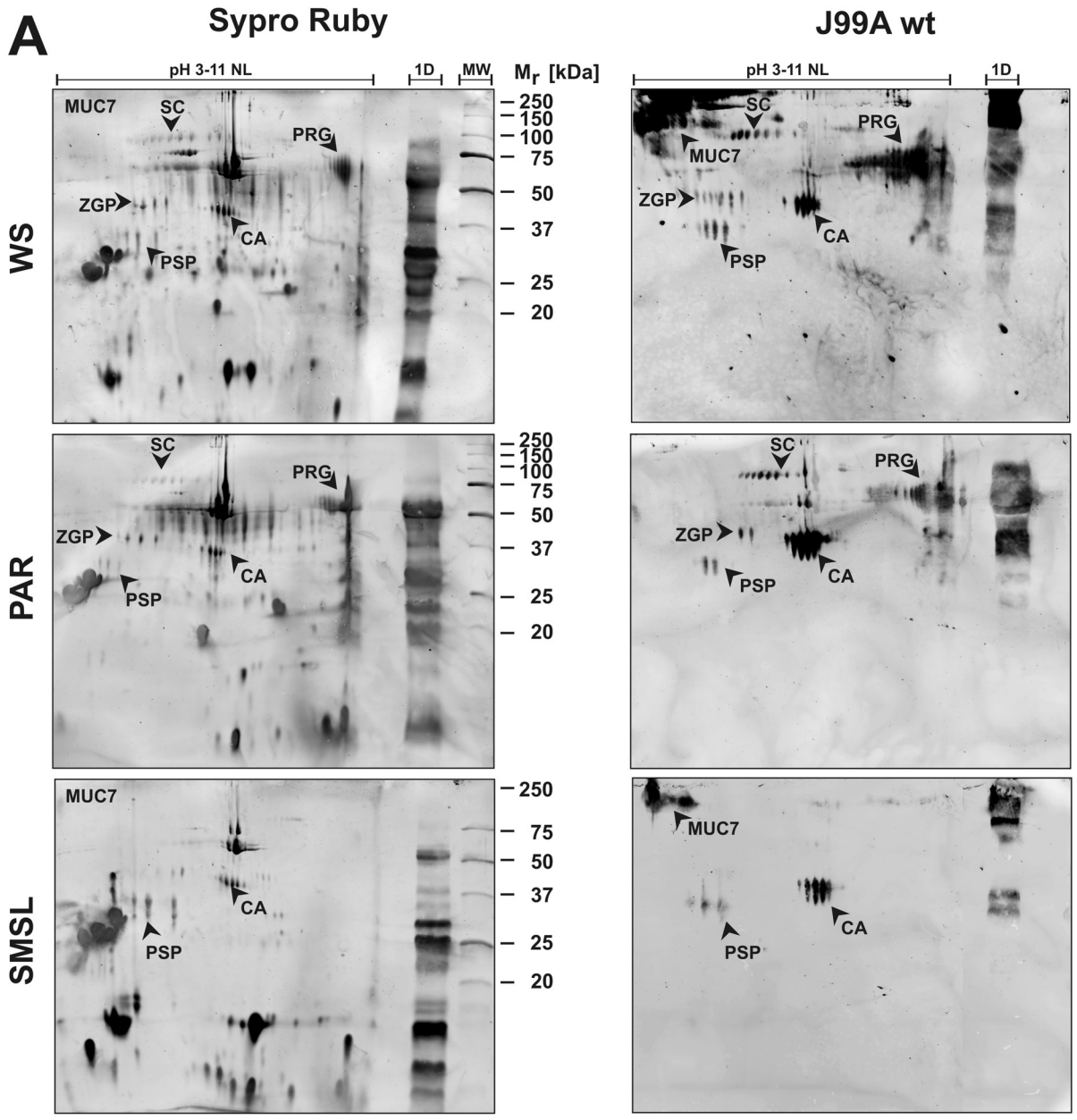
Fig. 3*: MS analyses of MUC5B, MUC7 and gp-340 identified as salivary receptors for* H. pylori*-adhesins. Peptide mass fingerprint spectra were acquired by MALDI-TOF analysis of tryptic peptides from bands cut out of the 1-D gel shown in Figure 1 B. Results were verified by additional MALDI-TOF/TOF analysis. Amino acid sequences identified by MALDI-TOF as well as MALDI-TOF/TOF of the corresponding proteins are displayed in the Supplemental Data.*

An additional band half-way in between the bands of MUC5B and MUC7 was bound by *H. pylori* wt J99A to different extents in WS and glandular secretions. This band was bound by the *sabA*-deficient mutant M917B but not recognized by the *babA*-deficient mutant J99*babA*. A band excised from the Pro-Q-Emerald®-stained gel at the corresponding molecular range (see arrow 2 in Fig. 1) was identified by MALDI-TOF and MALDI-TOF/TOF as the salivary agglutinin (gp-340, gi|55962155) (Fig. 3). A very strong almost confluent signal involving a large group of bands in the molecular range between 30 and 100 kDa was seen after overlay with *H. pylori* wt J99A in parotid secretion and in WS. Similar strong binding in WS and parotid saliva was found in the same range after overlay with the *sabA*-deficient mutant M917B. In SMSL secretion, considerably less binding in this range was detected by the wt strain J99A but a prominent band at a molecular range of about 40 kDa remained to be bound and was also recognized by *babA*-deficient mutant J99*babA* in all three salivary secretions. With the *sabA*-deficient mutant M917B no binding in SMSL secretion could be detected in this range. An attempt to identify components within this conglomeration of putative receptor molecules by MALDI-MS failed because of overlaps between different protein bands. Significantly, the *babA/sabA*-double mutant did not recognize any bands in either secretion.

BabA- and SabA-dependent recognition of salivary glycoproteins on 2-D transfers

The bacterial overlay was performed on transfers of 2-D gels from WS, parotid and SMSL secretions to allow the identification of salivary receptors for *H. pylori* that could not be separated on 1-D overlays (Fig. 4). The original locations of protein spots on transfers of 2-D gels from WS, parotid and SMSL secretions were visualized by Sypro Ruby® blot stain prior to bacterial overlay (Fig. 4A). The spot patterns in all three secretions appeared in a reproducible fashion when compared to Coomassie-stained spot patterns mapped in a previous proteome analysis of human saliva [21] The locations of significant spots or spot groups are marked in Fig. 4A. After washing out the Sypro Ruby® blot stain, transfers were overlaid with *H. pylori* J99A wt (Fig. 4A). On 2-D transfers of WS, the wt strain bound to salivary mucin MUC7 (gi|22748665), the secretory component (poly-Ig-receptor, gi|514366), proline-rich glycoprotein (gi|41349488), zinc-α_2-glycoprotein (gi|4699583), carbonic anhydrase VI (gi|14530767), and parotid secretory protein (gi|16755850). Binding of the *H. pylori* J99A wt strain to these same components was also observed on transfers of glandular secretions but was dependent on their respective expression in parotid and SMSL secretions.

The adhesins responsible for binding to these identified receptor molecules were examined by the use of adhesin-deficient mutants in overlay experiments (Fig. 4B).


A
Sypro Ruby
J99A wt
pH 3-11 NL
1D
MW
Mr [kDa]
WS
PAR
SMSL
MUC7
SC
PRG
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CA
PSP
250
150
100
75
50
37
25
20

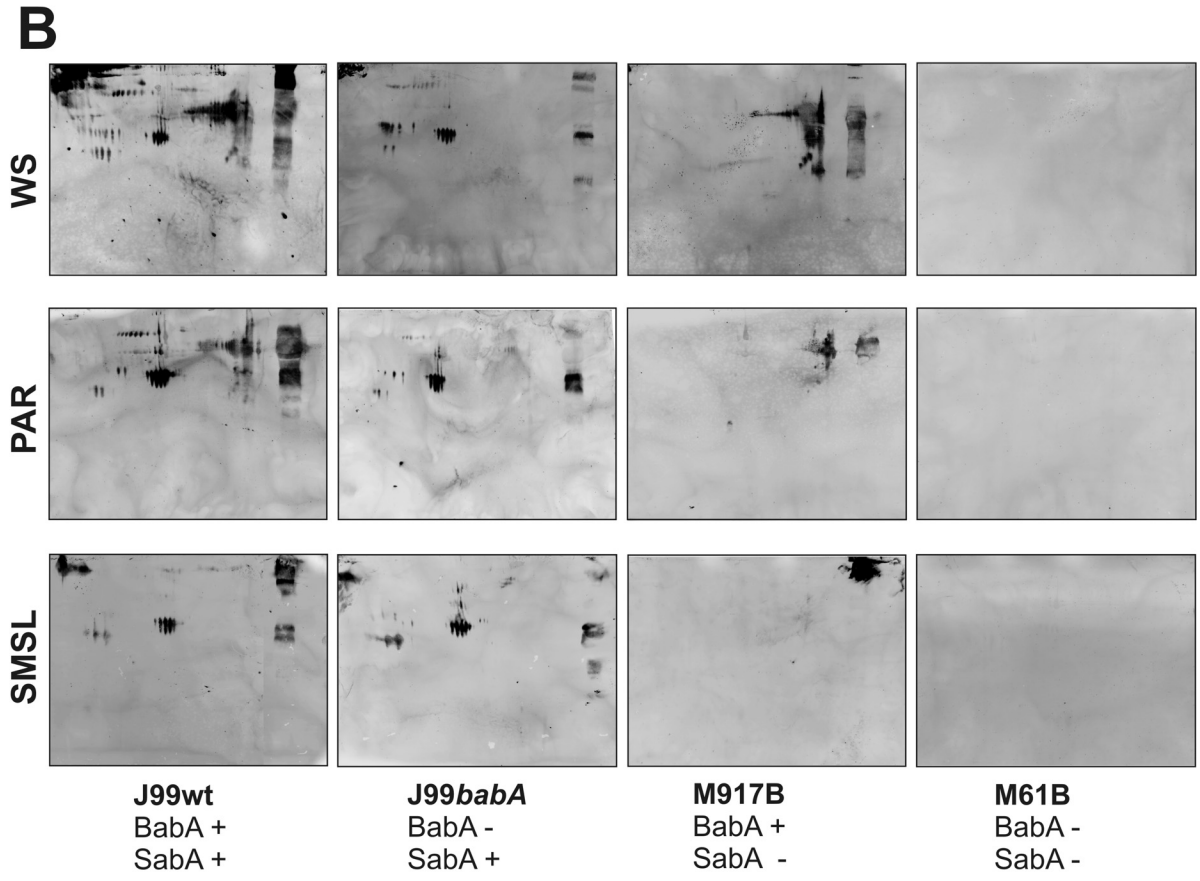

***Fig. 4**: Adhesion of FITC-labeled* H. pylori *J99A wild-type and adhesin-deficient mutants J99*babA*, M917B, and M61B to transfers of whole saliva (WS), parotid (PAR), and submandibular-sublingual (SMSL) secretions separated by 2-D gel electrophoresis. A, Nitrocellulose transfers were stained with SyproRuby® blot stain prior to bacterial overlay to visualize the locations of protein spots. After washing out the SyproRuby® stain, the same transfers were overlaid with FITC-labeled* H. pylori *J99A wild-type. Following removal of non-adherent organisms, bound bacteria were detected by a fluorescence scanner. Spots previously identified by MALDI-MS and MALDI-MS/MS as MUC7, secretory component of the poly-Ig-receptor (SC), zinc-α_2-glycoprotein (ZGP), parotid secretory protein (PSP), carbonic anhydrase VI (CA), and proline-rich glycoprotein (PRG) (21) are indicated by arrows. B, Transfers from all three salivary secretions were overlaid by the adhesin-deficient* H. pylori *mutants. The overlays with* H. pylori *wild-type J99A are identical to Fig. 4A and were included for easier comparison of bound salivary receptors. The presence or absence of adhesins BabA and SabA are indicated below the strain designations. A sample of the respective salivary secretion, separated only in the second dimension, was included on all gels as a control.*

The *babA*-deficient mutant strain J99*babA* bound to zinc-α_2-glycoprotein, carbonic anhydrase VI, and parotid secretory protein in all three salivary secretions. Binding to MUC7 was only

observed in WS and SMSL secretion, whereas binding to the secretory component (poly-Ig-receptor) was observed only in WS and parotid secretions. No binding of the *bab*A-deficient mutant strain to the proline-rich glycoprotein could be detected. The *sabA*-deficient mutant strain M917B retained binding only to the proline-rich glycoprotein but failed to bind MUC7, the secretory component (poly-Ig-receptor), zinc-α_2-glycoprotein, carbonic anhydrase VI, and parotid secretory protein. In SMSL secretion, the *sabA*-deficient mutant strongly bound to a high molecular weight component in the 1-D lane but no corresponding spot could be detected in the 2-D separation. This strong interaction was consistently observed only on the 1-D lane and may be due to binding of the BabA adhesin to the high molecular weight mucin MUC5B that can be easily visualized on the origin of the gel in 1-D PAGE (see Figs. 1 and 2) but due to its high molecular weight so far does not appear after 2-D PAGE [21]. Significantly, no binding to any component in any of the three salivary secretions could be detected by overlay with the *babA/sabA*-deficient mutant M61B.

Discussion

Because the oral cavity is the most feasible entry point for gastric *H. pylori,* a legitimate interest exists, to explore whether *H. pylori* simply passes through the oral cavity, or whether this bacterium may interact with components of saliva, possibly resulting in a modulation of its infectivity. The aim of this investigation was to use a novel combination of proteomics techniques with the bacterial overlay to comprehensively search for salivary receptors for *H. pylori* adhesins on a previously established salivary proteome map and to assign these receptors to the binding activities of the responsible adhesins by probing with adhesin-deficient isogenic mutants. It was now possible to assign previously reported binding of *H. pylori* to salivary mucin MUC7 exclusively to the activity of the SabA-adhesin, binding to the salivary agglutinin gp-340 exclusively to the activity of the BabA-adhesin, and binding to the high-molecular weight salivary mucin MUC5B mainly to the activity of the BabA adhesin and to a lesser degree to that of the SabA-adhesin. In addition, binding of *H. pylori* to the proline-rich glycoprotein was newly detected by the 2-D bacterial overlay and could be assigned to the activity of the BabA adhesin, whereas the SabA adhesin was found responsible for binding to additional newly detected receptor molecules including salivary carbonic anhydrase VI, secretory component (poly-Ig-receptor), parotid secretory protein, and zinc-α_2-glycoprotein.

Adhesive interaction of *H. pylori* with MUC5B has been previously reported and was postulated to be based mainly on binding of a *H. pylori* neutrophil-activating protein (NAP) to

sulfated glycans on this mucin [11, 12]. In the present study, it could be demonstrated that binding of *H. pylori* to MUC5B is mediated mainly by the BabA adhesin implying that ABO/Lewis b blood group antigens that were found on this mucin [26] may serve as the prevailing receptor structures. This is supported by a previous characterization of the binding properties of *H. pylori* adhesins utilizing glycoconjugate arrays in which adhesion to salivary mucin MUC5B was mainly associated with the BabA-adhesin and to a much lesser extent with the SabA-adhesin [20] and agrees with reports that MUC5B carries both fucosylated blood group antigens and α2,3-linked sialic acids [27]. Only in few bacterial overlay experiments employing *sabA*-containing *H. pylori* mutants as probes on 1-D transfers from both, WS and SMSL secretion, weak binding to MUC5B could be observed (data not shown), further indicating that the interaction of SabA with sialic acid termini on MUC5B may play only a subordinate role. A requirement of additional adhesins for binding to MUC5B could not be supported by the present results and by previous use of glycoconjugate arrays because the *babA/sabA* double mutant did not bind to this mucin [20]. Analogous findings have been reported for the interaction of the BabA-adhesin with gastric mucin MUC5AC [28]. In future studies, it would be promising to explore also the secretions of the minor salivary glands to clarify the role of sulfatation of glycans for higher binding avidity to MUC5B because highest sulfatation was reported in a subpopulation of high molecular weight mucins, secreted from the palatine salivary glands [12].

In contrast to MUC5B, previously described binding of *H. pylori* to the lower molecular weight mucin MUC7 [13] was now found to be exclusively mediated by the SabA-adhesin, implying that α2,3-linked sialic acid termini may serve as the crucial receptor motifs. Sialic acid termini are known to be present on this mucin both in context with the sialyl-T antigen and the sialyl-Lewis x antigen [29] and the latter had been previously suggested to be involved in binding of *H. pylori* to MUC7 [13]. From the present results, combined with knowledge about the specificity of the SabA adhesin for terminal α2,3-linked sialic acids [20], it can now be postulated that not only sialyl-Lewis x but also the sialyl-T antigen may serve as receptor motifs on MUC7.

Salivary agglutinin (gp-340) is another component that had previously been reported to interact with *H. pylori* [13, 14]. This was confirmed in the present study and, by use of the adhesin-deficient mutants of *H. pylori*, binding to this molecule could now for the first time be clearly assigned to the activity of the BabA-adhesin. Although presence of sialic acid on gp-340 had been reported [30], an involvement of sialic acid epitopes on gp-340, that would mediate binding by the SabA-adhesin, could not be supported by the present results. It could

be hypothesized that somehow receptor activity of terminal sialic acid might be restrained by its linkage to subterminal oligosaccharides or that sialic acid epitopes might not be accessible to the adhesin for sterical reasons. In this respect it is noteworthy, that a recent study showed different densities of α2,3-linked sialic acids on gp-340 among different individuals [31]. Thus, a donor dependency in binding of the SabA-adhesin to gp-340 is possible and would deserve further study.

By the newly developed use of 2-D transfers for bacterial overlay, it was possible to identify several novel receptors for *H. pylori* in saliva. By comparison to previously established proteome maps of WS, parotid, and SMSL secretions [21], salivary carbonic anhydrase VI, the parotid secretory protein, zinc-α_2-glycoprotein, and the proline-rich glycoprotein were found to support binding of *H. pylori.* In addition, a group of several horizontal isoform spots in WS and parotid secretion at a molecular range of about 90 kD were also identified as receptors for *H. pylori*. Database search initially resulted in best alignment of the peptides with the poly-Ig receptor [21]. However because the poly-Ig receptor is a cell membrane-bound molecule, it is most likely that the protein that was found in salivary secretions rather represents the secretory component that becomes cleaved as the major part of the poly-Ig receptor upon release of secretory immunoglobulins, e.g. S-IgA, from the luminal side of glandular epithelial cells [32].

Binding of *H. pylori* to the proline-rich glycoprotein could be assigned to the activity of the BabA-adhesin. Although a presence of Lewis b antigens on the proline-rich glycoprotein was not shown in a structural analysis of its oligosaccharides, it is well known that this glycoprotein contains a high amount of α1,2-linked fucose termini [33]. The Lewis y antigen (Fucα1,2Galβ1,4GlcNAc[Fucα1,3]) which is the most abundant oligosaccharide structure on the proline-rich glycoprotein [33] was not recognized as a receptor for the BabA-adhesin when performing bacterial overlay on immobilized neoglycoproteins [20]. It might be, however, that purely by the high receptor density or by favorable sterical presentation provided on a natural glycoprotein, sufficient receptor activity could be gained to mediate bacterial binding. Binding of the *H. pylori* SabA-adhesin to carbonic anhydrase VI, formerly also known as gustin, is most likely based on the presence of di-, tri- and tetra-sialylated termini on the two *N*-linked oligosaccharide chains that had been described on this molecule whereas fucose was found only in an interior linkage [34, 35]. Similarly, SabA-mediated binding of *H. pylori* to the secretory component (poly-Ig-receptor) can be explained by its high sialic acid content including the availability of α2,3-linked terminal sialic acids [36]. An additional involvement of BabA-mediated binding to fucose-containing oligosaccharide

motifs as suggested in the past [16] could not be confirmed in this present assay system, although the presence of Lewis b epitopes has been described on the secretory component [16, 36]. Zinc-α_2-glycoprotein, that is also recognized by the *H. pylori* SabA-adhesin, was reported to contain 7% sialic acid and only few fucose (0.2%) but a more detailed structure of the oligosaccharides or the linkages of sialic acid on this protein is obviously not available [37, 38]. Unfortunately, no information about oligosaccharides on the parotid secretory protein, another novel receptor for the *H. pylori* SabA-adhesin, is available so far. Thus, a putative presence of sialic acid termini on this protein can only be proposed based on the findings of this study.

It can only be hypothesized what the biological consequences of binding of *H. pylori* to these glycoprotein receptors in human saliva might be. On one hand, adhesin-mediated binding to these receptors may allow *H. pylori* to attach to oral or dental surfaces coated with a film consisting mostly of salivary proteins, the so-called pellicle, or to colonize among the complex biofilm microbiota on teeth [39]. There is however conflicting opinion about the presence of *H. pylori* in the oral cavity and its possible role for oral-oral transmission or re-infection of the stomach [8]. On the other hand, because of the constant influx of saliva into the upper alimentary tract and the mixing with gastric juice [6], it could be that *H. pylori* once decorated with certain salivary glycoproteins changes its pathogenic properties in the stomach by e.g. better protection from the body's immune response or by a better resistance to gastric acid or proteolytic challenge. It has been reported that *H. pylori* can survive only for a few minutes in the hostile environment of gastric juice [40]. In this context it is of interest that an acid-protective barrier function of salivary mucins has been reported [41]. Furthermore, it is noteworthy, that 10-14 mg of salivary carbonic anhydrase VI that is bound by the *H. pylori* SabA-adhesin are swallowed during one day [42, 43] and that *H. pylori* by itself expresses carbonic anhydrases that were suggested to contribute to the adaptation of *H. pylori* to the harsh environment of the stomach [44].

The described combination of bacterial overlay with 2-D electrophoresis and proteomics techniques may be further employed for identification of receptor proteins recognized by any given bacterial adhesin for a variety of complex biological fluids, mucous secretions, or membrane preparations of eukaryotic cells.

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Chapter 7

Applicability of the Overlay Technique to Commensal Bacteria of the Oral Cavity Using *Streptococcus gordonii* and *Actinomyces naeslundii* as Probes

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Abstract

Previously performed bacterial overlay assays with fluorescence-labeled *Helicobacter pylori* (*H. pylori*) had been shown to be suitable for screening adhesin specificities of the bacterium on dot-blots. In addition, the adhesive recognition of salivary proteins by *H. pylori* could be shown on transfers of two (2-D)-dimensional gels. The purpose of this study was to apply these established overlay techniques to well-characterized commensal bacteria of the oral cavity with the aim to show the applicability of this method to other bacteria. Dot-blot arrays of natural glycoproteins and synthetic neoglycoproteins were produced. 2-D gels of human whole saliva were made and transferred onto nitrocellulose membranes. Dot-blot overlays were carried out with fluorescence-labeled *Streptococcus gordonii* DL1 and *Actinomyces naeslundii* WVU45 wild-type strains and their mutants (D102, WVU45M). With this method all known binding specificities of sialic acid/ *N*-acetylgalactosamine- (*S. gordonii*) and galactose/*N*-acetylgalactosamine- (*A. naeslundii*) reactive lectins could be confirmed. 2-D overlays were performed with fluorescence-labeled *Streptococcus gordonii* DL1. These revealed the expected interactions of this strain with salivary proteins as well as yet unknown interactions. Together, these results show the applicability of fluorescence-labeling in bacterial overlay experiments for different species of bacteria. In particular, the 2-D overlay proved to be a powerful method for screening diverse bacterial receptors in a complex biological fluid.

Introduction

Adhesion of bacteria to cell surfaces, and bacterial colonization of the host, respectively, are in many cases based on specific lectin-carbohydrate interactions [1]. In consequence, studying receptor specificity of lectin-like bacterial adhesins is useful to understand more about pathology and tissue tropism of certain bacteria.

In previous studies the bacterial overlay technique was used successfully for exploring lectin-dependent binding specificities of bacteria, independently of whether radiolabeled [2, 3] or biotinylated bacteria [4, 5] were used. An improvement of the bacterial overlay technique used fluorescence-labeled bacteria on dot-blot arrays. The dot-blots contained various structurally related carbohydrates ranging from high molecular weight glycoproteins to monosaccharide-neoglycoproteins. This approach was helpful to characterize adhesin specificities of *Helicobacter pylori* (*H. pylori*) [6]. The use of fluorescently-labeled *H. pylori* facilitated also the development of a high-resolution overlay on transfers of two-dimensional gels of salivary proteins [7] and, thus, the identification of salivary receptors for *H. pylori*.

Gram-positive bacteria, such as actinomyces or viridans streptococci are known to initiate bacterial colonization on teeth surfaces. This is in many cases mediated by lectin-carbohydrate interactions of the bacteria with components of the acquired enamel pellicle [8, 9]. Actinomyces express type 1 and type 2 fimbriae, depending on the respective strain [10]. Type 1 fimbriae (e.g. of A*ctinomyces viscosus* 5519) have adhesins recognizing certain amino acid sequences on proline-rich proteins (PRP) [11], while type 2 fimbriae (e.g. of *A. naeslundii* WVU45) have lectin-like adhesins recognizing saccharide motifs that contain either galactose (Gal) or *N*-acetylgalactosamine (GalNAc). These in turn, are responsible for coaggregation with certain streptococci [8, 12]. Viridans streptococci are known to adhere to saliva-coated hydroxyapatite, a model of the tooth surface, by interaction with sialic-acid containing receptors [13]. Binding of *Streptococcus gordonii* DL1 to such receptors depends on the Hs antigen, a glycosylated surface component, encoded by *hsa* [14]. In addition, it is known that *S. gordonii* strains coaggregate with bacteria expressing GalNAc-containing receptors on their surface [15].

The immediate aim of this study was to show that the improved overlay technique is applicable to commensal bacteria of the oral cavity, and other bacterial species. *S. gordonii* DL1, *A. naeslundii* WVU45 and clearly defined spontaneous mutants were used as probes.

Materials and Methods

Dot-blot array

Nitrocellulose membranes (Protran BA 85, pore size 0.45 µm, Schleicher & Schuell GmbH, Dassel, Germany) were spotted with 1 µl 0.9% NaCl (Braun, Melsungen, Germany) solution containing 1 µg (neo-)glycoprotein and 0.1% NaN_3 (Sigma, Steinheim, Germany). Glycoproteins and neoglycoproteins used in this study are listed in Table 1. A-C are glycoproteins of natural origin, D-I are synthetic neoglycoproteins.

***Table 1**: Glycoproteins and neoglycoproteins used in this study*

Array No.	(Neo-)Glycoprotein	mol sugar residues/ mol protein	Sugar chains
A1	Fetuin[1]		
A2	Asialofetuin[1]		
A3	Glycophorin A[1]		
A4	Asialoglycophorin A[1]		
A5	Laminin[1]		
A6	α-acid Glycoprotein[1]		
B1	Thyroglobulin[1]		
B2	Mucin (bovine submaxillary gland)[1]		
B3	Asialomucin[1]		
B4	Salivary mucin MUC5B[2]		
B5	Proline-rich protein-1 (PRP-1)[3]		
B6	Salivary α-Amylase[1]		
C1	Secretory immunglobulin A (S-IgA)[4]		
C2	Lactoferrin[1]		
C3	Fibronectin[1]		
C4	Lysozyme[1]		
C5	Bovine serum albumin (BSA)[1]		
C6	Human serum albumin (HSA)[1]		
D1	Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[5]	22	Lacto-*N*-tetraose
D2	Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[5]	23	Lewis a
D3	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[5]	25	H type 1
D4	Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[5]	22	Lewis b
D5	GalNAcα1-3[Fucα1-2]Galβ1-O-APE-HSA[5]	19	Blood group A

Array No.	(Neo-)Glycoprotein	mol sugar residues/ mol protein	Sugar chains
D6	Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[5]	12	sialyl-Lewis a
E1	Galβ1-4GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[5]	10	Lacto-*N*-neotetraose
E2	Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[5]	20	Lewis x
E3	Fucα1-2Galβ1-4GlcNAcβ-O-APE-HSA[5]	19	H type 2
E4	Fucα1-2Galβ1-4[Fucα1-3]GlcNAcβ-O-APE-HSA[5]	12	Lewis y
E5	Galα1-3[Fucα1-2]Galβ1-O-APE-HSA[5]	19	Blood group B
E6	Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4(Glc)-APD-HSA[5]	13	sialyl-Lewis x
F1	GalNAcβ1-3Galα1-4Galβ1-4(Glc)-APD-HSA[5]	8	Globo-*N*-tetraose
F2	Galα1-3GalNAcα-O-10 Å spacer-HSA[6]	20	
F3	GalNAcβ1-4Galβ-O-10 Å spacer-BSA[6]	20	
F4	Galβ1-3GlcNAcβ-O-10 Å spacer-BSA[6]	20	
F5	Galβ1-3GalNAcα1-O-APE-HSA[5]	17	T antigen
F6	GalNAcβ1-3Galα-O-10 Å spacer-BSA[6]	20	
G1	Galβ1-4GlcNAc-3 atom spacer-BSA[7]	11	*N*-Acetyllactosamine
G2	Fucα1-2Galβ1-4(Glc)-APD-HSA[5]	16	2'-Fucosyllactose
G3	Neu5Acα2-6Galβ1-4(Glc)-APD-HSA[5]	15	6'-Sialyllactose
G4	Neu5Acα2-3Galβ1-4(Glc)-APD-HSA[5]	6	3'-Sialyllactose
G5	Neu5Acα2-3Galβ1-4GlcNAc-3 atom spacer-BSA[7]	11	3'-Sialyl-*N*-acetyllactosamine
G6	Neu5Acα2-3Galβ1-4[Fucα1-3]Glc-3 atom spacer-BSA[7]	7	3'-Sialyl-3fucosyllactose
H1	Galα1-O-PAP-HSA[8]	10-20	α-Galactose
H2	Galβ1-O-PAP-HSA[8]	10-20	β-Galactose
H3	GlcNAcα1-O-PAP-HSA[8]	10-20	α-*N*-Acetylglucosamin
H4	GlcNAcβ1-O-PAP-HSA[8]	10-20	β-*N*-Acetylglucosamin
H5	GalNAcα1-O-PAP-HSA[8]	10-20	α-*N*-Acetylgalactosamine
H6	GalNAcβ1-O-PAP-HSA[8]	10-20	β-*N*-Acetylgalactosamine
I1	Galβ1-4Glcβ-O-10 Å spacer-BSA[6]	20	Lactose
I2	Fucα1-O-PAP-HSA[8]	10-20	α-Fucose
I3	Glcα1-O-PAP-HSA[8]	10-20	α-Glucose
I4	Manα1-O-PAP-HSA[8]	10-20	α-Mannose
I5	Manβ1-O-PAP-HSA[8]	10-20	β-Mannose
I6	Xylβ1-O-PAP-HSA[8]	10-20	β-Xylose

[1] Sigma

[2] kindly provided by M.J. Levine, Department of Oral Biology, SUNY, Buffalo, NY, USA

[3] kindly provided by Donald I. Hay, Forsyth Institute, Boston, MA, USA

[4] MP Biomedicals, Aurora, Ohio, USA

[5] IsoSep AB, Tullingen, Sweden

[6] Glycorex AB, Lund, Sweden

[7] Dextra, Reading, UK

[8] Diagnostic International, Schriesheim, Germany

Saliva

Sample collection was performed under cooling. Unstimulated whole saliva (WS) was freshly collected from one healthy male subject by expectoration. Samples were filtrated twice using a membrane filter (Minisart, Sartorius, Hannover, Germany) of 5 µm and 0.2 µm pore size. Saliva was subsequently concentrated with Amicon® Ultra (Millipore, Bedford, MA, USA) centrifugal filter devices (molecular weight cut-off, 5,000) at 3,000 x g for twice 30 min. Protein concentration of the sample was determined by the bicinchoninic acid (BCA) protein assay (Pierce) with BSA as a standard.

Two-dimensional gel electrophoresis and transfer

2-D PAGE of WS was performed with ready-made Immobiline Dry Strips (IPG strip, pH 3-10 NL, 18 cm; GE Healthcare Bio-Sciences, Freiburg, Germany) for the isoelectric focusing step as well as with precast Ettan DALT gels for the second dimension (12,5%T, 3%C, 255 x 196 x 1mm, GE Healthcare Bio-Sciences) as previously described [16]. Amount of proteins loaded was 300 µg. 100 µg of proteins of the same sample as applied to the IPG strip, was included as a 1-D separated control. Control saliva was denatured under reducing conditions, applied to small piece of filter paper and separated simultaneously with the proteins on the IPG strip in the second dimension. Transfer onto the nitrocellulose membrane (0.45 µm pore size, Schleicher & Schuell, Dassel, Germany) was carried out by electroblotting in a semi-dry transfer unit (Multiphor II with NovaBlot unit, GE Healthcare Bio-Sciences) with a transfer buffer containing 25 mM Tris (USB, Cleveland, OH, USA), 192 mM glycine (Merck, Darmstadt, Germany) dissolved in 20% v/v methanol (Merck). Settings were 0.8 mA/cm^2 for 2 h.

Visualization of 2-D-separated proteins

For displaying the complete 2-D pattern of transferred salivary proteins, the blot was first washed with 7% acetic acid (Merck) and 10% methanol (Merck) and was then stained with

Sypro Ruby® blot stain (Invitrogen, Karlsruhe, Germany). Staining was carried out according to the manufacturer's instruction at 4°C and protein spots were visualized by a Typhoon Imaging System (Sypro Ruby mode, GE Healthcare Bio-Sciences).

Bacterial overlay

The bacterial overlay procedure was performed at 4°C. Dot-blot membrane was washed for 5 min and the 2-D transfer blot for 10 min in Tris-buffered saline (TBS). Membranes were blocked in TBS containing 5% BSA (fraction V, Biomol, Hamburg, Germany), 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 2 h. Bacteria included in this study, were *S. gordonii* DL1 (Challis) and its spontaneous mutant D102 [14] as well as *A. naeslundii* WVU45 (ATCC 12104) and its mutant WVU45M [8]. Bacteria were grown overnight in complex medium as previously described [14]. They were then adjusted to a concentration of 1 x 10^8 bacteria/ml in phosphate-buffered saline (PBS) (equivalent to an optical density of 4). Optical density was recorded by measuring the absorption of the bacterial suspension in PBS with a photometer (Ultrospec 1000, Pharmacia Biotech, Cambridge, England). Bacteria were washed with PBS twice, and were subsequently labeled with 100 µg/ml fluorescein-5-isothiocyanate (FITC, Molecular Probes, Oregon, USA) for 30 min. After the labeling procedure bacteria were washed with PBS three times. *S. gordonii* strains were recovered by centrifugation at 3,000 x g and *A. naeslundii* strains at 1,500 x g for 5 min respectively. Bacteria were resuspended in 10 ml blocking buffer and were then added to the membranes. Final concentration of bacteria was 2.5 x 10^7 organisms per ml in a total volume of 40 ml for dot-blot membranes or 250 ml for 2-D transfers blots (~ 0.6 ml of bacterial suspension per cm^2 of nitrocellulose membrane). Incubation with the bacteria was carried out for 30 min in darkness, and membranes were subsequently washed three times for 5 min with TBS containing 0,05% Tween-20, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Bound bacteria were detected using a Typhoon imaging system (FITC mode, GE-Healthcare Bio-Sciences).

Results

Recognition of natural and synthetic glycoproteins by S. gordonii

The extended version of the established glycoconjugate array [6] contained sialic acid, Gal and GalNAc sugar structures in different linkages, and some more natural glycoproteins (Fig 1). As expected, the FITC-labeled DL1 wild-type strain of *S. gordonii* could be demonstrated to bind components of this array (Fig. 1). The sialic acid-dependent interactions of *S. gordonii* DL1 with fetuin (A1), glycophorin A (A3), laminin (A5), sialyl-Lewis x (E6), 3'-sialyllactose

(G4), 3'-sialyl-*N*-acetyllactosamine (G5) and to a lesser degree 3'-sialyl-3-fucosyllactose (G6) described previously [6] could be reproduced with the extended version of the array. In contrast, the *S. gordonii* mutant strain D102 that does not express a sialic acid binding adhesin, failed to bind these components. In addition, the incorporation of GalNAc-containing neoglycoconjugates showed the expected binding activity to GalNAc-containing receptors: *S. gordonii* DL1 bound to globo-*N*-tetraose (GalNAcβ1-3Galα1-4Galβ1-4Glc; F1), GalNAcβ1-3Galα-disaccharide (F6) and GalNAcβ-monosaccharide (H6). Weaker binding was noted for GalNAcα-monosaccharide (H5). Interestingly, GalNAcβ1-4Galβ-disaccharide (F3) was not recognized by this strain. The mutant D102 showed the same binding activities and bound also to GalNAc-containing receptors. Among natural occurring glycoproteins PRP-1 (B5) and fibronectin (C3) were bound by both, DL1 and D102, but with different intensities. α_1-Acid glycoprotein (A6), mucin from bovine submaxillary gland (B2), α-amylase (B6) and S-IgA (C1) were bound only by *S. gordonii* DL1, but not by D102.

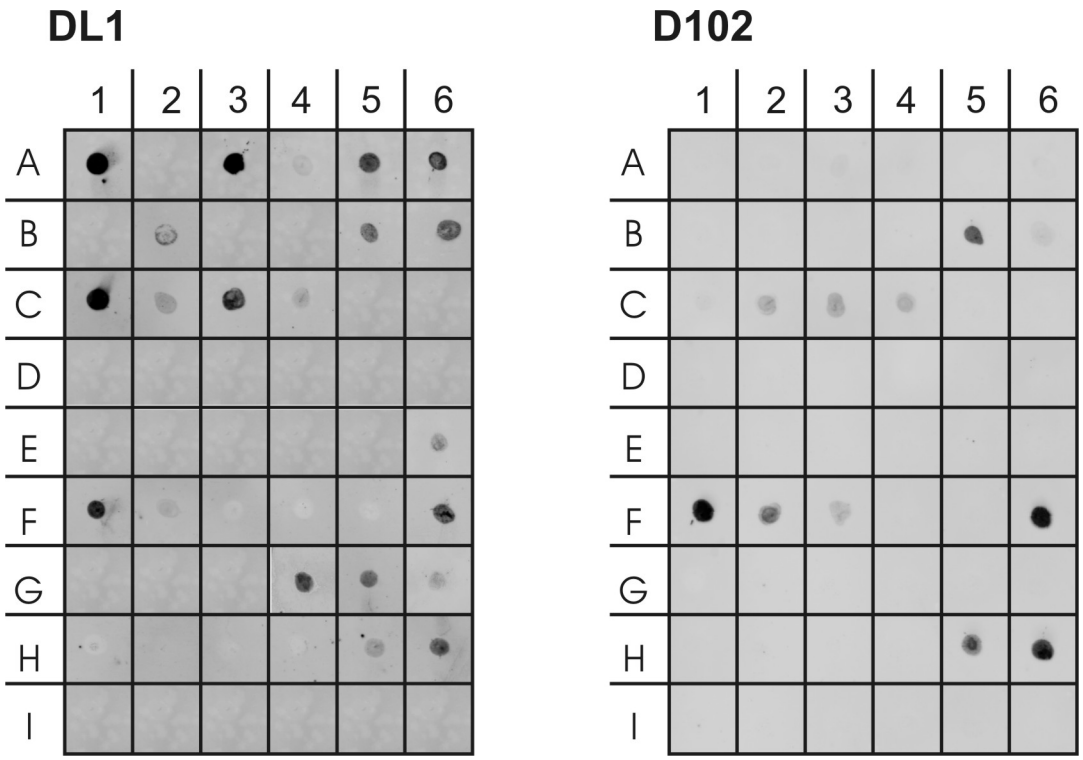

***Fig. 1**: Binding of* S. gordonii *wild-type strain DL1 and its mutant D102 to immobilized glycoproteins and neoglycoproteins. Nitrocellulose membranes, spotted with (neo-)glycoproteins (1 µg per spot), were incubated with FITC-labeled bacteria and fluorescent signals were recorded by a fluorescence scanner. Spots can be attributed to array numbers listed in Table 1.*

Recognition of natural and synthetic glycoproteins by A. naeslundii

The well-known Gal/GalNAc-dependent binding characteristics of *A. naeslundii* could be shown clearly by the bacterial overlay assay using the FITC-labeled WVU45 wild-type strain in comparison to the nonfimbriated mutant strain WVU45M (Fig. 2). *A. naeslundii* WVU45 bound to almost all Galβ- and GalNAcβ-containing neoglycoconjugates (D1, E1, F1, F3-F6, G1, H2, H6, I1) with the exception of Lewis a- and Lewis x-antigen, that show a fucose-side chain at the Galβ-terminus. In contrast, no binding of *A. naeslundii* to Galα- and GalNAcα-containing sugars could be observed. Furthermore, asialofetuin (A2), glycophorin A (A3), asialoglycophorin A (A4), thyroglobulin (B1), α-amylase (B6), S-IgA (C1) and lactoferrin (C2) were bound by WVU45. The spontaneous mutant strain WVU45M showed only negligible binding activities to some components on the array.

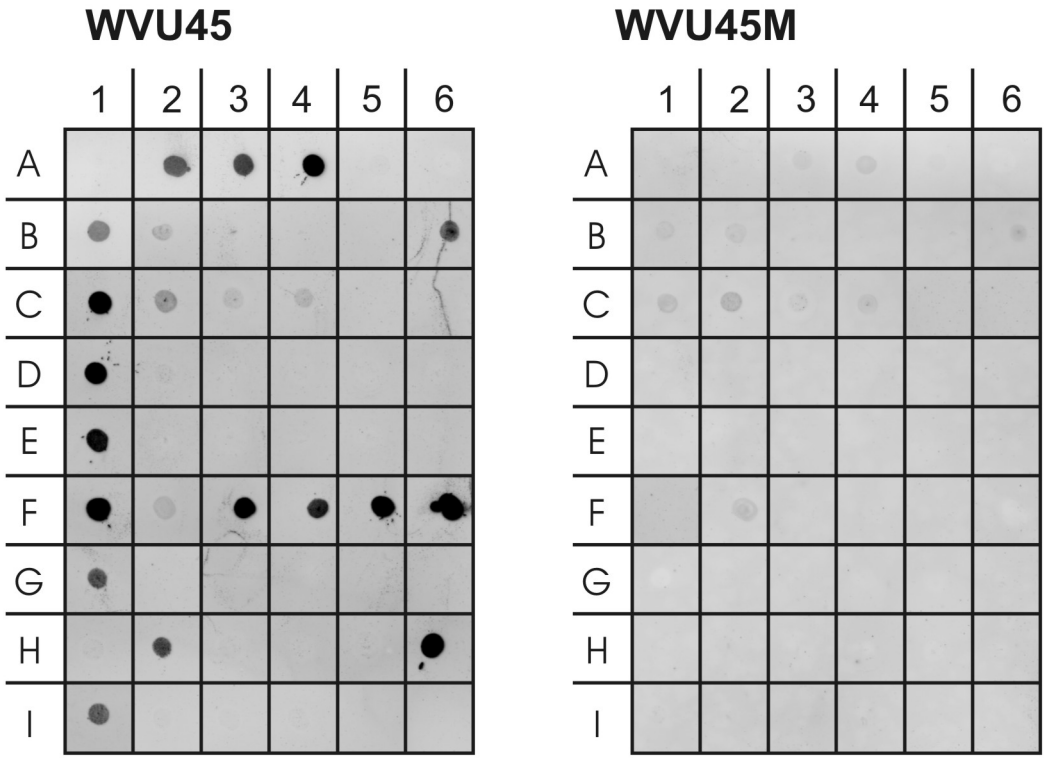

Fig. 2*: Binding of* A. naeslundii *wild-type strain WVU45 and its mutant WVU45M to immobilized glycoproteins and neoglycoproteins. Nitrocellulose membranes, spotted with (neo-)glycoproteins (1 µg per spot), were incubated with FITC-labeled bacteria and fluorescent signals were recorded by a fluorescence scanner. Spots can be attributed to array numbers listed in Table 1.*

Salivary receptors for S. gordonii *revealed by 2-D PAGE*

In order to test for the adherence of *S. gordonii* DL1 to salivary proteins, 2-D gels of WS were transferred onto nitrocellulose membranes and fluorescently labeled *S. gordonii* DL1 wild-

type strain was applied to the nitrocellulose membrane surface (Fig. 3B). The identification of salivary receptors of *S. gordonii* DL1 was achieved in the following steps: First, all WS proteins on 2-D transfers were detected using the Sypro Ruby® blot stain (Fig. 3A). Subsequently, colocalization of bacterial bound spots was determined. Identity of most protein spots was known from a previously performed proteome analysis of human WS [16]. *S. gordonii* DL1 bound to salivary mucin MUC7, the secretory component of the poly-Ig-receptor, zinc-α_2-glycoprotein, Ig-α2-heavy chain, carbonic anhydrase VI, and proline-rich glycoprotein. Furthermore, it recognized acidic and basic PRPs.

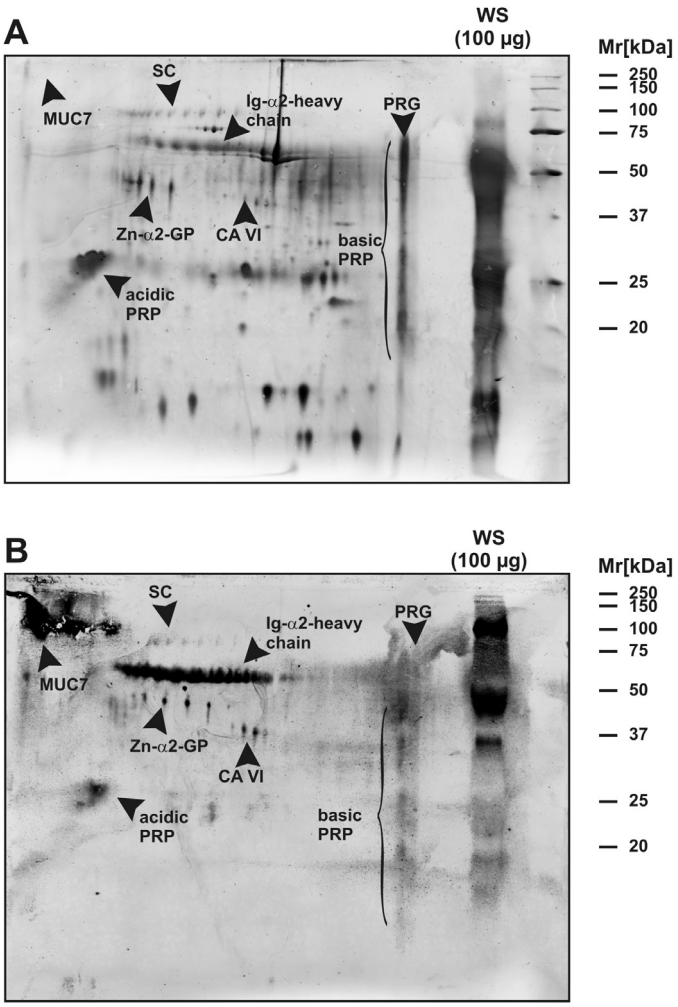

Fig. 3*: Binding of* S. gordonii *wild type strain DL1 to proteins of human whole saliva (WS). Proteins were separated by 2D-PAGE, transferred onto a nitrocellulose membrane, A. stained with Sypro Ruby ® blot stain and B. overlaid with FITC-labeled bacteria. Fluorescent signals were recorded by a fluorescence scanner. Proteins bound by DL1 were identified by comparison with a previously established 2-D map of WS [16]. SC: secretory component of the poly-Ig-receptor; Zn-α2-GP: zinc-α2-glycoprotein; CAVI: carbonic anhydrase VI; PRG: proline-rich glycoprotein; PRP: proline-rich protein;*

Discussion

Adhesion is a key event in bacterial colonization of a host [17]. Therefore, adhesion studies are an important method to obtain more information about the biology and pathology of bacteria. One possibility to study adhesin specificities of microbes is to perform bacterial overlays on immobilized proteins [2-5]. Previous methods for the investigation of bacterial adhesion used radiolabeled bacteria or biotinylated bacteria. Both approaches suffer from a number of disadvantages: radiolabeling cannot be performed in every laboratory, and biotinylation is time consuming. The use of FITC-labeled bacteria in overlay assays is easier and faster than the above methods. This technique has been used successfully to characterize binding specificities of *H. pylori* [6]. Because FITC is an amine-reactive fluorescein-derivate that labels proteins by covalent binding [18], the question arose, of whether or not this method works also with other bacteria that express other types of adhesins, or if FITC interferes in some way with the adhesion process. Examining this question was the immediate aim of this study.

S. gordonii DL1 is known to bind preferentially to receptors with α2,3-linked sialic acid termini [14]. Sialic acid-containing glycoproteins and neoglycoproteins were previously used in a glycoconjugate array for bacterial overlay with FITC-labeled DL1 [6], in which this adhesion property could be confirmed. This study reproduced the finding that *S. gordonii* DL1 binds α2,3-linked sialic acid on natural glycoproteins and on neoglycoproteins. We confirmed also that sub-terminal sugars might play a role in the recognition because sialyl-Lewis a and MUC5B were no substrates for binding.

Some glycoproteins and neoglycoproteins were newly investigated in this dot-blot-array for the first time, and were also recognized by *S. gordonii*. For the mucin from bovine submaxillary gland and α_1-acid glycoprotein a sialic acid-dependent interaction is very likely because *S. gordonii* DL1 bound these proteins whereas the mutant strain D102 did not. The mucin preparation contained 9-17% sialic acid (declaration of the manufacturer), which is probably recognized by the Hsa adhesin of *S. gordonii*. α_1-Acid glycoprotein is also known to be extensively sialylated and contains, amongst others, α2,3-linked sialic acids [19]. Thus, again, a Hsa-dependent adhesion could be suggested. S-IgA was strongly bound by *S. gordonii* wild-type strain DL1 whereas D102 did not bind. This result is consistent with previous observations that adhesion of *S. gordonii* to S-IgA is due to α2,3-linked sialic acids on the *O*-linked oligosaccharides of IgA1 [20].

Furthermore, an interaction of α-amylase with *S. gordonii* DL1 could be shown whereas the *hsa*-lacking D102 showed no interaction. This result was reproduced several times, and a sialic acid-dependent interaction might be possible. This is supported by the structure of α-amylase. α-Amylase contains 1 mol sialic acid *per* mol [21]. However, it was hypothesized that the sialic acid terminus is α2,6-linked. This sugar structure is known not to be recognized by *S. gordonii* DL1 [6]. In addition, *S. gordonii* DL1 did not bind salivary α-amylase on 2-D transfers of WS. This was also observed in a previous study for 1-D blots of salivary glandular secretions [4]. Assuming, that the interaction of *S. gordonii* with α-amylase is abolished by denaturing, as it takes place during the electrophoresis process, a protein-protein-interaction rather than a lectin-like interaction might be possible. This was also supposed for adhesion of *S. gordonii* to α-amylase coated hydroxyapatite [22]. In this regard, amylase-binding proteins AbpA and AbpB could be identified [23, 24]. Therefore, the question of why only *S. gordonii* wild-type strain but not the *hsa*-lacking mutant strain bound to spotted α-amylase has to be investigated in more detail.

PRP-1 is the sole protein on the array that is not glycosylated, with the exception of HSA and BSA. It belongs to the group of acidic PRPs and was investigated several times for adhesion of oral streptococci [4, 25-27]. On this array PRP-1 was bound by both, wild-type strain DL1 and mutant strain D102, indicating a sialic acid-independent interaction. This result confirms previous statements of a PRP-binding adhesin of *S. gordonii* that binds to peptide motifs of acidic PRPs [25].

Binding to fibronectin was noticed for *S. gordonii* wild-type strain DL1 and weaker for mutant strain D102. This result might be explained by two effects. On the one hand fibronectin contains sialic acid residues [28], that might be bound only by the wild-type strain. Residual binding as seen on the array of D102, in contrast, might be sialic acid-independent. Probably this adhesion is mediated by one of the fibronectin-binding proteins, FbpA or CshA, that were previously reported for *S. gordonii* [29].

Beside the sialic-acid dependent binding property of *S. gordonii* DL1, it is known that this strain coaggregates with other streptococci due to GalNAcβ1-3Gal-receptors presented by streptococcal cell wall polysaccharides, e.g. of *S. oralis* [15]. Results of the dot-blot overlay confirmed the preferred binding of *S. gordonii* DL1 to immobilized neoglycoconjugates with GalNAcβ1-3Gal termini as reported previously by using biotinylated bacteria [5]. The use of monosaccharide-neoglycoconjugates suggested, in addition, that the avidity of *S. gordonii* DL1 to GalNAcβ may be higher than to GalNAcα. It is also noteworthy that the linkage of

terminal GalNAc to Gal alone is not sufficient for binding of DL1, because this strain did not bind to GalNAcβ1-4Galβ. Thus, not only the order of saccharides but also their type of linkage is crucial for bacterial adhesion.

A. naeslundii WVU45 have type 2 fimbriae responsible for binding of Gal- and GalNAc containing receptors [8, 12] on certain streptococci and epithelial surfaces. In the present study, this property could be verified on immobilized neoglycoproteins not only concerning the type of saccharides but also the preference for a β-linkage of Gal and GalNAc, respectively. In addition, we found that *A. naeslundii* WVU45 bound to asialofetuin but not to fetuin. This property has been explained by the presence of terminal sialic acid on fetuin that blocks the recognition of subterminal galactose [20]. In the case of glycophorin A and asialoglycophorin A, it could be observed that *A. naeslundii* WVU45 bound independently of sialylation. On the asialoglycophorin A, WVU45 binds probably to terminal galactose of the *O*-glycan chains. On glycophorin A, it could bind to the few non-masked terminal Galβ1-4GlcNAc-epitopes of the asparagines-linked *N*-glycans [30]. Adhesion to immobilized S-IgA by *A. naeslundii* WVU45 was also confirmed. This interaction had been observed previously and was attributed to *O*-linked oligosaccharides at the hinge region of the IgA1 heavy chain [20].

FITC-labeled *H. pylori* has been used successfully on transfers of 2-D gels [7]. In this study we have used *S. gordonii* as a probe in order to test for the applicability of this technique to other bacteria, because *S. gordonii* initiates the development of oral biofilms by adhesion to various salivary proteins of the acquired pellicle [9]. This overlay experiment was carried out with *S. gordonii* DL1 on transfers of separated WS proteins. Among the detected salivary receptors some previously published interactions of *S. gordonii* DL1 with salivary proteins were confirmed, namely the recognition of salivary mucin MUC7 [4], acidic PRPs [4], IgA α_2 heavy chain [20] and proline-rich glycoprotein [4]. No information was available on the adhesion of *S. gordonii* to, or the interaction of *S. gordonii* with the secretory component of the poly-Ig-receptor, basic PRPs, zinc-α_2-glycoprotein, and carbonic anhydrase VI. As already mentioned, α2,3-linked sialic acid on *O*-linked oligosaccharides of the IgA1 heavy chain was thought to be responsible for the recognition of S-IgA by *S. gordonii* [20]. At present, only speculation is possible about the binding of *S. gordonii* DL1 to the secretory component, another part of S-IgA presenting only *N*-glycans [31]. Either the respective group of spots on the transfer is not the secretory component, or DL1 binds, additionally, other parts of S-IgA than only the sialic acid containing *O*-glycans of IgA1. Considering structural studies on the secretory component *N*-glycans, it seems likely that *S. gordonii* might also

interact with this part of S-IgA because over 70% of the glycans are sialylated. Sialic acid occurs on both forms, α2,3 and α2,6-linked [31]. Concerning the detected binding of *S. gordonii* DL1 to basic PRPs it can be hypothesized that this interaction is based on the recognition of the same or a similar peptide motif than in the interaction of *S. gordonii* with acidic PRPs. Previous studies indicated that the minimal segment of acidic PRP that promoted adhesion of *S. gordonii* was the carboxy-terminal dipeptide Pro-Gln [25]. This dipeptide was also found as carboxy-terminus in three of the basic PRPs [32]. Thus, it has to be investigated in more detail, if this is the responsible receptor epitope of basic PRPs for *S. gordonii*. Binding of DL1 to zinc-α_2-glycoprotein and carbonic anhydrase VI is most likely due to sialic acid present on these molecules [33, 34]. Further experiments using the *hsa*-deficient mutant D102 could solve this question.

It must be stated, that in this chapter only the Hsa-adhesin of *S. gordonii* was discussed in more detail because this is the best-characterized adhesin of *S. gordonii* that mediates a lectin-like interaction. However, there are several other adhesins of oral streptococci reported in the literature. Some of these have been mentioned above: the amylase-binding proteins A and B (AbpA and AbpB) [23, 24], the fibronectin binding adhesins CshA and FbpA [29] as well as the PRP-binding adhesin [25]. In addition, the antigen I/II proteins have been described as multi-functional adhesins, that bind human salivary glycoproteins as well as other microbial cells [35]. The *S. gordonii* suface proteins SspA and SspB belong to this group of proteins and are known to play a role in adherence to salivary proteins and in coaggregation with *A. naeslundii* [36]. First experiments with isogenic *sspA* and *sspB* mutants of *S. gordonii* [36] on glycoconjugate arrays were carried out. Because these mutants showed no loss of binding activity on the various sugar structures it was concluded, that non of these adhesins mediate a lectin-like interaction (data not shown).

Concluding Remarks

Fluorescence-labeling of microbes for bacterial overlay on immobilized proteins is an easy and powerful method to study adhesin specificities of bacteria because the type of saccharides and necessary linkages known from previous studies, were also recognized by FITC-labeled bacteria in this study. Glycoarrays with various glycoproteins and neoglycoproteins were in this regard very helpful. Finally, it could be shown that the 2-D overlay is suitable for different strains of bacteria and for testing of lectin-dependent and peptide-dependent bacterial interactions.

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Chapter 8

Summary and Conclusion

Since the discovery of *Helicobacter pylori* (*H. pylori*) in 1983 [1] enormous progress has been made in determining the pathogenesis of this microbe in gastric disease. While the way of transmission is still under dispute, it is generally accepted that *H. pylori* must reach the stomach *via* the oral cavity [2, 3]. However, there are only few studies about interactions of *H. pylori* with salivary components and no study about the influence of saliva on *H. pylori* exists so far (**chapter 1**). The immediate aim of this thesis was to search for possible interactions of *H. pylori* with glycosylated salivary proteins, by developing an appropriate methodological approach to identify such interactions and to characterize the molecular basis of such interactions.

Proteome analysis of human saliva

In order to utilize two-dimensional gel electrophoresis (2-DE) and subsequent transfer of separated proteins onto nitrocellulose membrane (blotting) for a high resolution bacterial overlay, a proteome analysis of human whole saliva (WS), parotid, and submandibular-sublingual (SMSL) secretions was performed. This was carried out by means of 2-D gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

In the first part of this thesis, 2-DE of human saliva was optimized in order to obtain protein maps as complete as possible with a good quality of the spot pattern (**chapter 3**). Testing and comparing different pH ranges in the first dimension, the optimal pH range for isoelectric focusing (IEF) of salivary proteins could be evaluated. It turned out that pH 3-10 was most suitable to display salivary proteins in a 2-D gel. pH 4-7 and 6-11, in contrast, showed only a limited range of protein spots. The way of sample application in the IEF step affected the number of spots and the quality of the spot pattern. The best result was achieved when the IPG strip was rehydrated directly with the saliva sample under application of voltage. WS was initially lyophilized and dialyzed and a first spot analysis of the resulting 2-D gel was carried out by MALDI-MS. In this gel, a total of 253 spots could be identified, whereof 138 spots were identified as keratin. It can be assumed that the keratin contamination (mainly keratin 4 and 13) originates from cellular debris. In addition, a number of other cellular proteins were identified that may originate from the oral epithelium. Thus, the use of saliva that is directly lyophilized turned out to be inapplicable for proteome analysis. Filtration of WS prior to gel electrophoresis improved the results by reducing the number of spots, including most spots that were identified as keratin. In addition, a new spot group that stained pink with Coomassie blue could be detected. It was hypothesized that these spots are

acidic proline-rich proteins (PRPs) - a major class of salivary proteins that were absent from most of the previously performed 2-DE studies on WS.

This hypothesis could be verified by the following proteome analysis of filtrated WS (**chapter 4**). In filtrated WS, 131 spots could be identified. Only two of these spots were keratin. The pink-stained spot group turned out to be acidic Db-type PRPs. In addition, basic PRPs and the glycosylated PRP could be identified. Beside the proteome analysis of filtrated WS, a proteome analysis of parotid secretion and, for the first time, of SMSL secretion was performed. In parotid secretion 43 spots and in SMSL secretion 53 spots were identified. Because the 2-D maps of WS and glandular secretions should be used as tools for adhesion studies on *H. pylori*, the extent of intra-individual and inter-individual variations of 2-D spot pattern was investigated. For intra-individual comparison, salivary 2-D gels from one individual collected at 3 different days were compared. These gels of WS showed a good reproducibility in their overall spot pattern but with some quantitative variations whereas glandular secretions showed no apparent differences in the spot pattern between the three collection days. For inter-individual comparison, salivary 2-D gels from four different individuals were compared. In this case it can be summarized that there was a consistency of most major spots. Again, whole saliva showed mainly quantitative differences. Glandular secretions appeared similar with only few distinct differences.

Glycoconjugate array

For adhesion studies on *H. pylori*, isogenic mutants were used, lacking either the BabA- and/or SabA-adhesins [4]. These mutants and their wild-type strain J99A could be very well characterized by overlay with fluorescence-labeled bacteria on immobilized (neo)glycoproteins (**chapter 5**). Interaction between the adhesin BabA and the H-1-, Lewis b- and related fucose-containing antigens could be confirmed [5]. The previously described interaction of *H. pylori* with terminal α2-3-linked sialic acids could be shown too [6]. The use of a *sabA* mutant and sialidase treatment of glycoconjugate arrays showed for the first time that the adherence of *H. pylori* to laminin is mediated by the sialic acid-binding adhesin, SabA. In addition, it could be shown that the adhesion to salivary mucin MUC5B is mainly associated with the BabA adhesin, and to a lesser extent with the SabA adhesin. It turned out that the adhesion of *H. pylori* to fibronectin and lactoferrin persisted in the *babA/sabA* double mutant. This binding could be abolished by denaturation but not by deglycosylation. Therefore, it was suggested that this interaction may depend on the recognition of unknown receptor moieties by one or more additional unknown bacterial surface components.

In order to show that the newly developed fluorescence-labeling of bacteria in the overlay experiment does not interfere with adhesion properties, a bacterial overlay on glycoconjugate arrays was carried out with two other well-defined species of oral bacteria (actinomyces and streptococci) (**chapter 7**). On these arrays all known binding specificities of sialic acid/*N*-acetylgalactosamine- (*S. gordonii*) [7, 8] and galactose/*N*-acetylgalactosamine- (*A. naeslundii*) [9] reactive lectins could be confirmed, providing evidence that fluorescence-labeling of bacteria may be a universally applicable method.

Adhesion studies on human saliva

Characterized *H. pylori* mutants were applied to blots of 1-D and 2-D gels of human saliva by bacterial overlay (**chapter 6**). Three receptor molecules of *H. pylori* detected by 1-D overlay could successfully be identified by MALDI-MS, confirming the binding of *H. pylori* to MUC5B, MUC7 and gp-340 [10]. Adhesion to MUC5B was associated with both the SabA and the BabA adhesins. Adhesion to MUC7 could now be associated with the SabA adhesin and adhesion to gp-340 was shown to be mediated by the BabA adhesin. The 2-D overlay revealed novel salivary receptors for *H. pylori*. Identification of these receptors was achieved by comparison of the overlay membrane with the established proteome maps of human saliva (**chapter 4**). Binding of *H. pylori* to the proline-rich glycoprotein was detected for the first time and assigned to the activity of the BabA adhesin. The SabA adhesin was found responsible for binding to other newly detected receptor molecules, including carbonic anhydrase VI, secretory component (poly-Ig-receptor), parotid secretory protein and zinc-α2-glycoprotein.

In order to show the applicability of the 2-D overlay technique for other species of bacteria, it was also carried out with *S. gordonii* DL1 (**chapter 7**). This revealed some expected interactions of *S. gordonii* DL1 with salivary receptors (e.g. MUC7, proline-rich glycoprotein, acidic PRPs and IgA α2-heavy chain) as well as so far unknown interactions including the secretory component of the poly-Ig-receptor, basic PRPs, zinc-α2-glycoprotein and carbonic anhydrase VI.

Conclusion

In conclusion, this thesis combined for the first time successfully a proteomics approach with the bacterial overlay technique. This method showed not only the binding of *H. pylori* to salivary proteins but facilitated the identification of respective receptor molecules considerably. In addition, this technique was found to be suitable for other bacteria. Thus, a

widely applicable tool for studying adhesion of bacteria was developed. To what extent the obtained results maybe of importance for bacterial pathogenesis has to be investigated in the future.

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Appendices

List of Abbreviations

1-D	one-dimensional
2-D	two-dimensional
2-DE	two-dimensional gel electrophoresis
AlpAB	adherence-associated lipoprotein A/B
APD	acetylphenyendiamine
APE	acetylphenylethyl
BabA	blood group antigen binding adhesin
BCA	bicinchoninic acid
BSA	bovine serum albumin
CagA	cytotoxin-associated gene A
CHAPS	3-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid
DTT	dithiothreitol
ECM	extracellular matrix proteins
FITC	fluorescein-5-isothiocyanate
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
H. pylori	Helicobacter pylori
HSA	human serum albumin
IEF	isoelectric focusing
IPG	immobilized pH gradient
LC	liquid chromatography
LTA	*Lotus tetragonolobus* agglutinin
MALDI-TOF	matrix-assisted laser desorption/ionization-time-of-flight
MALT	(mucosa-associated lymphoid tissue)-lymphoma
M_r	relative molecular mass (dimensionless)
MS	mass spectrometry
MudPIT	multidimensional protein identification technology
NeuNAc	*N*-acetylneuraminic acid
NL	non-linear
OMP	outer membrane protein
PAGE	polyacrylamide gel electrophoresis

PAP	*p*-aminophenyl
PBS	phosphate buffered saline
PCR	polymerase-chain-reaction
PFF	peptide fragmentation fingerprint
p*I*	isoelectric point
PMF	peptide mass fingerprint
PMN cells	polymorph nuclear leukocytes
PRG	proline-rich glycoprotein
PRP	proline-rich protein
SabA	sialic acid-binding adhesin
SDS	sodium dodecyl sulfate
S-IgA	secretory immunglobulin A
SMSL	submandibular-sublingual
SNAP	similarity-neighborhood approach
TBS	Tris-buffered saline
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
U	unit
UEA-I	*Ulex europaeus* agglutinin
WS	human whole saliva
wt	wild-type

Supplementary Material to Chapter 4

***Supplementary Figure**. Amino acid sequences of proteins newly mapped in 2-DE of human saliva. Amino acid sequences determined by MALDI-PFF are highlighted in dark gray and amino acid sequences determined by MALDI-PMF are highlighted in light gray.*

gi|1310882 **Cyclophilin B**

```
        10         20         30         40         50
GPKVTVKVYF DLRIGDEDVG RVIFGLFGKT VPKTVDNFVA LATGEKGFGY
        60         70         80         90        100
KNSKFHRVIK DFMIQGGDFT RGDGTGGKSI YGERFPDENF KLKHYGPGWV
       110        120        130        140        150
SMANAGKDTN GSQFFITTVK TAWLDGKHVV FGKVLEGMEV VRKVESTKTD
       160        170
SRDKPLKDVI IADCGKIEVE KPFAIAKE
```

gi|16116524 **CST1**

```
        10         20         30         40         50
MAQYLSTLLL LLATLAVALA WSPKEEDRII PGGIYNADLN DEWVQRALHF
        60         70         80         90        100
AISEYNKATK DDYYRRPLRV LRARQQTVGG VNYFFDVEVG RTICTKSQPN
       110        120        130        140
LDTCAFHEQP ELQKKQLCSF EIYEVPWENR RSLVKSRCQE S
```

gi|7239109 **HSPC059**

```
        10         20         30         40         50
MPHLLVTFRD VAIDFSQEEW ECLDPAQRDL YRDVMLENYS NLISLDLESS
        60         70         80         90        100
CVTKKLSPEK EIYEMESLQW ENMGKRINHH LQYNGLGDNM ECKGNLEGQE
       110        120        130        140        150
ASQEGLYMCV KITCEEKATE SHSTSSTFHR IIPTKEKLYK CKECRQGFSY
       160        170        180        190        200
LSCLIQHEEN HNIEKCSEVK KHRNTFSKKP SYIQHQRIQT GEKPYECMEC
       210        220        230        240        250
GKAFGRTSDL IQHQKIHTNE KPYQCNACGK AFIRGSQLTE HQRVHTGEKP
       260        270        280        290        300
YDCKKCGKAF SYCSQYTLHQ RIHSGEKPYE CKDCGKAFIL GSQLTYHQRI
       310        320        330        340        350
HSGEKPYECK ECGKAFILGS HLTYHQRVHT GEKPYICKEC GKAFLCASQL
       360        370        380        390        400
NEHQRIHTGE KPYECKECGK TFFRGSQLTY HLRVHSGERP YKCKECGKAF
       410        420        430        440        450
ISNSNLIQHQ RIHTGEKPYK CKECGKAFIC GKQLSEHQRI HTGEKPFECK
       460        470        480        490        500
ECGKAFIRVA YLTQHEKIHG EKHYECKECG KTFVRATQLT YHQRIHTGEK
       510        520        530        540        550
PYKCKECDKA FIYGSQLSEH QRIHRGEKPY ECKQCGKAFI RGSHLTEHLR
       560        570        580        590        600
THTGEKPYEC KECGRAFSRG SEHTLHQRIH TGEKPYTCVQ CGKDFRCPSQ

LTQHTRLHN
```

gi|40549418 **Lactoperoxidase; salivary peroxidase**

```
        10         20         30         40         50
MRVLLHLPAL LASLILLQAA ASTTRAQTTR TSAISDTVSQ AKVQVNKAFL
        60         70         80         90        100
DSRTRLKTAM SSETPTSRQL SEYLKHAKGR TRTAIRNGQV WEESLKRLRQ
       110        120        130        140        150
KASLTNVTDP SLDLTSLSLE VGCGAPAPVV RCDPCSPYRT ITGDCNNRRK
       160        170        180        190        200
PALGAANRAL ARWLPAEYED GLSLPFGWTP GKTRNGFPLP LAREVSNKIV
       210        220        230        240        250
GYLNEEGVLD QNRSLLFMQW GQIVDHDLDF APDTELGSSE YSKAQCDEYC
       260        270        280        290        300
IQGDNCFPIM FPPNDPKAGT QGKCMPFFRA GFVCPTPPYK SLAREQINAL
       310        320        330        340        350
TSFLDASFVY SSEPSLASRL RNLSSPLGLM AVNQEVSDHG LPYLPYDSKK
       360        370        380        390        400
PSPCEFINTT ARVPCFLAGD SRASEHILLA TSHTLFLREH NRLARELKRL
       410        420        430        440        450
NPQWDGEKLY QEARKILGAF VQIITFRDYL PILLGDHMQK WIPPYQGYSE
       460        470        480        490        500
SVDPRISNVF TFAFRFGHLE VPSSMFRLDE NYQPWGPEPE LPLHTLFFNT
       510        520        530        540        550
WRMVKDGGID PLVRGLLAKK SKLMKQNKMM TGELRNKLFQ PTHRIHGFDL
       560        570        580        590        600
AAINTQRCRD HGQPGYNSWR AFCDLSQPQT LEELNTVLKS KMLAKKLLGL
       610        620        630        640        650
YGTPDNIDIW IGAIAEPLVE RGRVGPLLAC LLGKQFQQIR DGDRFWWENP
       660        670        680        690        700
GVFTNEQKDS LQKMSFSRLV CDNTRITKVP RDPFWANSYP YDFVDCSAID
       710
KLDLSPWASV KN
```

gi|16116524 **Mucin 7**

```
        10         20         30         40         50
MKTLPLFVCI CALSACFSFS EGRERDHELR HRRHHHQSPK SHFELPHYPG
        60         70         80         90        100
LLAHQKPFIR KSYKCLHKRC RPKLPPSPNN PPKFPNPHQP PKHPDKNSSV
       110        120        130        140        150
VNPTLVATTQ IPSVTFPSAS TKITTLPNVT FLPQNATTIS SRENVNTSSS
       160        170        180        190        200
VATLAPVNSP APQDTTAAPP TPSATTPAPP SSSAPPETTA APPTPSATTQ
       210        220        230        240        250
APPSSSAPPE TTAAPPTPPA TTPAPPSSSA PPETTAAPPT PSATTPAPLS
       260        270        280        290        300
SSAPPETTAV PPTPSATTLD PSSASAPPET TAAPPTPSAT TPAPPSSPAP
       310        320        330        340        350
QETTAAPITT PNSSPTTLAP DTSETSAAPT HQTITSVTTQ TTTTKQPTSA
       360        370
PGQNKISRFL LYMKNLLNRI IDDMVEQ
```

gi|88462 **Proline-rich phosphoprotein (gene *PRH1*, Db allele)**

```
        10         20         30         40         50
QDLNEDVSQE DVPLVISDGG DSEQFLDEER QGPPLGGQQS QPSAGDGNQD
        60         70         80         90        100
DGPQQGPPQQ GGQQQQGPPP PQGKPQGPPQ QGGQQQQGPP PPQGKPQGPP
```

```
       110         120         130         140         150
QQGGHPPPPQ GRPQGPPQQG GHPRPPRGRP QGPPQQGGHQ QGPPPPPPGK
       160         170
PQGPPPQGGR PQGPPQGQSP Q
```

gi|41349486 **Proline-rich protein *Bst*NI, subfamily 1**

```
        10          20          30          40          50
MLLILLSVAL LALSSAQNLN EDVSQEESPS LIAGNPQGPS PQGGNKPQGP
        60          70          80          90         100
PPPPGKPQGP PPQGGNKPQG PPPPGKPQGP PPQGDKSRSP RSPPGKPQGP
       110         120         130         140         150
PPQGGKPQGP PAQGGSKSQS ARSPPGKPQG PPQQEGNNPQ GPPPPAGGNP
       160         170
QQPQAPPAGQ PQGPPRPPQG GRPSRPPQ
```

gi|20070125 **Prolyl 4-hydroxylase**

```
        10          20          30          40          50
MLRRALLCLA VAALVRADAP EEEDHVLVLR KSNFAEALAA HKYLLVEFYA
        60          70          80          90         100
PWCGHCKALA PEYAKAAGKL KAEGSEIRLA KVDATEESDL AQQYGVRGYP
       110         120         130         140         150
TIKFFRNGDT ASPKEYTAGR EADDIVNWLK KRTGPAATTL PDGAAAESLV
       160         170         180         190         200
ESSEVAVIGF FKDVESDSAK QFLQAAEAID DIPFGITSNS DVFSKYQLDK
       210         220         230         240         250
DGVVLFKKFD EGRNNFEGEV TKENLLDFIK HNQLPLVIEF TEQTAPKIFG
       260         270         280         290         300
GEIKTHILLF LPKSVSDYDG KLSNFKTAAE SFKGKILFIF IDSDHTDNQR
       310         320         330         340         350
ILEFFGLKKE ECPAVRLITL EEEMTKYKPE SEELTAERIT EFCHRFLEGK
       360         370         380         390         400
IKPHLMSQEL PEDWDKQPVK VLVGKNFEDV AFDEKKNVFV EFYAPWCGHC
       410         420         430         440         450
KQLAPIWDKL GETYKDHENI VIAKMDSTAN EVEAVKVHSF PTLKFFPASA
       460         470         480         490         500
DRTVIDYNGE RTLDGFKKFL ESGGQDGAGD DDDLEDLEEA EEPDMEEDDD

QKAVKDEL
```

gi|350217 **0508206A peptide PA (proline-rich peptide),saliva**

```
        10          20          30
GPGFVPPPPP PPYGPGRIPP PPPAPYGPGI FPPPPPQP
```

***Supplementary Table**: Spot counts and accession numbers of proteins identified by 2-DE and MS in whole saliva and glandular secretions.*

Spot No.	Spot Count			Protein Name	Accession [a]	Swissprot	Database Values		2-DE Values	
	WS	SMSL Saliva	Parotid Saliva				p*I*	MW	p*I*	MW
1	2	--	--	Actin	14250401	P02570	5.6	41.0	5.2	52.6-53.7
2	1	--	--	Calgranulin A	21614544	P05109	5.7	13.2	6.2	16.3
3	2	--	--	Calgranulin B	4506773		5.7	13.1	5.5-5.6	18.4-18.5
4	9	1	--	Carbonic anhydrase VI	14530767 (WS; S)	P23280	6.6	35.3	5.9	37.5
	--	3	4		58736977 (P, S)		9.1	35.3	5.9-6.2	36.4-38.6
5	4	--	--	Human Zinc-α-2-Glycoprotein	4699583 (WS)	P25311	5.7	31.6	5.2-5.3	41.4-56.3
	--	2	3		7246026 (P, S)		5.6	31.6	5.2-5.4	40.5-41.1
6	1	--	--	Cyclophilin B	1310882	P23284	9.2	19.7	9.1	22.2
7	1	--	--	Cystatin B	4503117	P04080	7.1	11.1	6.6	19.2
8	1	1	--	Cystatin C	14278690	P01034	9.2	12.3	7.9	19.0
9	1	--	--	Cystatin D	539565	P28325	7.6	16.1	6.2	19.2
10	3	--	--	Cystatin SA-III precursor of acquired enamel pellicle	235948		4.7	14.2	4.0-4.4	19.5-22.7
11	1	2	--	Cystatin SA	4503105	P09228	4.7	16.4	3.6-4.5	18.2-18.8

Spot No.	Spot Count			Protein Name	Accession [a]	Swissprot	Database Values		2-DE Values	
	WS	SMSL Saliva	Parotid Saliva				p*I*	MW	p*I*	MW
12	1	2	--	Cystatin SN precursor	19882251 (WS, S)	P01037	6.9	16.4	6.6	18.8
	--	--	1		2144579 (P)		7.0	16.4	6.6	19.1
13	2	--	--	Cytokeratin 1	17318569	P04264	8.2	66.1	7.3	24.5
14	2	--	--	Enolase 1	4503571	P06733	7.0	47.2	6.6	43.3
15	32	6	10	Human salivary amylase	1633119	P04745	6.2-6.3	55.9-57.8	5.9-6.3	52.6-57.6
16	3	--	--	Ig alpha-1 chain	113583	P01876	5.6	37.8	5.6-5.8	56.3-57.6
17	2	--	--	Keratin 10	40354192	P13645	5.1	58.8	6.0	48.3
18	1	--	--	Lactoferrin	494252	P02788	8.5	75.9	8.1	82.8
19	3	4	--	Parotid secretory protein	16755850		5.5	27.1	4.9-5.3	29.8-32.8
20	1	--	--	Phosphoglycerate kinase	4505763	P00558	8.3	44.6	8.1	44.0
21	3	2	4	poly-Ig receptor	514366	P01833	5.3	75.5	5.5-6.1	86.1-93.6
22	2	4	--	Prolactin-inducible protein	4505821	P12273	8.6	16.6	4.5-4.8	17.1-20.5
23	30	8	6	Proline-rich phosphoprotein (gene *PRH1*, Db allele)	88462	sim P02810	4.8	17.5	3.2-6.2	24.3-35.4
24	1	--	3	Proline-rich protein *Bst*NI, subfamily 1, isoform 3	41349486	sim P04280	10.8-11.3	17.7	5.6-9.0	22.5-27.2

Spot No.	Spot Count			Protein Name	Accession [a]	Swissprot	Database Values		2-DE Values	
	WS	SMSL Saliva	Parotid Saliva				p*I*	MW	p*I*	MW
25	2	--	1	Proline-rich protein *Bst*NI, subfamily 3	41349488	sim P10163	10.8	35.1	9.5	57.6
26	1	--	--	Prolyl 4-hydroxylase	20070125	P07237	4.8	57.1	5.0	56.3
27	12	3	3	Serum albumin	4389275	P02768	5.5-5.7	65.2-66.4	5.5-5.7	69.8-72.1
28	1	--	--	Thioredoxin	230939	P10599	4.8	11.4	4.9	18.4
29	6	--	--	Transferrin	4557871	P02787	6.8	77.1	6.2-6.5	79.8
P1	--	--	1	HSPC059	7239109		8.8	70.8	6.1	26.4
P2	--	--	4	Ig alpha-2 heavy chain	184761		5.7	36.4	5.5-5.6	57.6
P3	--	--	4	Ig κ light chain VLJ region	21669311		5.5	27.7	7.1-7.6	26.1-26.4
P4	--	--	1	Lactoperoxidase, salivary peroxidase	40549418		8.9	80.3	7.2	79.8
P5	--	--		Salivary acidic proline-rich phosphoprotein 1/2 precursor	131008	P02810	4.5	17.0	4.1	26.8
S1	--	2	--	CST1	16116524		6.9	16.4	6.1-7.2	18.9-19.0
S2	--	4	--	Cystatin S	4503109	P01036	5.0	16.2	4.0-4.4	19.5-22.7
S3	--	4	--	Histatin 1	4504529	P15515	9.1	7.0	6.2	16.3

Spot No.	Spot Count			Protein Name	Accession [a]	Swissprot	Database Values		2-DE Values	
	WS	SMSL Saliva	Parotid Saliva				p*I*	MW	p*I*	MW
S4	--	1	--	Mucin 7	22748665		9.0	39.2	3.1	120.7
S5	--	1	--	0508206A peptide PA, proline-rich peptide	350217		5.0	3.8	8.9	18.2

[a] WS, whole saliva; P, parotid saliva; S, submandibular-sublingual saliva.

Supplementary Material to Chapter 6

Supplementary Figure. *Amino acid sequences used for identification of MUC5B, MUC7, and gp-340 as determined by MALDI-PMF. Amino acid sequences additionally determined by MALDI-PFF are indicated in bold letters.*

MUC5B (gi|51470790)

m/z calib.	m/z theor.	Error [ppm]	Protein	Sequence
722.3906	722.4201	-40.9	Mucin 5	TSVFIR
830.4343	830.4484	-17	Mucin 5	NREQVGK
893.4259	893.4368	-12.3	Mucin 5	EELPYSR
956.4508	956.4477	3.3	Mucin 5	TFDGDVFR
973.5236	973.5107	13.2	Mucin 5	TWLVPDSR
1036.5907	**1036.5903**	**0.3**	**Mucin 5**	**AQAQPGVPLR**
1091.4588	1091.4944	-32.6	Mucin 5	DPCPAmTANPFR
1107.5469	1107.5434	3.2	Mucin 5	LTDPNSAFSR
1140.6601	1140.6853	-22.1	Mucin 5	GLVGSRPVVTR
1258.7133	1258.7159	-2.1	Mucin 5	LTPLQFGNLQK
1325.6536	**1325.649**	**3.4**	**Mucin 5**	**AAYEDFNVQLR**
1388.7358	1388.7388	-2.2	Mucin 5	AVTLSLDGGDTAIR
1434.7589	1434.7051	37.5	Mucin 5	DMOxAKTWLVPDSR
1463.7832	1463.7892	-4.1	Mucin 5	NGVLVSVLGTTTMOxR
1695.792	1695.8188	-15.8	Mucin 5	SEQLGGDVESYDKIR
1725.7273	1725.7536	-15.2	Mucin 5	DDCPAmLQRDGTTAASCPAmK
2118.1065	2118.1347	-13.3	Mucin 5	VDIPALGVSVTFNGQVFQAR

MUC7 (gi|22748665)

m/z calib.	m/z theor.	Error [ppm]	Protein	Sequence
788.4503	788.4783	-35.6	Mucin 7	QKPFIR
814.4303	**814.4537**	**-28.7**	**Mucin 7**	**FLLYMK**
830.4274	**830.4486**	**-25.5**	**Mucin 7**	**FLLYMOxK**
870.4136	**870.4334**	**-22.8**	**Mucin 7**	**HHHQSPK**
954.46	954.4757	-16.5	Mucin 7	ERDHELR
1026.5257	**1026.5348**	**-8.8**	**Mucin 7**	**RHHHQSPK**
1060.5734	**1060.5788**	**-5.1**	**Mucin 7**	**LPPSPNNPPK**
1061.555	1061.5532	1.7	Mucin 7	FPNPHQPPK
1538.7551	1538.7868	-20.6	Mucin 7	FPNPHQPPKHPDK
1617.8382	**1617.8178**	**12.6**	**Mucin 7**	**SHFELPHYPGLLAH**
2387.2266	2387.2777	-21.4	Mucin 7	SHFELPHYPGLLAHQKPFIR

gp-340 (gi|55962155)

m/z calib.	m/z theor.	Error [ppm]	Protein	Sequence
730.3345	730.3848	-68.9	gp340	LVNGDGR
734.4004	734.4565	-76.5	gp340	VTVIFR
778.4074	778.4463	-50.1	gp340	VEVLYR
820.3731	820.4139	-49.8	gp340	MOxTIHFR
881.4705	**881.4885**	**-20.5**	**gp340**	**FPSVYLR**
904.4614	904.4793	-19.8	gp340	AFHFLNR
991.5614	991.5689	-7.5	gp340	GRVEVLYR
1005.5603	1005.5733	-13	gp340	INLGFSNLK
1075.5536	1075.5536	0	gp340	FISDHSITR
1115.5557	1115.5485	6.4	gp340	QIFTSSYNR
1436.6567	1436.648	6	gp340	GSFTSSSNFMOxSIR
1457.6712	1457.6548	11.2	gp340	DDTYGPYSSPSLR
1459.7465	**1459.7545**	**-5.5**	**gp340**	**FGQGSGPIVLDDVR**
1488.7339	1488.7017	21.6	gp340	QLGCGWATSAPGNAR
1525.7281	1525.7109	11.3	gp340	CPAmVWEIEVNSGYR
1538.7185	1538.7062	8	gp340	CVWDIEVQNNYR
1560.7597	1560.7415	11.7	gp340	QLGCGWAMOxLAPGNAR
1631.7786	1631.7786	0	gp340	QLGCPAmGWAMOxLAPGNAR
2452.0767	2452.0767	0	gp340	DVQLEGGCNYDYIEVFDGPYR
2737.3034	2737.2898	5	gp340	SDISFQNTGFLAWYNSFPSDATLR

Curriculum vitae

Name:	Anke Walz
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Education:

09/84-02/87	Elementary School, Ulm-Lehr
02/87-08/88	Elementary School Bad Abbach
09/88-07/97	Von-Müller-Gymnasium, Regensburg

Professional Training and Experience

11/97-04/02	Study of Pharmacy at the University of Regensburg, Regensburg
10/00-02/01	One semester studies at the Swiss Federal Institute of Technology, Zürich, Switzerland, Prof. Hans P. Merkle
04/02	University degree in pharmaceutical science
05/02-10/02	Traineeship at "Apotheke im Donaueinkaufszentrum", Regensburg
11/02-04/03	Traineeship at the hospital pharmacy of the medical centre of the University of Regensburg
07/03	Approbation as pharmacist
08/03-present	PhD at the Department of Restorative Dentistry and Periodontology, Medical Faculty, University of Regensburg, Regensburg, Prof. Stefan Ruhl
10/05	First Prize Award in the 19th BZÄK/DGZMK/ DENTSPLY Student Clinician Competition

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Walz A, Odenbreit S, Mahdavi J, Borén T, Ruhl S. Identification and characterization of binding properties of *Helicobacter pylori* by glycoconjugate arrays. Glycobiology (2005); **15:** 700-708. **(Chapter 5)**

Walz A, Stühler K, Wattenberg A, Hawranke E, Meyer HE, Schmalz G, Blüggel M, Ruhl S. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis. Proteomics (2006); **6:** 1631-1639. **(Chapter 4)**

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Engel K, Aspholm M, **Walz A**, Ruhl S, Faller G, Haas R, Borén T, Odenbreit S. Interrealtions between AlpAB-, BabA-, and SabA-dependent adherence activities of *Helicobacter pylori*. 2. Gemeinsamer Kongress von DGHM und VAAM, Göttingen, Germany, 2005 [BIOspektrum, **Sonderausgabe 2005**, GIV002]

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