Mucin-bacterial interactions in the human oral cavity and digestive tract

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Mucins are a family of heavily glycosylated proteins that are the major organic components of the mucus layer, the protective layer covering the epithelial cells in many human and animal organs, including the entire gastro-intestinal tract. Microbes that can associate with mucins benefit from this interaction since they can get available nutrients, experience physico-chemical protection and adhere, resulting in increased residence time. Mucin-degrading microorganisms, which often are found in consortia, have not been extensively characterized as mucins are high molecular weight glycoproteins that are hard to study because of their size, complexity and heterogeneity. The purpose of this review is to discuss how advances in mucus and mucin research, and insight in the microbial ecology promoted our understanding of mucin degradation. Recent insight is presented in mucin structure and organization, the microorganisms known to use mucin as growth substrate, with a specific attention on *Akkermansia muciniphila*, and the molecular basis of microbial mucin degradation owing to availability of genome sequences.

Introduction

Mucus is present at the interface between many epithelial surfaces and their extracorporeal environments, including the oculo-rhino-otolaryngeal tracts, the respiratory tract, the gastrointestinal (GI) tract and the reproductive tract. Mucus is a complex viscous secretion, which often forms a continuous layer. The mucus is a highly hydrated gel (~95% water) formed by large glycoproteins, called mucins, which further contains salts, lipids[1] and proteins that are involved in defense such as defensins, growth factors, immunoglobulins, lysozym and trefoil factors and many other intestinal proteins.[2] Humans secrete large volumes of mucus that amounts to approximately 10 liters of mucus per day.[3] Mucus has many roles and can act as: (1) a lubricant, as in the airways and when facilitating the passage of food in the intestine;

(2) a selective barrier by allowing passage of low molecular weight components, such as nutrients to the epithelial cells; and (3) a defense system that protects the underlying epithelial cells from mechanical damage or entrance of harmful substances of either chemical nature, such as drugs, toxins or heavy metals or biological nature, such as luminal pepsin, organic acids, pathogenic bacteria, viruses or parasites.[4-7] Another important function of mucus, which has come to light more recently, is to serve as a substrate for the growth, adhesion and protection of the trillions of microbial cells that are present in the lumen in the GI tract. This review addresses specifically this microbial dimension and summarizes recent insight in mucus structure and organization, the microbes known to use mucin as growth substrate, and the molecular basis of microbial mucin degradation.

Mucus

Mucus is constantly produced, secreted, and shed, a process that can take from a few minutes to several hours depending on the organ and the situation, such as invasion by pathogens that requires a fast response of the epithelium.[8] The thickness and dynamics of the mucus layer differ highly among organs. The oral cavity is covered by a salivary film consisting of water, mucins, and other proteins. Assuming that the saliva is distributed evenly over the tissues the calculated thickness of the salivary film is 70 to 100 µm.[9] The stomach presents a thicker mucus layer (approximately 300 µm), which protects the underlying gastric epithelium against the hostile acidic conditions.[10] The small intestine is mainly covered by a loosely attached mucus layer (150–400 µm), which is thinnest in the jejunum, where the major nutrient uptake takes place.[10] In the colon, the mucus layer thickness follows a gradient from the caecum to the rectum from thin to a thick mucus layer, respectively, reaching a thickness of 800–900 µm in the distal colon.

Histochemical analysis of human biopsies revealed two different mucus sub-layers, as described in the colon.[11] The outer layer designated as a mobile or non-adherent layer, is largely soluble and constantly removed, and acts as a lubricant by expulsing potentially dangerous agents (like microorganisms and viruses) trapped in this layer.[10] The underlying inner layer, also termed adherent layer, is firmly adherent to the epithelial surfaces and not soluble in water. This latter sub-layer acts as a selective barrier,

Table 1. Classification of human mucins: Length of the tandemly repeated amino acid sequence (TR), location of protein expression and chromosomal localization

MUC protein	Amino acids in TR	Main location of expression	Chromosome locus	References
Secreted:				
MUC2	23/16	small intestine, colon, tracheobronchial tissue	11p15.5	128–131
MUC5AC	8/5	stomach, respiratory tissue, Brunner's glands	11p15.5	128, 132–135
MUC5B	29	salivary glands, tracheobronchial tissue, Brunner's glands, endocervix, gall bladder, pancreas	11p15.5	21, 70, 128, 136, 137
MUC6	169	stomach, gall bladder, Brunner's glands	11p15.5	70, 128, 138, 139
MUC7	23	salivary glands	4q13.3	140–142
MUC8	13/41	tracheobronchial tissue	12q24.3	143, 144
MUC19 (predicted protein)	7/7/15/16/9/8/5	salivary glands, tracheobronchial tissue	12q12	145
Membrane-bound:				
MUC1	20	all epithelia	1q22	146–148
MUC3A	17/375	small intestine, colon, gall bladder	7q22	129, 149–151
MUC3B	17/375	small intestine, colon	7q22.1	149–151
MUC4	16	virtually all epithelia	3q29	152, 153
MUC12	28	colon	7q22.1	154
MUC13	15	trachea, small intestine, colon	3q21.2	155
MUC15	none	spleen, small intestine, colon, prostate, lung	11p14.2	156
MUC16	156	ovarian tissue, ocular tissue	19p13.2	157–159
MUC17	59	pancreas, small intestine, colon	7q22.1	20, 35, 160
MUC20	19	renal tissue	3q29	161

and allows only passage of smaller molecules.[4] After removal by suction of the loosely attached outer mucus layer from the firm inner layer, the loosely attached layer is replenished quickly as was studied in tissue of live animals.[2,10] The relative thickness of the outer and inner mucus layer depends on the organs, e.g., in stomach and colon the inner layer is thick (100–150 μm), whereas the firm inner layer is hardly existent in the jejunum.[10] Recently, the inner mucus layer that adheres closely to the epithelium and the more loosely attached outer mucus layer were described in detail in mice.[2] It appears that in the colon the firm inner layer contains hardly any bacteria, but has a similar composition than the outer 'sloppy' layer, which contains a high concentration of bacteria. In both sub-layers, the mucin Muc2 was invariably the main structural constituent, yet the transition from the one to the other layer is abrupt. It is not yet known what defines both sub-layers.[2]

Disturbance of the structure and function of the mucus layer is characteristic for the pathology of many diseases of the respiratory tract, such as asthma, chronic obstructive pulmonary diseases or cystic fibrosis, which are all characterized by over-production of mucus.[12] A disturbed mucus layer integrity is also invariably part of the pathology of disorders of the GI tract like Crohn disease and ulcerative colitis. In case of ulcerative colitis, the mucus layer was found to be thinner in the inflamed part of the tissue, whereas in Crohn disease, the mucus thickness was normal or even higher than usual.[13] The protecting role of the colonic mucus layer has been recently demonstrated in genetically engineered mice deficient in Muc2, which is the dominant structural mucin of intestinal mucus in human and mouse.[14,15] These mice spontaneously develop colitis in the absence of the Muc2 glycoprotein, and also Muc2^{+/-} heterozygous mice have a significantly increased sensitivity towards the colitis-inducing agent dextran sulfate sodium.[16] Thus, the integrity of the mucus layer is crucial to ensure the protection of the underlying epithelium, at least in the mouse colon.

Mucins

Mucus layers consist mainly of gel-forming mucin-type glycoproteins, which are heavily O-glycosylated molecules. These mucins are usually produced in specialized mucous cells of glandular tissues and in goblet cells of the GI tract.[17-21] Biophysically, mucins are responsible for the visco-elastic properties of the mucus-gel, and are characterized by 50–90% O-linked glycans (by weight of the mucin molecules), which are attached to the protein backbone (apomucin). This resuls in very high molecular weight complexes of many millions of Daltons, which are to date impossible to measure accurately. To date 17 human mucin genes have been assigned to the MUC gene family, according to the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC; www.gene.ucl.ac.uk/nomenclature/index.html) (**Table 1**). Based on sequence homology, several mucin families can be distinguished: such as mucus-forming mucins at human chromosome locus 11p15, which have probably evolved through gene duplication of one ancestral gene,[22] and a number of
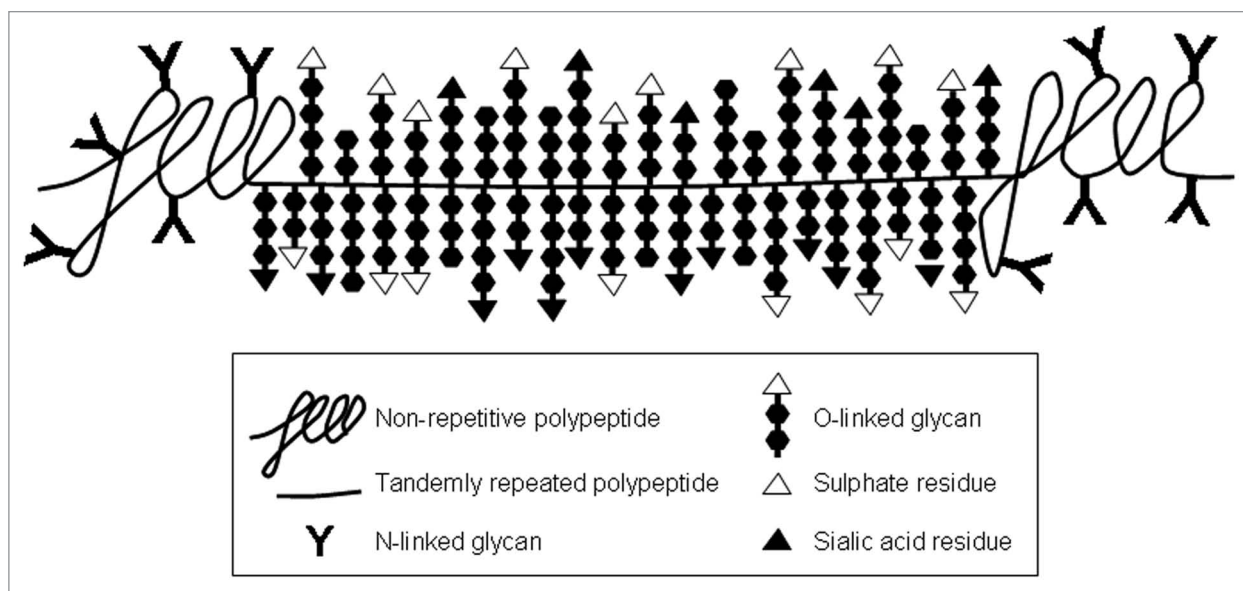
Figure 1. Schematic representation of a mucin molecule. The part of the polypeptide where the O-linked glycans are concentrated is not drawn to scale. Based on electron micrographs of isolated mucin molecules, this glycopeptide comprises at least 70% of the length of the mucin molecule.[40]

membrane-bound mucins at locus 7q22, which might also share a common ancestral gene.[23] Genes encoding mucin-type glycoproteins have appeared at different moments and different locations in our ancestral genome, as excellently (reviewed in ref. 24) Lang et al. Mucin polypeptides have distinct domains: a central domain on which O-glycosylation is concentrated; the so-called 'PTS region' (i.e., region rich in the amino acid residues, proline, threonine and serine, **Fig. 1**). Within the PTS region there is usually a repetitive array of relatively short amino acid sequences, in tandem repeats, which are often variable in number among individuals. The central part of the mucin peptide core is bordered at both ends with amino acid sequences referred as 'unique regions', which sequences differ from the central or PTS region, and have a more normally distributed amino acid composition (**Fig. 1**).

To the tandemly repeated amino acids sequences of the polypeptide a very large number of O-linked carbohydrate chains are added in the Golgi apparatus during biosynthesis. These are linked to the apoprotein via N-acetylgalactosamine (GalNac) that is coupled to the hydroxyl group of either a serine or threonine residue via an O-glycosidic linkage (**Fig. S1**). Then galactose and/or N-acetylglucosamine (GlcNAc) residues are added to this initial GalNAc to form the various core structures, of which there are eight different forms, and 4 of these are the most common on mucins (**Fig. S1**). The backbone region of these glycans consists of successive additions of galactose and either GalNac or GlcNac residues, which may come in three types: type-1 chain (Galβ1-3GlcNAc), type-2 chain (Galβ1-4GlcNAc) or type-3 chain (Galβ1-4GalNAc). The peripheral sugars of the oligosaccharide chains often have an arrangement identical to those found in the ABH histo-blood group antigens present on red blood cells (**Fig. S1**).[25] Other terminal groups may constitute, e.g., the Lewis histo-blood group structures (by addition of fucose residues), and other more rare blood group structures. For example,

human salivary MUC5B glycosylation reflects the ABH and Lewis histo-blood group antigen status of individual.[26,27] Further modification of the glycan structure of mucins often occurs via the addition of sialic acid and/or sulfate residues that give the mucins their overall negative charge and which contributes to the specific functions of mucin. The O-glycosylation that can be found on a single MUC-type mucin from one source is usually very heterogeneous. For example human colonic MUC2 contains more than 100 different O-linked glycans, which range in size from to 2 to 12 monosaccharides, most of which are based on the core 3 structure.[28] Strikingly, the spectrum of these O-glycans was very uniform among human individuals.[28] In the oral cavity, it was demonstrated that the glycosylation of the salivary mucins MUC5B and MUC7 is heterogeneous and can differ between individuals even with the same blood group. These different mucin glycoforms are secreted in the oral cavity by physically separated salivary glands.[29-32] The production and presence of these various glycoforms of the mucins could well play a role in the adhesion of the various oral bacteria that also show a preferred localization at the various sites in the oral cavity (see below).

Based on their structure, mucins are usually subdivided into two classes: secretory and membrane-bound mucins. Secretory mucins are apically secreted by specialized cells, such as the goblet cells in the intestines and airways, and characterized by their high molecular weight, and their ability to form a viscous gel. Membrane-bound mucins, in contrast, are synthesized by epithelial cells, such as MUC1 and MUC3 by enterocytes in the intestine, and integrated into their apical plasma membranes. Secretory mucins contain cysteine-rich sequences, located in the N- and C-terminal regions, which allow the formation of disulfide bridges to form either filamentous multimers,[33,34] or more complex network-like covalent structures as demonstrated for human MUC2.[35] Membrane-bound mucins, such as MUC1 or

MUC3, do not form covalent multimers and are not gel-forming, but do contain a C-terminal trans-membrane anchor.[36] Membrane-bound mucins have been thought to play a role as cell surface receptors and sensors, which translate information about external conditions into cellular responses including proliferation, differentiation, apoptosis or secretion of specialized cellular products.[37]

Depending on the sugar composition, mucins can be differentiated bio- and histochemically as neutral or acidic mucins. Acidic mucins contain substantial amounts of sialic acid ('sialomucins') and/or sulfate residues ('sulfomucins') giving a strong negative charge to the mucin molecule. This distinction between sialomucins/sulfomucins and acidic/neutral mucins is hardly ever absolute. Most mucins carry at least some of these negatively charged groups and at thus not neutral in the strict sense. Also many mucins might carry both sulfate—and sialic acid residues, and the name sialo/sulfomucins in fact only indicates the predominance of one or the other negatively charged group. The sulfation has been proposed to provide extra protection of the underlying epithelium against degradation by the high density of bacteria, because sulfation confers relative resistance to most bacterial mucin-degrading enzymes.[38,39] There is usually also a substantial amount of N-glycosylation found on mucins (e.g., 40 chains per MUC2 molecule), but relative to the O-glycosylation this makes a relatively small contribution to the molecular size of the mucin molecules.[33] The extensive glycosylation of mucins strongly affects their physical properties, giving the molecules, e.g., a very high buoyant density of around 1.4 g/ml, and extending the monomeric mucin molecules into very long filaments.[34,40] The glycosylation can mediate specific binding of immune cells, and pathogenic and commensal microbes, and plays a role in inflammation and cancer metastasis.[37,41-43] Several studies indicate that changes in mucin-structure change their protective properties. When in mice the O-glycosylation is altered, by knocking out either a specific core 3 type glycosyltransferase or a set of three core 2 type glycosyltransferase, the mice are rendered more susceptible to development of colitis.[44,45] A similar effect is caused by knocking out the intestinal sulfate transporter, which leads to under-sulfated intestinal mucin, which also increases the sensitivity to small intestinal infections and the development of experimental colitis in these mice.[46]

In diverse intestinal infection models in the mouse it was clearly demonstrated that mucin production as well as mucin structure was influenced by the presence of pathogens. One example is the intestinal infection of mice by the intestinal nematode *Nippostrongylus brasiliensis*, which remains in the gut lumen for several weeks. These studies show that the predominant secretory mucin of the small intestine Muc2 is specifically upregulated, but also that the structure of the mucins change in a specific pattern over time during the infection.[47,48] For the duration of the infection by these nematodes, increasing numbers of mucin-producing goblet cells contain sulfomucins, and also transient modifications in the terminal sugars were observed,[49] indicating the adaptability of the epithelium as well as the increased necessity to produce mucins in order to expel the nematodes. Another example is intestinal infection in mouse by rotavirus; a virus that replicates within intestinal epithelial cells, thereby destroying the enterocytes in a process that is self-limiting in a few days. Upon rotavirus infection there is an increase in Muc2 synthesis shortly after virus infection. During the following phase of villus atrophy, the Muc2 synthesis is largely maintained, despite severe pathology.[50] Also in these studies of rotavirus infection it appears that the Muc2 structure changes, since the mucins produced contain much less sulfation than in under non-infected situations.[50]

Mucus and Mucin-associated Microbes

The mucus layer is considered the first line of defense between the bacteria in the lumen and the host cells, and also serves as the initiation surface for host-microbe interactions. The GI-tract contains a complex ecosystem that is composed of trillions of microbial cells. Bacteria associated with mucus probably gain an advantage over the luminal or planktonic bacteria. Indeed, bacteria colonizing the mucus gel are less susceptible to elimination by the passage of luminal contents, and have increased access to carbon sources provided by the mucus layer, compared to luminal bacteria.[51] The mucus has a dual role in relation to microbiota; it protects the underlying mucosa from undesired interactions with microbes such as pathogens; besides it provides an initial adhesion site, nutrient source, and matrix on (and/or in) which bacteria can proliferate and thrive (**Fig. 2**). This dualistic role of mucins, i.e., keeping the bacteria at bay and at the same time provide attachment sites, has been noted earlier by Van Klinken et al.[19]

In general, commensal GI bacteria benefit from the mucus-layer by (1) attaching to mucus (retention at mucosal surface), (2) 'hiding' in the outer mucus-layer (which needs some form of mobility of the individual bacterium by the diverse mechanism as recently reviewed by Jarrell and McBride,[52] or a form of 'mixing' of the bacteria with the luminal contents) and (3) using the mucin as nutrient source (which needs specific enzymes, and requires cooperation among consortia). From the point of view of our body, the mucus-layer thus has two main purposes. First, it serves as a protective secretion; a physical layer. Second, mucins are provided to the luminal GI bacteria as a kind of 'prebiotic' to (at least partly) manage the growth of intestinal luminal microbiota. Mucins have no known or recorded anti-bacterial activity. If mucins were purely a defensive layer, as postulated in early mucus research, these molecules would probably have acquired such a function during evolution. Thus, their partial consumption by bacteria seems at least a part of their physiological functions. As mucins vary widely in their extent and form of glycosylation, this could well constitute a means of the body to stimulate bacterial growth in an organ-specific manner. Interestingly, the 'protective' and 'prebiotic' functions of the mucins collide, since both are affected by changes in glycan structure, which will both affect physical properties (as in gel-formation), but also the abilities for degradation by bacterial enzymes. However, these two roles can be balanced, i.e., separately regulated, by the type of mucin (different MUC genes in different organs, and regulation of the levels of their respective productions), by physico-chemical composition of the mucin molecules that is determined by type

of glycosylation and extent of oligo-merization of the mucins, and by cellular localization: i.e., secretory vs. membrane-bound mucins.

It was shown in a model reactor with an artificial mucus layer that the microbial community living inside the mucus matrix differed phylogenetically and metabolically from the luminal community.[53] Luminal mucosa-associated bacteria varied inter-individually, and showed intra-individual similarity on different sites of the colon.[54-56] The mucosa-associated bacteria are in close contact with/proximity to the epithelial cells, and therefore can have a major impact on these cells. However, a study based on fluorescent in situ hybridization (FISH) with a combination of a general 16S rRNA oligonucleotide probe targeting all bacteria, and specific probes targeting particular groups of bacteria, showed that there was no specific population present in the mucus layer, relative to commensal bacteria as located in the lumen.[57] In contrast, human and animal studies using FISH, light and scanning electron microscopy, showed bacteria embedded in the mucus layer.[7,58] Johansson et al. investigated the presence of bacteria in the inner and outer mucus layer of colonic tissues. Semi-quantitative PCR and FISH using a general 16S rRNA probe showed that very low numbers of bacteria were present in the inner mucus layer, while large numbers of bacteria were detected in the outer mucus layer, suggesting that the inner mucus layer acts as a major physical barrier against penetration of bacteria into the epithelium.[2]

Mucin Binding by Bacteria

Carbohydrate structures present on mucins are extremely diverse (**Figs. 1 and S1**). As a result, mucins offer an 'oasis' of binding sites for microbial (pathogens and commensals) adhesion. By offering binding sites similar to those of epithelial cells, mucin can prevent pathogen adhesion to the underlying epithelial cells, and further translocation into the mucosa. Human saliva, e.g., contains two mucin-type glycoproteins: the high molecular weight, gel-formin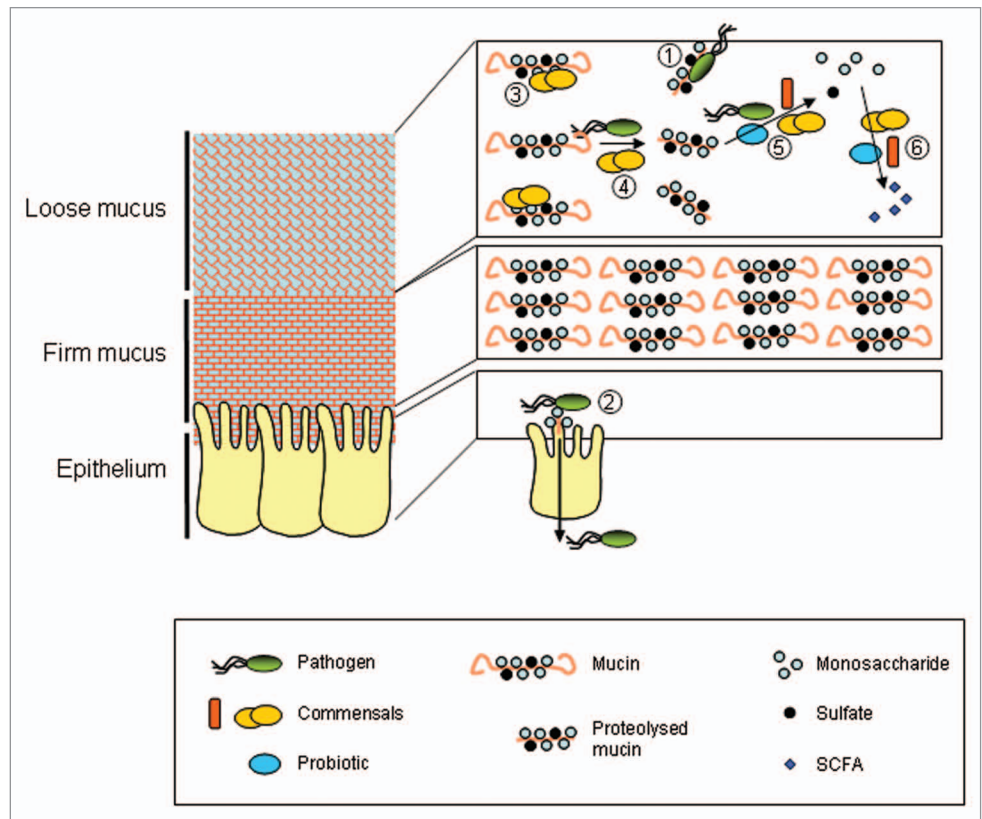g mucin MUC5B, and the low molecular weight, soluble mucin MUC7. Secretions of the human submandibular, sublingual and minor salivary glands contain MUC5B and MUC7.[59] Several investigations have demonstrated that MUC5B and MUC7 alone or together, interact with oral microorganisms in order to protect the underlying epithelium (summarized in **Table 1S**). Another example of this 'scavenging' function of mucins can be found for MUC1 from human breast milk.[60] MUC1 adheres to pathogenic microorganisms, such as rotavirus, Campylobacter, *Escherichia coli* (ETEC), which interferes with their colonization in the infant GI tract.[61-63] The ability to adhere to mucins has been suggested to be one of the criteria for the selection of lactic acid bacteria with probiotic activity.[64,65]

Figure 2. Schematic representation of the different associations of gel forming mucins with intestinal bacteria. Pathogens can be bound by proteolytically degraded mucin in the lumen (1), as mucins contain similar carbohydrate structures as found on the epithelium and thus be expelled. Similarly pathogens might be bound by the mucins in the mucus layer and therefore not reach the epithelium. When pathogens reach the epithelium they can bind to receptors (often carbohydrate structures) and might translocate across the epithelium (2). Normally, this will primarily occur when the mucus layer is gone as in severe pathology or concern microbes that can achieve mobility through the mucus layer, such as *H. pylori* in gastric mucus. Commensal bacteria can be present as planktonic bacteria in the lumen, live bound to the mucins in the mucus layer or live protected within the mucus without direct interactions (3). Commensal bacteria might compete with pathogens for binding sites on the mucins, thereby denying access to the pathogens (1 vs. 3). Some commensal as well as some pathogenic bacteria are able to proteolytically degrade mucins (4). Commensals, pathogens and probiotics are able to use mucins as energy source by degrading the O-glycans (5); the latter could happen within the mucus layer as well as in the lumen. The released monosaccharides are amongst others converted by commensal and probiotic bacteria into secondary metabolites (6), such as short chain fatty acids (SCFA), which are, e.g., essential for the colonic epithelium as an energy source. Please notice the scale of the components of the drawing. Mucin molecules (polymers) are several micrometers long, and are thus in the same range of size as the bacteria. In contrast, the epithelial cells are about 20 μm tall, whereas the mucus layer in, e.g., the colon can reach up to 800 μm in thickness.

However, as comparative studies on the efficiency of probiotic strains are scarce, the validation of this attribute has to be confirmed. For this type of study, the proper procedure would be to isolate the genuine mucin(s) from the organ of interest. As indicated above, even pure mucins (e.g., human colonic MUC2[28]) are very heterogeneous in nature due to their extensive and variable O-glycosylation, and the glycosylation varies from organ to organ even for one specific MUC-type mucin. In other words, only mucins from the organ of interest will do as appropriate targets for bacterial adhesion and/or degradation studies. Thus, identification of relevant bacterial-mucin interactions needs to be performed 'pair-wise,' i.e., by using the proper mucin from the organ of interest in combination with (a community of) the relevant bacteria known to reside in that organ. These essential studies have as yet not been done, largely hampered by lack of proper (human) mucin-sources, which would allow for isolation of sufficient amounts of native mucins to enable these experiments.

The molecular mechanism by which binding to mucins may occur is receiving increasing attention. Several genomes of probiotic lactic acid bacteria contain genes with predicted mucin-binding activity.[66] One of them, the protein GroEL of *Lactobacillus johnsonni* was shown to bind to mucins and aggregate *Helicobacter pylori*, suggesting that the protein could facilitate clearance of this pathogen during mucus flushing.[67] The carbohydrate structures on mucins can also promote the invasion of specialized pathogens by providing them a first attachment site that facilitates further access to epithelial cells. The recent study of Celli et al. showed that the pathogenic bacterium *H. pylori*, responsible of gastric ulcers, achieves motility by modifying the rheological properties of the mucus layer.[68] Previously, it was demonstrated in the antrum of *H. pylori*-infected individuals, that *H. pylori* co-localizes in situ with the extracellular mucin MUC5AC (the predominant gel-forming mucin in the human stomach) as well as with the apical domain of MUC5AC-producing cells of the superficial gastric epithelium.[69] We showed that the histo-blood group antigen Lewis b, present on gastric mucin MUC5AC, is the primary adhesion site for *H. pylori* in the stomach.[70] This is in line with the earlier work of Boren et al. who showed that Lewis b structures mediated the attachment of *H. pylori* to human gastric epithelium, when assayed on tissue sections.[71] The *H. pylori* adhesin, which interacts with Lewis b, has been cloned and was designated as blood group antigen-binding adhesin (BabA).[72] Recently, it was discovered that one of the most widely consumed probiotic lactic acid bacteria, *L. rhamnosus* GG, contains cell-envelope bound filaments, known as pili. These pili contain the SpaC pilus protein, which is able to mediate strong binding to host mucins.[73] This finding might explain the ability of the probiotic strain to persist in the GI tract as well as to stimulate the host immune system.

Mucin Degradation by Bacteria

Besides providing attachment sites to bacteria, mucins can be an important factor for bacterial colonization by providing an energy source. Mucin is an important carbon source for bacteria, mainly in the distal colon where the availability of carbohydrates is limited.[74] The carbohydrate structures account for about 80% of mass the mucin molecules and hence constitute a significant endogenous carbon and energy source for intestinal microbes able to cleave the relevant glycosidic linkages.

Mucin degradation. Mucin degradation by bacteria is often regarded as an initial stage in pathogenesis, since it would disturb the protection of the host mucosal surfaces. The fact of mucin degradation also offers ecological advantages to certain bacteria that scavenge released products from mucin degradation such as oligosaccharides or sulfate. One example is the relation between sulfate reducing-bacteria (SRB) and mucin degraders. Sulfomucins offer a potential source of sulfate for subsequent reduction into hydrogen sulfide, which has been shown to be highly toxic for the intestinal epithelium, promoting cell proliferation.[75] When fecal microbiota was inoculated into a three-stage continuous culture system supplemented with mucin, stimulation of the growth of SRB, especially Desulfovibrio sp. and subsequent production of sulfide were observed.[76] Interestingly, a co-culture of *B. fragilis* and *D. desulfuricans* enhanced sulfide production.[77] Hence, mucin degradation is sometimes viewed as negative effect, although this might hold only for the activities of specific bacteria. Mucin degradation has been recognized as a normal process of mucus turn-over in the GI tract, starting a few months after birth.[78] Midtvedt et al. studied the establishment of the mucin-degrading microbiota from 30 Swedish children from birth to the age of two years old, by analyzing their fecal samples. They found that the establishment of mucin-degrading bacteria starts during the first months of life, and is completed when the children are around two years old. Interestingly, a relation with diet was also observed: breast-fed babies showed a delay in the mucin degradation profile as compared with babies fed with formula milk. An explanation might be that mucins and the other mucin-like glycoproteins present in the breast milk compete with endogenous GI mucins as microbial substrates.[79] A specific transcriptional response to mucin-type oligosaccharides was recently observed in Bifidobacterium spp. in breast fed babies, confirming the reaction of intestinal microbiota to mucin.[80]

Mucin degradation has also been found to affect the host. The epithelium of germ-free mice differs morphologically from that of conventionally raised animals: non-degraded mucin is found in feces, their goblet cells are smaller and less abundant, the colonic mucus layer is approximately two times thicker and the weight of the cecum can reach up to eight times that of conventional animals.[81-84] The swelling of the cecum in germ-free animals is due to the accumulation of mucus, and the resulting retention of water, due to the absence of mucin-degrading bacteria.[85] In addition, when fecal suspensions or pure cultures of Clostridium, Bacteroides or Peptococcus were introduced into germ-free rodents, the cecum showed a striking reduction to its normal weight,[86,87] suggesting the beneficial workings of mucin-degrading bacteria. In the study of Schwerbrock et al. on the conventionalization of germ-free mice, the intestinal production of the major intestinal mucin Muc2 was carefully characterized and quantified as a function of time after colonization.[82] In healthy control mice the level of Muc2 production was steady after the conventionalization, however over time there was a

Figure 3. Phylogenetic tree derived from the 16S rRNA sequence data of isolated and cultured mucin-degrading bacteria in the human digestive tract. Bar represents 10% divergence.

marked increase in the level of sulfation of the Muc2 molecules. These results were compared to an IL10 knockout mouse, which develops spontaneous colitis when colonized by bacteria. The IL10 knockout mice have lower level of Muc2 synthesis in germ-free conditions and react to the conventionalization by a quick upsurge in Muc2 production, which however quickly falls to very low levels. At the same time the level of sulfation of the Muc2 molecules produced in the colon of the IL10 knockout mouse decrease to barely detectable levels. The results indicate that bacteria in normal and pathological conditions can have quite opposite effects on mucin production and on mucin structure, as exemplified by the level of sulfation.[82]

Isolation of mucin-degrading microbes. Enrichment of dental plaque microbiota on saliva-containing culture media demonstrated that cell-bound microbial enzymes almost completely degrade salivary proteins, including mucins.[88] Further studies showed that oral Streptococcus species were capable to grow in defined medium containing pig gastric mucin and used this as a nutritional substrate.[89]

In a series of pioneering studies, Hoskins and co-workers studied the adult fecal microbiota, which was able to degrade pig gastric mucin in vitro. They first quantified the microbiota using most probable number analysis using mucin-based medium after fecal inoculation.[90] This study revealed that 1% of the total fecal

Table 2. Bacterial mucin-degrading enzymes identified in the human digestive tract

Organisms	Enzymes	References
Oral cavity		
Streptococcus anginosus	β-*N*-acetyl-D-glucosaminidase, α- and β-D-galactosidase	89
Streptococcus mitis	β-*N*-acetyl-D-galactosaminidase, β-*N*-acetyl-D-glucosaminidase, α- and β-D-galactosidase, α-L-fucosidase, neuraminidase	89
Streptococcus mutants	β-*N*-acetyl-D-glucosaminidase, α- and β-D-galactosidase	89
Streptococcus oralis	β-*N*-acetyl-D-galactosaminidase, β-*N*-acetyl-D-glucosaminidase, α- and β-D-galactosidase, α-L-fucosidase, neuraminidase, protease	88, 89
Streptococcus sanguinis	β-*N*-acetyl-D-galactosaminidase, β-*N*-acetyl-D-glucosaminidase, α- and β-D-galactosidase, α-L-fucosidase, protease	88, 89
Streptococcus sobrinus	β-*N*-acetyl-D-glucosaminidase, β-D-galactosidase	89
Gastrointestinal tract		
Akkermansia muciniphila	α- and β-D-galactosidase, α-L fucosidase, α- and β-*N*-acetylgalactosaminidase, β-*N*-acetylglucosaminidase, neuraminidase, sulfatase.	162
Bacteroides fragilis	protease, α-*N*-acetylgalactosaminidase, β-galactosidase, β-*N*-acetyl-D-glucosaminidase, α-L-fucosidase, neuraminidase, sulfatase	163–165
Bacteroides thetaiotaomicron	α-fucosidase, β-galactosidase, α-*N*-acetylgalactosaminidase, β-*N*-acetylglucosaminidase, neuraminidase, sulfatase	166, 167
Bacteroides vulgatus	α- and β-galactosidase, α-fucosidase, β-*N*-acetyl-D-glucosaminidase, α- and β-*N*-acetylgalactosaminidase, neuraminidase	168
Bifidobacterium sp., *Bifidobacterium bifidum*	α-L-fucosidase, α-*N*-acetylgalactosaminidase, galactosyl-*N*-acetylhexosamine phosphorylase	169
Clostridium cocleatum	β-galactosidase, β-*N*-acetylglucosaminidase, α-*N*-acetylgalactosaminidase, neuraminidase	170
Clostridium septicum	β-Galactosidase, β-*N*-acetyl-D-glucosaminidase, glycosulfatase, neuraminidase	171
Helicobacter pylori	Glycosulfatase	172
Prevotella sp. RS2	Sulfoglycosidase, glycosulfatase	173, 174
Ruminococcus torques	α-*N*-acetylgalactosaminidase	175
Streptomyces sp.	α-L-fucosidase	176
Vibrio cholerae	Neuraminidase, β-*N*-acetylhexosaminidase, proteinase.	177

microbiota was able to use mucin as carbon source, including the genera Ruminococcus and Bifidobacterium, although complete degradation of mucin required the action of a specific consortium.[77] Bacteroides species were also shown to ferment mucins.[91] Very recently, the use of a basal medium supplemented with mucin as single carbon source as an alternative to the use of complex and rich media for the isolation of new intestinal strains has resulted in the isolation of novel bacteria, i.e., *Akkermansia muciniphila* from human fecal samples and *Enterorhabdus mucosicola* from ileal samples from mouse.[92] This suggests that there are many more bacteria that can use mucin as primary carbon-source, which wait to be discovered. A phylogenetic overview of cultivated mucin-degrading bacteria present in the human digestive tract is depicted in **Figure 3**.

Isolation of *Akkermansia muciniphila* and its abundance in humans and animals. Recently, the use of a targeted cultivation approach allowed the isolation of a novel microorganism, *Akkermansia muciniphila*, capable of growth on mucin as sole carbon and nitrogen source.[93] This is the first intestinal member belonging to the recently discovered Verrucomicrobia phylum. Members of this bacterial phylum have been detected using molecular techniques in a variety of ecosystems, including soil,

water and GI tract and increasing numbers of isolates.[94] Using periodic acid-Schiff staining (that stains mucin molecules) on *A. muciniphila* cultures growing on mucin, it was estimated that 80% of the mucin was degraded and metabolized in a growing culture, indicating the efficient breakdown of the mucin molecule (Derrien M et al., unpublished results). A variety of enzymes targeting a wide range of mucin carbohydrates have been identified in grown cultures of *A. muciniphila* (**Table 2**). The available genome sequence is being now analyzed and will provide new information about the genetic capacity of *A. muciniphila* to degrading mucin (**Table 3**). In addition to genetic information, 16S rRNA-based surveys, including clone libraries, showed that *A. muciniphila* was detected as a dominant bacterium in the GI-tract. The study of Wang et al. reported that the 16S rRNA gene sequence of *A. muciniphila* was detected in the distal ileum, ascending colon and rectum with levels of 5, 6 and 9% of the clone libraries from a healthy 54-year-old female, respectively.[95] In a larger scale study from Eckburg et al. in which 13,355 prokaryotic ribosomal RNA gene sequences were examined from various colonic mucosal sites and feces of three healthy subjects, *A. muciniphila* was detected in every site from one subject, from the ascending colon, descending colon and stool of another

Table 3. Mining for candidate mucin-degrading enzymes in seven sequenced genomes

Phylum	Species name[1]	Size (Mbp)	# CDS	# Signal P[2] (%)	# UF[3] (%)	GH	P	Su	Si	Total
Verrucomicrobia	*A. muciniphila*	2.7	2176	576 (26)	270 (47)	35	13	11	2	61
Bacteroidetes	*B. vulgatus*	5.2	4076	1200 (29)	510 (43)	145	42	4	7	198
	B. thetaiotaomicron	6.3	4816	1574 (33)	777 (49)	171	30	11	9	221
	P. gingivalis	2.4	2090	451 (22)	248 (55)	9	28	0	1	38
Proteobacteria	*H. pylori*	1.6	1494	311 (21)	141 (45)	0	6	0	0	6
Firmicutes	*S. mutans*	2.0	1960	331 (17)	140 (42)	4	10	0	0	14
Actinobacteria	*Bif. longum* subsp. *infantis*	2.8	2486	462 (19)	181 (39)	6	7	1	0	15

The header "Candidate mucin-degrading enzymes[4]" spans columns GH, P, Su, Si, Total.

[1]The accession numbers for the different species are NC_010655 (*A. muciniphila*), NC_009614 (*B. vulgatus*), NC_004663 (*B. thetaiotaomicron*), NC_010729 (*P. gingivalis*), NC_000921 (*H. pylori*), NC_004350 (*S. mutans*), NC_011593 (*B. longum* subsp. *infantis*). [2]These numbers (percentages) represent the protein coding genes that encode a putative signal peptide. [3]These numbers (percentages) represent the protein coding genes that encode a putative signal peptide and lack a predicted function. [4]The candidate mucin-degrading enzymes are categorized into four broad categories; glycosyl hydrolases (GH), proteases/peptidases (P), sulfatases (Su) and sialidases/neuraminidases (Si). For details of the list, see **Table S2**.

subject, but was not detected in appreciable numbers in the third subject.[58] Sequences corresponding to *A. muciniphila* and related species have been detected in 16S rRNA clone libraries, originating from human biopsies, feces derived from healthy adults,[58,96,97] and inflammatory bowel disease patients.[98] A recent molecular inventory revealed that Akkermansia species are widely distributed amongst wild and zoo mammals, with a strong predominance in herbivores.[99] Other studies also reported the presence of *A. muciniphila* in mice,[100] herbivores[101] and hamsters.[102]

Specific 16S rRNA primers and probes have brought new information about this microorganism. Its abundance over life time has been measured using qPCR on fecal samples from babies, children, adults and elderly people. FISH revealed that *A. muciniphila* is a common member of the human intestinal tract with a high prevalence and variable abundance, and that its colonization starts in early life and develops within a year to a level close to that observed in adults (10[8] cells/g feces), but decreases in the elderly. Interestingly we also found that the 16S rRNA sequence of *A. muciniphila* contains two mismatches with the eubacterial probe (EUB-338, commonly used as positive control) and that *A. muciniphila* cells showed only a weak signal after EUB-338 hybridization. With the increase of availability of 16S rRNA sequences, it was shown that EUB-338 does not cover all bacterial phyla, such as the Planctomycetales and Verrucomicrobia. Therefore, two additional probes, EUB-338-II and EUB-338-III, targeting these two groups respectively were designed. When combined, these three probes allow for detection of all the phyla of the bacteria domain.[103] The EUB-338-III is 100% complementary only to members of the divisions 1 to 4 of the Verrucomicrobia phylum.[104] Nevertheless, in intestinal studies, this specific probe has not been often employed, suggesting that *A. muciniphila* has been missed in most analyses so far and thus its presence was underestimated. The widespread occurrence of *A. muciniphila* in the intestine of diverse hosts supports its importance among the members of intestinal consortia. An obvious advantage of mucin-degraded bacteria over food-dependent bacteria is their ability to survive in more extreme GI conditions, such as lack of food or severe diarrhea. A recent study on cecal

microbiota of fasted Syrian hamsters reported that *A. muciniphila* significantly increased, supporting its ability to survive over species belonging to Firmicutes and Bacteroidetes. This ability also offered advantage to bacteria belonging to Proteobacteria due to released products from mucins degradation and most likely the release of sulfate from the mucins.[102] Many authors hypothesized that mucin-degraders are related to pathogenicity in the intestine as they might undermine the protective nature of the mucus layer. However, *A. muciniphila* has so far not been correlated to any disease or sign of pathogenicity. Its high prevalence in diverse gut ecosystems also supports its benign nature and probably also its importance for the microbial ecosystem. Moreover, colonization of germ-free mice with *A. muciniphila* did not reveal any sign of discomfort or pathology (Derrien et al. manuscript submitted). Moreover, a recent study from Swidsinski reported that it was negatively correlated with acute appendicitis, together with *Faecalibacterium prausnitzii, Eubacterium rectale* and *Bacteroides* spp.[105]

Molecular analysis of mucin-degrading bacteria. The last decade, the advent of molecular approaches based on the 16rRNA has allowed insight into the unseen microbiota and a more precise description of the intestinal microbiota. In an in vitro fermentation system mimicking the human intestine, Macfarlane et al. reported that a heterogeneous bacterial community was colonizing mucin.[53] These authors used a combination of cultivation and visualization of the bacterial community by FISH, based on specific and general 16S rRNA probes. They observed that this community consisted of members of the *Bacteroides fragilis* group, bifidobacteria, enterobacteria and clostridia. Further studies based on construction of 16S rRNA genes of enriched fecal mucin-degrading bacteria revealed the diversity of mucolytic consortia.[93] Mucolytic bacteria were enriched from fecal samples inoculated on a mucin-limited medium and monitored by denaturing gel gradient electrophoresis of 16S rRNA gene amplicons. This molecular fingerprint approach showed that the profiles from different subjects were highly diverse, with a similarity index of 57.4 ± 9.2%, indicating that the mucin-degrading bacterial community is roughly similar within the whole

population, but that there is still substantial individual variation. In addition, cloning and sequencing of 16S rRNA genes revealed that the majority (69%) of these genes derived from not-yet cultivated species. The 16S rRNA gene sequences that showed similarities higher than 98% to known database entries were related to the Gram-positive genera Clostridium and Ruminococcus (Clostridium clusters IV, XIVa and XVIII), whereas the remaining ones were related to species belonging to Verrucomicrobia and Proteobacteria.[93] In addition, recently Leitch et al. studied the colonization of insoluble substrates including pig gastric mucin by fecal bacteria in an anaerobic in vitro continuous flow system. They showed by a cloning—and sequencing strategy and FISH based on 16S rRNA gene, that Bifidobacteria were the principal species (*Bif. bifidum* and *Bif. breve*), followed by bacteria belonging to Clostridium cluster XIVa (41% of the mucin-associated sequences) including *Ruminococcus lactaris* and a closely related group of uncultured bacteria. Bacteria belonging to Bacteroidetes such as *B. vulgatus* were also detected on mucin.[106] These molecular based studies provided evidence for the fact that fecal samples contain a diverse population of mucin-associated bacteria, the composition of which differs from one person to the other.

Mucin degrading enzymes. Mucin degradation is achieved by a combination of mainly saccharolytic enzymes from the bacteria and proteolytic enzymes from the host and bacteria. As discussed above, the composition of O-linked glycans on the mucins, their size, linkages and terminal sugar-residues differs along the GI tract, being more neutral in the upper part, while more acidic in the lower part. Mucin-degrading bacteria can adapt to the host mucins by producing specific enzymes, which are able to degrade the histo-blood group antigens (oligosaccharides).[107-109] Due to their high complexity and diversity, mucins can only be completely degraded by a panel of diverse enzymes, including proteases, glycosidases, sialidases and sulfatases, together designated as 'mucinases'.[109,110] Mucin degradation in vivo starts probably with cleavage of the non-glycosylated regions of the polypeptide backbone performed by proteolytic enzymes (**Fig. 2**). Subsequently, the oligosaccharide chains are degraded by a panel of diverse glycosidases and finally followed by proteolytic degradation of the exposed protein core. Proteases are secreted by both host and bacteria, whereas glycosidases capable of degrading mucin-type O-linked glycans are only secreted by bacteria and not by the host tissues. The bacterial glycosidases include mainly β-N-acetyl-D-galactosaminidase, β-N-acetyl-D-glucosaminidase, α- and β-D-galactosidase and α-D-mannosidase; whereas the latter plays only a minor role in degradation of mucins that are relatively poor in N-linked glycans (mannose is only present in N-linked glycans). Sialidases (neuraminidases), sulfatases and α-fucosidases act on the terminal ends of the oligosaccharide chains and will often act as initiators of mucin oligosaccharide degradation, as sulfate, sialic acids and α-fucose (the latter in particular in the diverse blood group structures) form the usual terminal structures on the mucin-type O-glycans (examples in **Fig. S1**). Obviously, mucin degradation requires the subsequent actions of several microbial enzymes, mainly glycosidases, each having the specificity to degrade a specific glycoside linkage. Only a few bacteria produce all the enzymes necessary for complete mucin degradation. Several bacteria belonging to the genera *Streptococcus*, *Helicobacter*, *Akkermansia*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Prevotella*, *Ruminococcus*, *Streptomyces* produce one, several or all mucin-degrading enzymes (**Table 2**). The ratio of production and degradation of mucin is usually stable in healthy individuals, leading to a dynamic mucus-layer with over time a stabile thickness, composition and consistency. However, this status quo can be affected in pathogenesis as seen in tracheobronchial diseases, e.g., chronic obstructive pulmonary diseases and cystic fibrosis, in which the mucus layer is abnormally thick and the mucus has a very pronounced viscosity, disabling the proper clearance of mucus and bacteria from the airways. Also in intestinal pathology the mucin production and mucus layer is affected, mucin mRNA expression has been shown to be disturbed for several MUC genes in ileal and colonic mucosal biopsies from inflammatory bowel diseases patients such as ulcerative colitis and Crohn disease.[111] Changes in the properties of mucins and mucus thickness have been noted in ulcerative colitis and Crohn disease, such as decrease of the length of the O-glycans, a decrease in the degree of sulfation and increase of sialylation of the oligosaccharide chains.[112] Moreover, the thickness of the mucus layer is altered in these patients, leading to a weakness in the protective barrier and thus contributing to the pathology by increasing the vulnerability of the underlying epithelium.[13,41,113] In ulcerative colitis, the mucus layer is thinner, the epithelium harbors a reduced proportion of goblet cells and there are increased levels of fecal mucin-degrading enzymes, including sulfatases, as compared to healthy individuals, contributing to a general dysfunction of the mucosal barrier. The rate of mucin turn-over can be modified by bacterial colonization,[114,115] short chain fatty acids,[116] starvation,[117] and intake of fibers and other oligosaccharides.[116,118-123] Colonic fermentation (including that of mucins) has a major impact on our human metabolism,[99,124] thus the selective retention of and the presence of metabolic substrates for, bacteria are important mechanism to maintain body homeostasis. Also changed microbiota has been associated with changed health status such as obesity,[125] indicating that specific bacterial substrates such as endogenous mucins, are probably very important to regulate the microbial balance in the gut.

Genome mining for candidate genes involved in mucin degradation. Besides the purification and study of specific mucin-degrading enzymes, the increasing number of genome sequencing projects is providing a glimpse into the genetic basis of mucin breakdown. However, the complex nature of mucins frustrates the straightforward identification of the range of genes responsible for its degradation. Identifying candidate mucinases from the genetic repertoires of sequenced bacterial genomes relies partially on certain prediction tools, since for many species experimental data for these activities are lacking. Due to the large molecular size of mucins, mucin-degrading enzymes are expected to be either secreted proteins or proteins associated with the outside of the cell wall and using dedicated in silico secretion predictions tools, candidate mucin-degrading enzymes can be listed for a range of mucin-degrading bacteria (**Table 3**).

Genome-wide predictions of signal sequences, indicative of secretion via the classical pathway,[126] reveal general characteristics

of a genome's capacity for mucin degradation. As an example, a well-studied intestinal bacterium that can thrive on mucins is *B. thetaiotaomicron*, a common inhabitant of the human and mouse GI tract. The pioneering work of Salyers et al. in the 1970s has brought about information on the capacity of this organism to use host glycans and mucins in vitro.[91] Later studies from Gordon and coworkers revealed that *B. thetaiotaomicron* is a very well adapted bacterium to the intestinal environment, with a flexible ability to switch to mucins when polysaccharides are depleted from the diet.[127] Of the analyzed genomes, *B. thetaiotaomicron* shows the largest number of genes that encode putative signal sequences (1,574, 32.7% of the protein coding repertoire). It also contains the largest number of putatively secreted genes with glycosidase, sialidase, protease or sulfatase activities. Comparable numbers of these genes are encountered in *Bacteroides vulgatus*. Other known mucin-degrading GI bacteria, such as *Helicobacter pylori, Streptococcus mutans* and *Bifidobacterium longum* subsp. *infantis* show lower counts of these genes, and each is lacking at least one category of the functionalities for complete mucin degradation. The recently identified and characterized Verrucomicrobium *Akkermansia muciniphila*, which was isolated by dilution-to-extinction on mucin as a single carbon and nitrogen source, has predicted secreted genes for glycosidase, sialidase, protease and sulfatase activities. With this repertoire of functionalities in its relatively small genome, *A. muciniphila* seems therefore to be specialized in mucin degradation; more so than the Bacteroides spp., which have much larger genomes. On the other hand, the large numbers of unknown functions in all seven of these predicted secretomes may hold a plethora of activities towards mucin degradation.

Conclusion and Perspectives

Mucus overlying the epithelium in the oral cavity and the GI tract and the bacteria residing in these organs maintain intimates relationships. The combined information in this review has brought new insight in the specific bacterial community present in the digestive tract. Many members of this community are able to associate with mucins and an increasing number of bacteria are found to be able to degrade mucins that offer alternative nutrients during changes of diet, and is an integral part of the bacterial survival strategy in this complex ecology. The increasing availability of sequenced genomes of mucin-degraders helps us to understand the mucin degradation and its relevance for microbial ecology. The high degree of diversity of the mucin oligosaccharide chains, with their many potential binding sites and metabolic substrates for bacteria, is likely an important determinant in the site-specific colonization of bacteria along the digestive tract. The mucus layer and more specifically the constituting mucins are thus a major determinant of bacterial colonization. This notion is an important extension of the views advocated in the early years of mucin research that state that the mucus layer has only a barrier function. Now that it is becoming more and more clear that important commensal and probiotic bacteria can degrade mucins and use these as substrates, it seems that bacterial fermentation of mucins is an important feature to selectively favor the growth of certain classes of bacteria. Thus, aside from their well-described role in host mucosal defense, the mucins of the digestive tract could be designated as endogenous 'prebiotics'. The lumen/mucus interface and the mucus-layer itself harbors many ecological niches, which are sought for by beneficial commensal bacteria as well as by pathogenic organisms. We are still far from being able to understand these many and complex interactions between microbes and the mucus and mucins. Yet, the examples in this review indicate that there is a battle fought over the mucins by the oral and intestinal microbiota on a daily basis in GI tract, and that the outcome is an important factor in human health.

Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/DerrienGUT1-4.sup.pdf

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