Aus dem Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Expression and characterization of pancreatic secretory granule membrane major glycoprotein GP2 of animal origin

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
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vorgelegt von
Rafał Kolenda
Tierarzt aus Rojew, Polen

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mbvberlin | mensch und buch verlag

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Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
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Deskriptoren (nach CAB-Thesaurus):
pancreatic cells; Salmonella; glycoprotein; gene expression; serotypes; adhesins; polymerase chain reaction; SDS-PAGE; fluorescence in situ hybridization

Tag der Promotion: 06.02.2018

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-878-8
Zugl.: Berlin, Freie Univ., Diss., 2018
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

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1. Introduction

1.1. *Salmonella*

1.1.1. Epidemiology of *Salmonella*

Salmonella as a causative agent of a disease was first identified by Karl Joseph Ebert, when he described a bacillus as a possible cause of typhoid fever in humans. His results were confirmed following the isolation of pure cultures by Georg Theodor August Gaffky in 1884. The generic name *Salmonella* was introduced by Joseph Leon Lignières in 1900 to describe a bacterium discovered in Daniel Salmon's lab in 1885 (Barrow and Methner 2013). The genus *Salmonella* consists of two species: *Salmonella bongori* and *Salmonella enterica*. *S. enterica* is divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *indica*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *salamae* (Tindall et al. 2005). *Salmonellae* are differentiated into serovars according to the White-Kauffmann-Le Minor scheme by determination of somatic antigen (O), flagellar antigen (H) and biochemical properties (Grimont and Weill 2007). To better understand the epidemiology of *Salmonella* other techniques like phage typing, antimicrobial resistance measurement, pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) are also used (Foley et al. 2009; Switt et al. 2015). As these methods often do not offer sufficient discriminatory power to support epidemiological investigations and surveillance, whole genome sequencing (WGS) is the perfect tool for that and it will become a standard epidemiological technique in the near future (Wong et al. 2016).

The present number of serovars is 2659 according to the last supplement (no. 48) of the White-Kauffmann-Le Minor scheme (Issenhuth-Jeanjean et al. 2014). The majority of serovars belong to *S. enterica* subsp. *enterica* (1586), but only 50 of them have any significance as human or animal pathogens (Uzzau et al. 2000). *Salmonella* serovars can be divided by host range and clinical signs into host-restricted and host-adapted specialists and host-unrestricted generalists (Stevens et al. 2009). Host-restricted serovars like *S*. Typhi or *S*. Gallinarum cause systemic infection in one host (called typhoid fever and fowl typhoid/ pullorum disease, respectively) and do not cause disease in any other host (Shivaprasad 2000; Dougan and Baker 2014). Host-adapted specialists like *S*. Choleraesuis and *S*. Dublin mainly cause systemic disease in pigs and cattle, respectively, but can sporadically cause asymptomatic infections in other hosts (Chiu et al. 2004; Nielsen 2013). Most of the isolated serovars belong to a host-unrestricted group and are able to infect multiple hosts, with the outcome of their pathogenic actions usually being gastroenteritis (Majowicz et al. 2010).

According to the World Health Organization (WHO), non-typhoidal salmonellosis is one of the main foodborne diseases, globally affecting nearly 79 million people and resulting in death of 60 000 cases per year (Havelaar et al. 2015). Two most often studied host-unrestricted non-typhoidal serovars are *S*. Typhimurium and *S*. Enteritidis, since they are most frequently isolated from humans (Osimani et al. 2016). In the European Union (EU) for example, *S*. Enteritidis and *S*. Typhimurium were isolated from 44.4% and 17.4% human cases of salmonellosis in 2014, respectively (European Food Safety Authority and European Centre for Disease Prevention and Control (ECDC) 2015). As human non-typhoidal salmonellosis is a foodborne illness, animals are common reservoirs for these bacteria. *S*. Typhimurium was the most often isolated serovar from living pigs and cattle, and pig and bovine meat. In the case of the prevalence of *Salmonella* serovars in poultry it is important to mention, that implementation of National Control Program for *Salmonella* in EU changed the prevalence of serovars, which have now been brought under control through surveillance schemes and vaccination programmes (Dewaele et al. 2012). As a result, the most often isolated serovars from faeces of broiler flocks and laying hens are *S.* Infantis and *S*. Mbandaka. Interestingly, this does not correlate with the prevalence of these serovars in broiler meat, eggs and human salmonellosis cases. It has been shown that *S*. Enteritidis has properties, which make this serovars more successful in surviving in chicken-related products and therefore posing higher transmission rates to humans in comparison to other serovars (Gantois et al. 2009).

S. Choleraesuis was the dominant serovar isolated from pigs in 1950-70s in Europe (Sojka et al. 1977). Currently this serovar is very rarely isolated from pigs in Europe, but still can often be found in herds in the United States (Pedersen et al. 2015; European Food Safety Authority and European Centre for Disease Prevention and Control (ECDC) 2015). Human infections rarely occur through this serovar since they are highly adapted to their porcine hosts. The only exceptions were from a few Asian countries where *S*. Choleraesuis was frequently isolated from humans as a foodborne disease (Su et al. 2014; Domingues et al. 2014). *S*. Choleraesuis is divided by its biochemical properties into biotypes: *sensu stricto*, varies Kunzendorf and var. Decatur according to the White-Kauffmann-Le Minor scheme (Issenhuth-Jeanjean et al. 2014). Another host-adapted serovar, *S*. Dublin is associated with cattle (Nielsen 2013). In Europe, it is the most often isolated serovar from cattle and one of the most often isolated serovars from cattle around the world (Barrow and Methner 2013; European Food Safety Authority and European Centre for Disease Prevention and Control (ECDC) 2015). Human infections with *S*. Dublin are rarely reported (Fang and Fierer 1991; Foley and Lynne 2008; Funke et al. 2017).

Both aforementioned host-adapted serovars are mainly transmitted horizontally by the feco-oral route, but in the case of *S*. Choleraesuis aerosol transmission has been shown as another transmission route (Turk et al. 1992). Vertical transmission possibilities were observed in the case of *S*. Dublin (Counter and Gibson 1980).

S. Gallinarum represents a small group of host-restricted *Salmonellae*. *S*. Gallinarum was a major epidemiological concern in the 1950-60s worldwide (Barrow and Neto 2011). Currently, this serovar is no longer a problem in developed countries but is still a major concern in developing countries of South America, Africa and Asia (Sato et al. 1997; Kwon et al. 2010; Pulido-Landínez et al. 2014). Of importance in *S*. Gallinarum epidemiology is the possibility of both horizontal (feco-oral) and vertical transmission (infection of embryos and eggs in the ovary or oviduct) of the pathogen (Berchieri et al. 2001). *S*. Gallinarum is divided by its biochemical properties into two biotypes: Gallinarum and Pullorum according to the White-Kauffmann-Le Minor scheme (Issenhuth-Jeanjean et al. 2014).

1.1.2. Host specificity of *Salmonella*

Salmonella as a separate genus evolved from a common ancestor with *Escherichia coli* around 100-160 million years ago (Bäumler 1997). The evolution of this pathogen as an "invasive specialist" was divided by Bäumler et al. (1998) into three stages. It has been proposed that acquisition of the *Salmonella* pathogenicity island 1 (SPI-1) was the first step in the evolution of *Salmonella.* Type three secretion system 1 (T3SS-1), encoded by SPI-1, is present in all *Salmonella* serovars, but absent in other closely related organisms. Therefore, it was mentioned as a first divergence point of *Salmonella* from other Enterobacteriaceae. It allowed bacteria to invade epithelial cells, induce inflammation in the intestinal lumen and was the first step in the development of a successful strategy to evade a host's immune system (Haraga et al. 2008). A second milestone in the evolution of *Salmonella* was the acquisition of SPI-2. It was also the point at which the genus *Salmonella* was divided into two species: *enterica* and *bongori*. SPI-2 encodes type three secretion system 2 (T3SS-2), which enabled *Salmonella* to survive inside macrophages, constituting another step forward in establishing *Salmonella* as a specialist in evading host immune responses (Figueira and Holden 2012). The last phase of *Salmonella* evolution includes branching of *Salmonella enterica* into six subspecies accompanied by a change in host repertoire, with *Salmonella bongori*; *Salmonella enterica* subsp. *indica*, *hauntae*, *salamae*, *arizonae* and *diarizonae* tending to be associated with

cold-blooded animals, while *Salmonella enterica* subsp. *enterica* are mostly associated with warm-blooded animals (Fookes et al. 2011). This step is coincident with the evolution of host immune systems. Birds and mammals present most complex evolutionary immune systems, and the complexity of their primary and secondary lymphoid tissues in comparison to other vertebrates was the main obstacle for invasive *Salmonella* (Boehm et al. 2012; Boehm 2012). The emergence of new immune structures like lymph nodes and increasing specificity of antigen receptors in birds and mammals required from *Salmonella* additional genomic rearrangements in order to thrive in the new hostile immune environment.

The evolution of *Salmonella enterica* subsp. *enterica* as a pathogen of warm-blooded animals led to the development of two infection patterns: most of the serovars infect a wide range of hosts (generalists), while some of them are associated with only one/few host/-s (specialists) (Uzzau et al. 2000). As both patterns of infection require a different approach, the goal remains the same: survival and successive transmission to the highest number of new hosts possible. Generalist infection is usually limited to the gastro-intestinal tract, where bacteria divide intensively within a short period of time to be present in stool in very high loads that can increase the possibility of fecal-oral transmission to the next host. During infection, bacteria induce inflammation in the intestinal lumen of the host to outcompete commensal microbiota in a battle over shared resources (Stecher et al. 2007; Rivera-Chávez and Bäumler 2015). In the case of infection by a *Salmonella* specialist, which causes systemic infection, bacteria pass through the intestinal barrier without causing inflammation and spread to systemic organs (Barrow and Neto 2011; Dougan and Baker 2014). Systemic spread allows for the establishment of a chronic carrier organ or niche within the host. Dependent on the specialist, different organs act as a niche for chronic carriage of *Salmonella*: gall bladder (Gunn et al. 2014), reproductive tract (Wigley et al. 2001), spleen or lymph nodes (Nielsen et al. 2004). A chronic carrier state leads to long, intermittent shedding of the pathogen to the environment and thus increases the probability of transmission to another host from the same species.

Generalists and specialists show different approaches in their interaction with the immune system. While generalists can be detected by the immune system during the early stages of infection, specialists can “hide” for longer periods of time from being recognised by the immune system. Development of inflammatory diarrhoea during generalist infection is mediated by recognition of pathogen-associated molecular patterns (PAMPs) like curli amyloid fibrils and flagella by toll-like receptors (TLRs) 2 and 5 (Tükel et al. 2005; Nishimori et al. 2012; Atif et al. 2014). Another factor contributing to the development of inflammatory

response during generalist infection is inflammasome activation by cytosolic *Salmonella* in epithelial cells (Knodler et al. 2010; Sellin et al. 2014). It has been also shown that T3SS-1 effector proteins like SipA and SopE contribute to intestinal inflammation by activating nucleotide-binding oligomerization domain-containing protein (NOD) 1 and 2 (Keestra et al. 2011; Keestra and Bäumler 2014). *Salmonella* LPS is recognized by the complement system, which together with previously mentioned mechanisms leads to an influx of neutrophils into the intestinal lumen- a hallmark of *Salmonella* generalist- induced gastroenteritis (Santos et al. 2009). As could be predicted, *Salmonella* specialists evade the innate immune response and cause no inflammation in the intestinal phase of infection. In the case of *S*. Typhi, spatial segregation of virulence gene expression leads to a situation, where *S*. Typhi in the intestinal mucosa is non-flagellated, expression of T3SS-1 effector proteins is repressed and the complement system activation is blocked by expression of the Vi antigen (Winter et al. 2010b; Keestra-Gounder et al. 2015). Since not all *Salmonella* specialists express the Vi antigen, other immune evasion mechanisms must be exploited by other specialists (Pickard et al. 2003). In the case of *S*. Gallinarum, the non-expression of flagella helps in immune response evasion (de Freitas Neto et al. 2013).

It was proposed that host specialists were able to improve their fitness to one niche, which impaired their ability to survive in other environments. This hypothesis can be supported by different nutritional requirements of specialists and generalists, as it has been shown that specialists are auxotrophic to certain amino acids and vitamins (Uzzau et al. 2000). Adaptation to nutrients abundant in one host, but limited in other hosts or environment leads to decreased ability to survive outside the host (McMeechan et al. 2005; Matthews et al. 2015). Generalists rely on multiple metabolic pathways as a source of energy or building blocks during infection. It seems that in the case of specialists, that loss of redundancy in some metabolic pathways (e.g. D-glucarate degradation in *S*. Dublin) is a part of an association to a specific lifestyle, and that reduction of these pathways serves as a removal of unwanted energetic burden (Langridge et al. 2015). Generalists possess the ability to process terminal electron acceptors generated in the inflamed intestine by anaerobic respiration and this gives them a growth advantage over the gut microbiota in the inflamed gut (Winter et al. 2010a). Specialists, whose pathogenic actions take place outside intestinal lumen, are not able to utilise terminal electron acceptors like nitrate or tetrathionate (Nuccio and Bäumler 2014).

In the era of next-generation sequencing, it is considerably easy to compare the genomes of *Salmonella* specialists and generalists. The first major difference that can be found is in the

organisation of both genomes. Generalists and *E. coli* have a high degree of conservation in chromosomal gene order. It has been suggested that any genome arrangement alterations may lead to decreased multiplication of bacteria and as a consequence of that, a lower transmission rate to another host (Matthews et al. 2010; Bäumler and Fang 2013). Since specialists do not depend on multiplication at a maximal rate to outcompete other microbes, genomic rearrangements are a popular feature of specialists genomes (Helm et al. 2003; Liu et al. 2006; Thomson et al. 2008). Another feature found in all specialists when compared to generalists is genomic decay (Sabbagh et al. 2010; Matthews et al. 2015). It has been shown that specialists remove "unnecessary" burden by gene deletions and pseudogene formation. As an example, the genes involved in survival in an inflamed intestine are very important for host generalists, but can be forgone through deletion by hosts specialists (Nuccio and Bäumler 2014). Another factor important in successful intestinal colonisation is adhesion. As might easily be predicted *Salmonella* host generalists possess more functional adhesins than specialists, which gives them an advantage in colonisation of intestines of various hosts (Yue et al. 2012). Horizontal gene transfer was found to be a major driver of *Salmonella* evolution (Porwollik and McClelland 2003; Thomas and Nielsen 2005). Laterally acquired genomic regions found in clusters within *Salmonella* genomes called *Salmonella* pathogenicity islands (SPIs) serve as important determinants of *Salmonella* virulence and can play a different role in specialists and generalists (Eswarappa et al. 2008). Moreover, some of this SPIs like SPI-7 in *S*. Typhi are an important part of advantage in the colonisation of hosts and are thus strongly associated with their host-restricted lifestyle (Winter et al. 2009). It is important to mention that not only the presence or absence of certain virulence factors, in particular serovars, is associated with their host range, but also single nucleotide polymorphism (SNP) within a particular gene can lead to a change in tissue specificity (Kuźmińska-Bajor et al. 2012; Yue and Schifferli 2014).

All the aforementioned examples of host specificity are associated with the course of disease in immunocompetent hosts. The recent emergence of highly invasive *S*. Typhimurium Sequence Type 313 (ST313) and *S*. Enteritidis isolated from an immune-compromised human population shows the ability of *Salmonella* to adapt to their new environments (Okoro et al. 2015; Feasey et al. 2016). These bacteria have genome degradation and distinct infection patterns similar to host specialist, as they change their environment from intestinal to extra-intestinal, but their pathogenic actions are not restricted to one host (Parsons et al. 2013).

1.1.3. Type 1 fimbriae of *Salmonella*

Type 1 fimbriae (T1F) are one of the most common adhesive organelles in the family of Enterobacteriaceae and important adhesion factors in *Salmonella* intestinal pathogenicity. The *fim* fimbrial cluster is one of seven (among *bcf*, *stb*, *sth*, *std*, *saf* and *sti*) most abundant fimbrial clusters of *Salmonella* spp. (Yue et al. 2012). The *fim* operon of *Salmonella* consists of 10 genes and tRNA-Arg (Kisiela et al. 2013). FimW, FimY, FimZ and STM0551 proteins are responsible for regulation of T1F expression (Wang et al. 2012). FimA, FimF and FimH build the pili, FimC and FimD serve as chaperone and usher proteins during the assembly of T1F. The role of FimI is not yet fully understood. T1F are assembled by the chaperone-usher pathway of pilus biogenesis (Waksman and Hultgren 2009). All proteins needed for assembly (FimA, FimC, FimD, FimF and FimH) contain signal peptides for transport to the periplasm. FimC acts as a chaperone protein for FimA, FimF and FimH in periplasm: prevents from polymerization in the periplasm, and takes part in folding and assembly of the fimbriae. FimD is an usher outer-membrane protein, which facilitates subunit assembly into fimbria and export of the proteins trough the outer membrane. FimA, FimF and FimH contain hydrophobic N(and C-)-terminal extension (-s) that binds to a complementary part in FimC. In the FimD-usher, all proteins that constitute T1F are polymerised together by these extensions and this process is called donor strand exchange. T1F assembly starts with the FimC-FimH cognate substrate complex binding to the FimD usher protein. FimC-FimF complex is next bound into the FimD pocket. FimC bound to the N-terminal extension of FimH is exchanged for the C-terminal extension of FimF. In the next step, donor strand exchange is repeated with FimA and further elongation of the fimbrial shaft is continued with the FimA protein. One pilus comprises a FimH protein at the top bound to one FimF protein, with the fimbrial shaft built out of 500-3000 FimA subunits. Though it has been speculated that the *fimI* gene is required for regulation of length and mediation of adhesion, the mechanism of this process is so far not known (Rossolini et al. 1993). In contrast to the T1F of *E. coli*, deletion of one of *fimA*, *fimF*, *fimH* genes results in no fimbriae production (Zeiner et al. 2012), thus ascribing a shared role for all of them.

T1F in *Salmonella* were first described in the work of Duguid and Gillies (1958). These observations focused mainly on the ability of different *Salmonella* serovars and isolates to produce fimbriae and conditions inducing or inhibiting T1F production. Moreover, agglutination of red blood cells from different animals by T1F-positive (T1F+) *Salmonella* was also tested. For the first time, it was shown that *S*. Gallinarum produces T1F that do not agglutinate red blood cells. Follow-up studies conducted by Duguid contained information

about 149 serovars and 1442 isolates and showed mannose-dependent agglutination of red blood cells for the first time (Duguid et al. 1966). Like in the first study, induction of T1F in static liquid culture led to pellicle formation and multiple passages of bacteria in these conditions usually led to an increase in the percent of T1F+ bacteria. Growth on plates resulted in nearly no T1F+ *Salmonella*. Fimbriae of serovars not agglutinating red blood cells were initially called type 2 fimbriae, but genetic and microscopic analysis showed that they all belong to T1F (Crichton et al. 1989; Kisiela et al. 2005). In another study, adhesion mediated by T1F to red blood cells, leucocytes and epithelial cells was observed. Induction of T1F expression in *Salmonella* was associated with growth in aerobic static conditions for 24-48h, with multiple passages leading to an increase in the percentage of T1F+ bacteria (Old and Duguid 1970).

The role of T1F in *Salmonella* pathogenesis was studied intensively in various animal models. The first study with *S*. Typhimurium showed, that T1F+ strain was more infectious and virulent in a mouse model (Darekar and Duguid 1972). Mice infected with T1F+ bacteria also excreted *S.* Typhimurium in their faeces for longer periods. Similar results were obtained with follow-up studies (Duguid et al. 1976). A drawback of the aforementioned publications is that *Salmonella* strains used for testing (T1F-negative (T1F-) strain) was a "natural" derivative of T1F+ strain or strains "induced" or "not-induced" for T1F production. Other studies, which used transposon mutagenesis or the Datsenko-Wanner method for generation of T1F-non expressing mutants of *S*. Typhimurium or *S*. Enteritidis in a mouse infection model, obtained different results. In such studies, T1F- *S*. Typhimurium was more virulent than the original T1F+ strain (Lockman and Curtiss 1992). The authors hypothesised that the lower virulence of T1F+ strain was due to sequestration of T1F+ bacteria in liver, spleen and kidneys. However, the higher virulence of the T1F- *S*. Typhimurium strain in comparison to its parental isogenic strain was confirmed in another study, a result probably due to the activation of other virulence genes in absence of T1F (van der Velden et al. 1998). In a study analysing a *fimH* gene deletion mutant of *S*. Enteritidis, it was proposed that the T1F+ strain adhered to the intestinal lumen better, and therefore arguing for the expression of adhesive T1F being a limiting factor in the spread of bacteria outside the intestinal tract. The higher virulence of the T1F- strain compared to the TIF+ strain has been attributed to its relatively higher systemic spread (Kuźmińska-Bajor et al. 2015). In a 1-day-old chicken model of colonisation, the T1F- *S*. Enteritidis strain (which was also mutant for the SEF14 and SEF17 fimbriae) had lower ability to colonise the spleen, liver and caeca after 24h of infection, in comparison to the *S*. Enteritidis wild-type strain (Dibb-Fuller and Woodward 2000). In another study, T1F- *S*. Enteritidis (*fimA* gene single mutant)

did not show any significant differences in colonisation of the spleen and liver, and shedding of bacteria in faeces of 5-day-old chickens (Rajashekara et al. 2000). The only difference found in the T1F- strain was a higher amount of bacteria in the caecum 14 days post infection. Another study with chickens (laying hens) investigated the influence of *Salmonella* T1F on systemic infection (De Buck et al. 2004). The T1F- strain (Δ*fimD* deletion mutant) was present in blood, caeca and oviducts for a longer time than the WT strain, though the WT strain was more frequently isolated from eggs. Similarly, long-term infection studies of rats challenged with an *S*. Enteritidis mixture of WT and Δ*fimD* deletion mutants revealed that the T1F gene gives advantage at the early stages of infection in intestines, though T1F- strains are isolated in higher counts from intestines after 6 days post infection (Naughton et al. 2001).

The role of the T1F on adhesion to cells and cell lines has been extensively studied. Jones and Richardson investigated adhesion and infection of *S*. Typhimurium to HeLa cells and showed that *Salmonella* adhesion and invasion of HeLa cells occurred in a mannose-resistant manner, and was therefore probably not mediated by the T1F (Jones and Richardson 1981). Other publications with the HeLa cell line and *S*. Typhimurium or *S*. Braenderup have shown that T1F+ strains adhered to and invaded HeLa cells in higher numbers than non-fimbriated strains (Horiuchi et al. 1992; Bäumler et al. 1996a). Many studies with HEp-2 cells have shown that *Salmonella* adheres to cells in a mannose-sensitive manner (mediated by T1F) (Tavendale et al. 1983; Old et al. 1986; Ernst et al. 1990). However, contrasting results were presented in the study of Bäumler et al. (Bäumler et al. 1996a) whereby the T1F did not contribute to adhesion and invasion of HEp-2 cells. The variability of results obtained with the HEp-2 and HeLa cell lines was further shown in the study of Hancox et al. (1997), where *S*. Typhimurium WT bound to both cell lines better than their isogenic *fimH* gene mutant. The variation in adhesion assays with HEp-2 and HeLa cell lines may stem from experimental procedures during cultivation of cells and assays or use of various *S*. Typhimurium strains for assays, as well as different non-fimbriate strains. A major finding that the T1F FimH protein is responsible for binding and DNA sequencing enabled investigations for the role of sequence variation in adhesion. It has been shown that *fimH* gene allelic variation in two *S*. Typhimurium strains leads to different phenotypes in HEp-2 adhesion assays (Boddicker et al. 2002).

In another work, *S*. Typhimurium adhesion to and infection of isolated rat small intestine enterocytes occurred in a mannose-sensitive manner (Lindquist et al. 1987). The direct involvement of T1F in the intestinal phase of infection was later analysed in a rat infection model, where by immunohistochemistry, expression of T1F in *S*. Enteritidis and *S*.

Typhimurium adhering to enterocytes was proven (Ewen et al. 1997). T1F+ *S*. Typhimurium was tested for mannose-sensitive adhesion to isolated intestines of 1-day old chicks and the same results were obtained like in the case of isolated rat enterocytes (Oyofo et al. 1989). When the *S*. Enteritidis strain and its *fimD* gene deletion mutant were checked for adhesion to chicken duodenal explants, no significant difference between these two strains was found (Allen-Vercoe and Woodward 1999). But in the case of human buccal cells and mouse small intestine epithelial cells of *S*. Enteritidis, adhesion of *S*. Enteritidis was mediated by T1F and blocked by mannose preincubation of bacteria (Aslanzadeh and Paulissen 1990). In INT-407 and Caco-2 cell line models, three T1F- *S*. Enteritidis strains adhered to and invaded cells lower than its parental T1F+ strains (Dibb-Fuller et al. 1999). Dendritic cells (DC) can send dendrites in between enterocytes, which enables direct contact of DC with bacteria. Therefore a possible role of the interaction between murine bone marrow-derived DCs and T1F+ was investigated and it was shown that T1F+ *S*. Typhimurium can bind to DCs in a mannose-sensitive manner (Guo et al. 2007).

The discovery of T1F-dependend, mannose-sensitive haemagglutination of guinea pig red blood cells led to a hypothesis, that carbohydrates containing mannose are receptors for T1F. The carbohydrate specificity of *Salmonella* T1F was first tested by inhibition of agglutination with linear and branched oligosaccharides or glycosides of D- mannose. T1F of *Salmonella* displayed different carbohydrate specificities compared to T1F of *E. coli* (Firon et al. 1983, 1984).

One of the intriguing issues concerning T1F of *Salmonella* is/are possible receptor/-s for this adhesin expressed on host cells. It is known that most of the T1F+ *Salmonella* bind to glycoproteins (an exception from this rule is *S*. Gallinarum). Leusch et al. (1991) analysed binding of various *Salmonella* serovars to glycoproteins expressed in the intestine, egg white, blood, spleen and bile, and *S*. Typhi bound with the highest affinity to carcinoembryonic antigens (CEAs) and an unknown glycoprotein called in this publication “NCA-55”. In another study, laminin- extracellular matrix protein- was found as a receptor of *S*. Enteritidis and *S*. Typhimurium T1F, and T1F bound to the glycan part of laminin in a mannose-sensitive manner (Kukkonen et al. 1993). Another possible receptor for T1F is a 60-kDa glycoprotein, that was isolated from brush border of rat small intestine, but the name of this protein remains unknown (Ghosh et al. 1996). *S*. Enteritidis possesses the ability to infect chicken eggs during egg production. It was proposed that T1F might mediate binding of bacteria to the isthmus of the chicken reproductive tract. Binding of T1F+ *S*. Enteritidis to isthmus sections and secretions

was blocked by mannose and mediated by mannosylated glycoproteins, which were detected in isthmus using lectins (De Buck et al. 2003). Pancreatic secretory granule membrane major glycoprotein GP2 (GP2) has been identified as a transcytotic receptor of M cells for T1F+ *Salmonella* and this interaction is explained in detail in the chapter dedicated to GP2 (Hase et al. 2009). As the T1F-mediated adhesion to different cell types and cell lines varies among serovars with various host ranges, it was proposed, that they target different receptors. A study analysing adhesion of FimH proteins from *S*. Enteritidis, *S*. Choleraesuis, *S*. Dublin and *S*. Abortus-ovis to cell lysates from epithelial cell lines from various hosts presented different patterns of adhesion, dependent on the host range. FimH from generalist *S*. Enteritidis bound to surface membrane proteins of about 130 kDa, while FimHs from host specialists bound to a protein of about 55 kDa. The only non-glycan-mediated binding of *S*. Typhimurium T1F to a protein-receptor was published in the case of plasminogen (Kukkonen et al. 1998). Moreover, binding of *S*. Typhimurium T1F in this study was blocked by lysine analogue and not mannose.

The discovery that *fimH* gene allelic variations in two *S*. Typhimurium strains in positions 61(glycine->alanine) and 118 (phenylalanine->serine) lead to different phenotypes in binding assays (low->high), started a new era in investigations concerning T1F (Boddicker et al. 2002). Next, a low binding variant of *S*. Typhimurium was compared with FimH variant of *S*. Enteritidis, and it was proposed that *S*. Enteritidis FimH variant binding phenotype is low due to the presence of glycine at position 61 and phenylalanine at position 118 (Kisiela et al. 2006). Follow-up studies on this topic with *S*. Enteritidis FimH showed that only F118S substitution contributes to a change from low to high binding phenotype (Grzymajlo et al. 2010). It has been previously mentioned, that T1F of *Salmonella* can mediate binding to HEp-2 cells and DCs. As it turned out, variation within the FimH sequence in position 158 also modulates interaction with HEp-2 cells and DCs (Guo et al. 2009). Studies employing random mutagenesis and 3D structure predictions for *S.* Typhimurium FimH showed that not only changes in the predicted binding pocket, but also mutations in various parts of the *fimH* gene sequence can lead to a change in binding properties. It was proposed that shear force during binding of FimH can lead to activation of allosteric properties in FimH variants, which can alter binding properties of these FimH variants in comparison to binding under static conditions (Kisiela et al. 2011). Screening of various *Salmonella* serovars for FimH variation and HEp-2 cell line adherence, mannose-sensitive binding and biofilm formation further confirmed the variability in the mode of action of *Salmonella* FimHs (Dwyer et al. 2011). The “opus magnum” study on the role and association of *fimH* gene allelic variation in the pathogenesis of *Salmonella* serovars with

different host ranges was published by Kisiela et al. (2012). The authors investigated the role of FimH sequence variations on binding to standard glycoproteins, epithelial and macrophage-like cell lines and associated FimH high- and no-binding-phenotype with host specialists (host-restricted and host-adapted serovars) and low-binding phenotype with host generalists (host-unrestricted serovars). A recently published study investigated FimH variation among *S*. Typhimurium isolated from different hosts and FimH variant mediated host-specific adherence in vitro (Yue et al. 2015). Analysis of 580 isolates of *S*. Typhimurium revealed that presence of valine in position 245 of FimH was more often found in human isolates and alanine in the same position was more often found in bovine isolates. Moreover, cell line adhesion assay showed that V245A substitution changes affinity to cell lines of human and bovine origin. Results from the same study revealed that FimH variants from *Salmonella* host specialists mediated binding to cell lines in a host-specific manner in *in vitro* adhesion assays.

The loss of mannose-sensitive binding in T1F of *S*. Gallinarum was explained by a single amino acid change in position 78 of the FimH protein from threonine to isoleucine, since this protein is normally responsible for a mannose-resistant phenotype in its wild-type state (Kisiela et al. 2005). It has been discussed, whether the mannose-resistant phenotype of *S*. Gallinarum FimH confers the inactive phenotype of T1F unable to bind to any receptors or whether mannose-resistant phenotype leads to changes in receptor specificity. The study of Guo et al. (2009) provided proof, that the latter is true and could demonstrate that *S*. Gallinarum T1F mediate binding to chicken leukocytes. Another study investigating the role of FimH variation on the pathogenesis of *S*. Gallinarum showed that exchange of mannose-resistant *fimH* gene to mannose-sensitive *fimH* gene (from *S*. Enteritidis) in *S*. Gallinarum leads to decreased/no colonization of liver, spleen and caecal tonsils, thus showing a significant role of FimH variation in bacterial host specificity (Kuźmińska-Bajor et al. 2012). Investigations by Guo et al. (2009) and Kuźmińska-Bajor et al. (2012) revealed that T1F variation can confer a significant advantage for *S*. Gallinarum during pathogenesis in chickens, an observation that could explain such a drastic change like the switch from mannose-sensitive to mannose-resistant phenotype. Another example of an association of FimH variation with recognition of host proteins can be found in the aforementioned study of Grzymajlo et al. (2013), which identified substitutions in positions L57P and N101S in FimH as altering receptor specificity and possibly contributing to changes in host range of *Salmonella* serovars.

1.2. Payer's patches and M cells

1.2.1. Development and function in intestines

Gastrointestinal tract mucosa has the largest surface area of an animal's body- the total surface area varies between 30-400 m^2 and represents potential entry routes for many pathogens (Owen et al. 2013; Helander and Fändriks 2014). The lymphoid tissue responsible for the defence of the gastrointestinal tract is called gut-associated lymphoid tissue (GALT). GALT in the intestinal wall of mammals comprises organized lymphoid tissue i.e. Peyer's patches (PP), colonic or caecal patches and solitary intestinal lymphoid tissue (SILT) represented by cryptopatches and isolated lymphoid follicles (ILFs), but also loose clusters of cells like T and B lymphocytes, macrophages and dendritic cells (Herbrand et al. 2008; Coombes and Powrie 2008). The role of GALT is to protect against pathogens and ensure tolerance towards commensals. Of all the lymphoid tissue that comprise the GALT, the role of PP in these immune processes has been extensively studied. The organisation of PP in intestines differs between species considerably. In cattle and pigs, there are 20-50 jejunal PPs and one long ileal PP (up to 3m in cattle), which undergoes involution (after 15-24 months of life) and is replaced by isolated lymphoid follicles (Liebler-Tenorio and Pabst 2006). In human, there are around 50-100 jejunal PPs and 60-120 ileal PP (Cornes 1965). A large part of ileal PPs forms the lymphoid ring (Van Kruiningen et al. 2002). In mice, the number of PPs can vary between strains and usually there are between 5 to 10 PPs in the small intestine (Kelsall 1946). Histologically, PP consists of following regions: B-cell follicles, follicle-associated epithelium (FAE), subepithelial dome and T-cell zones between the follicles and FAE (Reboldi and Cyster 2016). PP are responsible for sampling of intestinal antigens (Schulz and Pabst 2013). Antigens delivered through FAE are captured by dendritic cells or macrophages in the subepithelial dome and presented to naive B cells in follicles. Activated B cells can stay in the follicles as a part of the germinal centre or move into extrafollicular areas, where they differentiate into IgA-producing plasma cells (Suzuki et al. 2010). IgAs produced by plasma cells protect from infection by pathogens or toxins produced by bacteria and help maintain homoeostasis between host and microbiota (Suzuki and Nakajima 2014).

Normal intestinal mucosa functions as a natural barrier against pathogens and antigens. FAE, which covers PP and provides contact with the intestinal lumen, differs from intestinal mucosa and contains cells specialised in the transport of luminal antigens and bacteria- membranous cells (M cells) (Williams and Owen 2015). Other cells that can be found in FAE are similar to intestinal mucosa and these are enterocytes, goblet cells and intraepithelial lymphocytes

(Edelblum and Turner 2015). The area of FAE covered with M cells can vary between 10 and 50% and is species dependent (J L Wolf and Bye 1984). M cells can be already detected in Payer's patches during fetal development (Bockman and Cooper 1975; Moxey and Trier 1978). The origin and differentiation of M cells were for a long time subjects of discussion, but recent advances in this field showed that M cells originate from Lgr5 positive stem cells, which give rise to all epithelial cell types in the small intestine (de Lau et al. 2012). The turning point for these cells on their way to becoming fully differentiated M cells is the activation of receptor activator of NF- κB (RANK) by the RANK ligand (RANKL) (Knoop et al. 2009). This leads to expression of Marcsl1 and annexin A5 in early stages of development and Spi-B, which is crucial to complete maturation of M cells (Kanaya et al. 2012). Other factors contributing to M cell differentiation are epithelium-intrinsic microRNAs (Nakato et al. 2016). It has been shown that presence or absence of external factors can influence the amount of M cells in Peyer's patches (Kanaya and Ohno 2014). For example, the intestinal microbiota can influence the amount of M cells in PP. Experiments with germ-free mice show that they have smaller PP with less M cells in comparison to non-germ-free mice and conventionalization of germ-free mice leads to increase of PP and M cells (Pickard and Chervonsky 2010). Another external factor influencing M cell differentiation is the B lymphocytes. Absence of B cells leads to decrease in PP size and a decrease in the number of M cells (Mach et al. 2005).

The morphology of M cells differs considerably in comparison to enterocytes. Firstly, M cells lack brush border and the apical membrane is not covered by a thick layer of glycocalyx and mucus. Another characteristic feature of M cell morphology is invagination of the basolateral membrane. Within this hollow, various immune cells can be found e.g. T and B cells, dendritic cells and macrophages (Brayden et al. 2005). As M cells specialise in antigen sampling, it was hypothesised they could express certain receptors that mediate antigen uptake. Several studies identified pattern recognition receptors (PRR) as mediators of antigen uptake by M cells. Various TLRs expression was found in mouse M cells and TLRs can be expressed exclusively on the surface of M cells (e.g. TLR1, 2, 4) or at a higher level than in other types of cells in the intestines (e.g. TLR8 and 9) (Tyrer et al. 2006; Cashman and Morgan 2009). In swine, expression of TLR2 and 9 on M cells was confirmed (Shimosato et al. 2005; Tohno et al. 2005). Unfortunately, there is no information about TLRs expression in human M cells. It has been observed in mice that M cells can take up sIgA and probably IgA-antigen complexes, but the receptor for sIgA remains unknown (Mantis et al. 2002). Cellular prion protein (PrP^c) is expressed specifically on the surface of M cells where it mediates transcytosis of Hsp60-

expressing bacteria (Nakato et al. 2012). M cells are able to take up T1F+ bacteria because they express the pancreatic secretory granule membrane major glycoprotein GP2 (Hase et al. 2009). GP2 expressed on the surface of M cells is also a receptor for botulinum toxin A (Matsumura et al. 2015).

1.2.2. M cells as entry route for pathogens

The role of M cells as a part of the GALT is antigen uptake and transcytosis, which further leads to the production of protective sIgAs. Interestingly, many examples show that pathogens are able to exploit the transcytosis by M cells as a gateway from the intestinal lumen to its underlying tissues onward to systemic spread. In this chapter, pathogenic mechanisms used by bacteria on M cells will be discussed.

Shigella flexneri can cause severe inflammatory diarrhoea. During the initial phase of infection, *Shigella* uses M cells to traverse the intestinal barrier (Fig. 1). The nature of translocation of *Shigella* through M cells is not elucidated, but it has been shown, that strains with a 140 kb virulence plasmid are transported more efficiently than the same strains without a virulence plasmid (Wassef et al. 1989; Sansonetti et al. 1996). Probably, virulent *Shigella* strains are inducing active transport through M cells via T3SS, in contrast to the avirulent strain, which is translocated passively by M cells. Translocation of *Shigella* through M cells is crucial for the next stages of infection because *Shigella* is only able to infect enterocytes from the basolateral side of cells (Mounier et al. 1992). After translocation through M cells, *Shigella* first encounters phagocytic cells, macrophages and DCs, in which it survives and induces apoptosis. After being released from these phagocytic cells, *Shigella* invades enterocytes from the basolateral side and replicates in the cytoplasm. Multiplication of bacteria, toxin production and release of proinflammatory cytokines lead to the destruction of the intestinal epithelium and FAE (with M cells), and inflammatory diarrhoea (Schroeder and Hilbi 2008).

M cells play also an important role in the pathogenesis of *Yersinia pseudotuberculosis* and *Y. enterocolitica* (Fig. 2). Both *Yersinia* species cause gastroenteritis, and depending on disease development infection with *Yersinia* can lead also to lymphadenitis, appendicitis, fever and abdominal pain (Fredriksson-Ahomaa et al. 2006; Long et al. 2010). In contrast to *Shigella*, a receptor and mechanism of internalisation for *Yersinia* is well known. *Yersinia* expresses invasin, which mediates binding to β_1 integrin. β_1 integrin is expressed on the apical and baso-

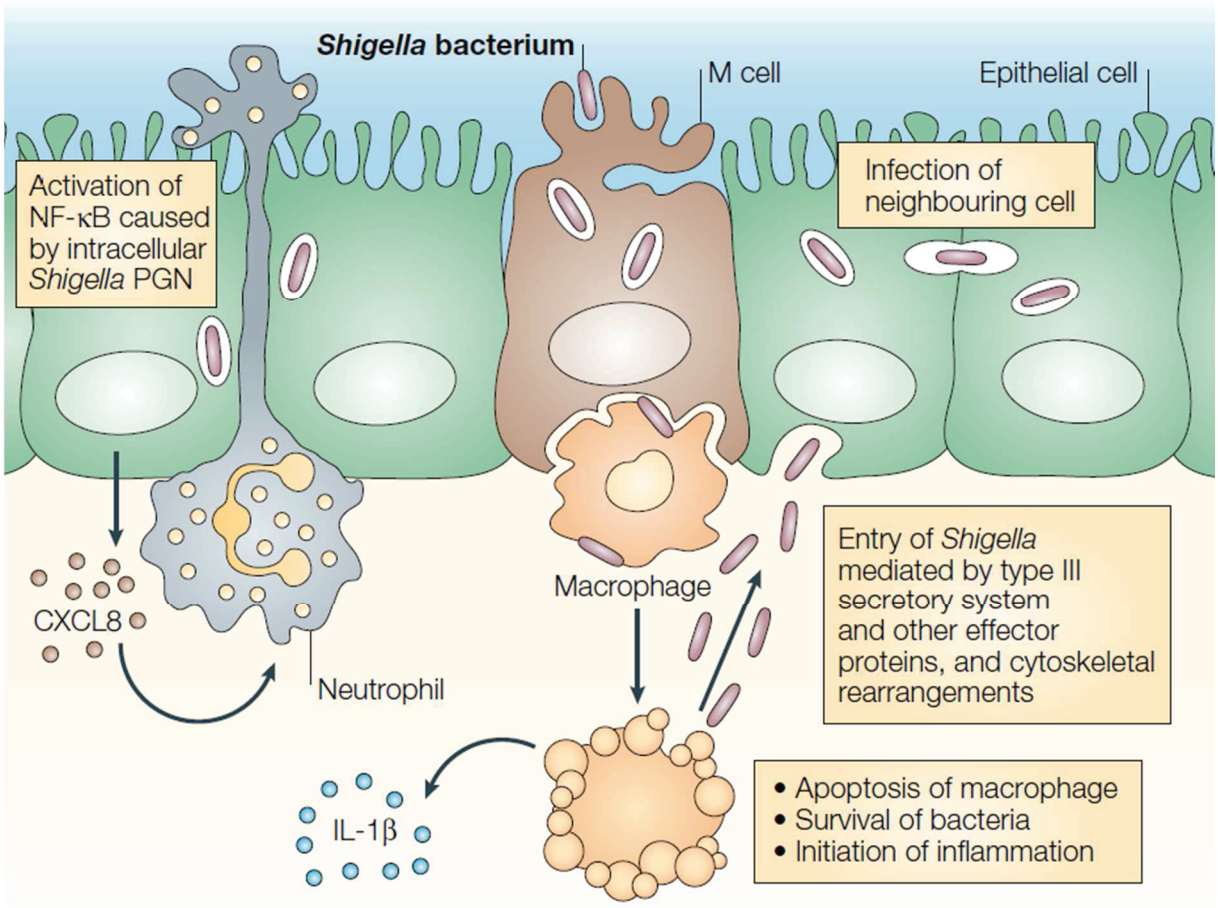

Figure 1. Schematic representation of *Shigella* interaction with M cells.
This image is taken from Sansonetti (2004) (Copyright License No. 4216980892119). *Shigella* traverses the intestinal barrier through M cells. After translocation through M cells, *Shigella* first encounters phagocytic cells, macrophages and DCs, in which it survives and induces apoptosis. After being released from these phagocytic cells, *Shigella* invades enterocytes from the basolateral side and replicates in the cytoplasm. Multiplication of bacteria, toxin production and release of proinflammatory cytokines lead to the destruction of the intestinal epithelium and FAE (with M cells), and inflammatory diarrhoea.

lateral membranes of M cells, but only on the basolateral part of enterocytes (Clark et al. 1998a). Invasin deletion mutants are impaired in their translocation ability through M cells (Handley et al. 2005). The binding of invasin to β_1 integrin leads to activation of the non-receptor protein tyrosine kinase (PTK) Fak and Cas. This causes actin rearrangement, and transcytosis of *Yersinia* through M cells (Bruce-Staskal et al. 2002). Similar to *Shigella*, *Yersinia* in the subepithelial dome has to challenge and bypass phagocytic cells, albeit by the use of a different mechanism. *Yersinia*, using the T3SS effector proteins, avoids internalisation by blocking actin rearrangement and killing by phagocytes (Pizarro-Cerdá et al. 2016). The survival of bacteria in PP leads to the destruction of PP and in some cases allows bacteria to spread to mesenteric lymph nodes, a process which can be extended by a systemic spread in rare cases.

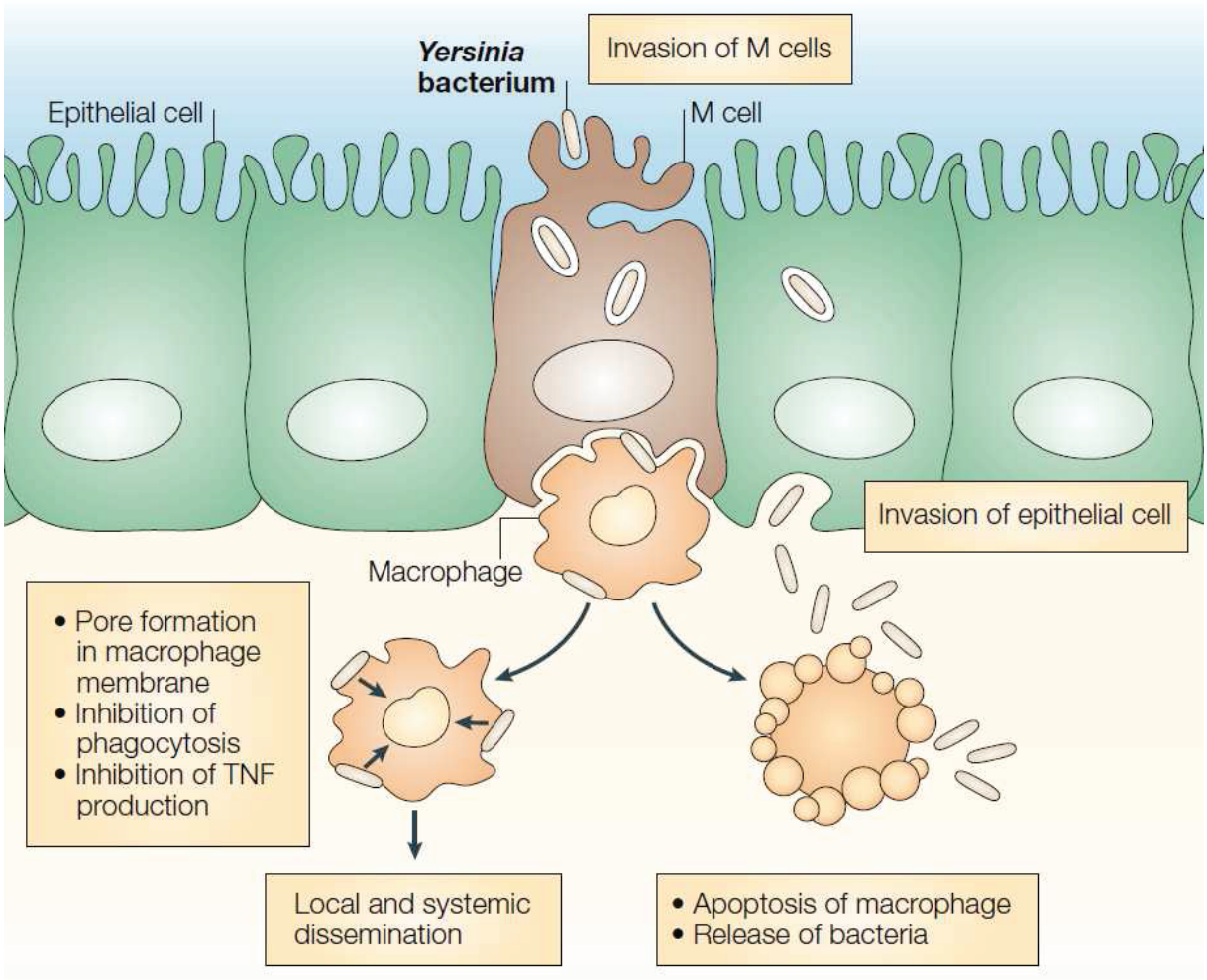

Figure 2. Schematic representation of *Yersinia* interaction with M cells.
This image is taken from Sansonetti (2004) (Copyright License No. 4216980892119). *Yersinia* traverses the intestinal barrier through M cells. *Yersinia* has to challenge and bypass phagocytic cells in the subepithelial dome. *Yersinia* uses the T3SS effector proteins and avoids internalisation by blocking actin rearrangement and killing by phagocytes. The survival of bacteria in PP leads to the destruction of PP and in some cases allows bacteria to spread to mesenteric lymph nodes, a process which can be extended by a systemic spread on rare occasions.

E. coli binding to M cells depends on pathotype and probably on the host. Enteropathogenic *E. coli* (EPEC) do not confer any genes targeting only M cells during infection (Miller et al. 2007). Specific binding of rabbit diarrheagenic *E. coli* (RDEC) to M cells was reported before, and the interaction was shown to be mediated by the AF/R1 pili expressed by RDEC (Inman and Cantey 1983; Cantey et al. 1999). Enterohaemorrhagic *E. coli* (EHEC) was able to target PP and cause attaching/effacing lesions in bovine and human intestines (Phillips et al. 2000). Moreover, EHEC is able to translocate across M cells and survive within macrophages. It has been proposed that translocation of EHEC through M cells is an important step in the development of haemolytic-uremic syndrome (HUS) (Etienne-Mesmin et al. 2011). Crohns disease-

associated *E. coli* (CDEC) was shown to be adherent and invasive to M cells (Roberts et al. 2010). Long polar fimbriae of CDEC are mediators of the binding between M cells and bacteria (Chassaing et al. 2011).

The interaction of *Salmonella* with M cells has been thoroughly investigated before (Carter and Collins 1974; Clark et al. 1994), especially the involvement of T3SSs in transcytosis through M cells. The first report on *Salmonella invA* and *invG* mutants show that the process of translocation through M cells is T3SS-1 independent (Clark et al. 1996). Other results were shown in the work of Penheiter et al. (1997), wherein *Salmonella* deficient in different components of T3SS (including *invG*) was not able to infect M cells. The discrepancies in results from these studies might be caused by different media used for bacteria in ligated ileal loops, as has been shown by Clark et al. (1998b). Different media composition could alter expression of virulence factors by *Salmonella*, leading to changes in interaction with M cells. Studies on the M cell-like cell line model has shown that translocation through M-like cells is T3SS-1 and T3SS-2 independent for *S*. Typhimurium (Martinez-Argudo and Jepson 2008). Since *Salmonella* can be translocated in a T3SS-independent manner, adhesion to M cells should be possible by other factors than the SPI-1 effector proteins (Fig. 3). First, long polar fimbriae (lpf) have been identified as a factor mediating adhesion to M cells in a murine model, but the receptor on the surface of M cells remains unknown (Bäumler et al. 1996b). Most recently, T1F have been shown to take part in adhesion to M cells and the M cell receptor for T1F+ bacteria was shown to be GP2 (Hase et al. 2009). After translocation through M cells, *Salmonella* encounters phagocytes in the subepithelial dome. *Salmonella* is capable of surviving and multiplying in phagocytes, which serve as vehicles for systemic spread or establishment of carrier state depending on the *Salmonella* serovar and host immune status (Figueira et al. 2013).

The role of M cells in the pathogenesis of bacteria from other families other than Enterobacteriaceae have been also studied. *Mycobacterium avium* subsp. *paratuberculosis* causes Johne's disease in ruminants (Bannantine and Bermudez 2013). Numerous studies show, that M cells can be infected by *M. paratuberculosis,* and that binding to M cells is mediated by a bridge formed between a fibronectin attachment protein homologue, fibronectin and integrins expressed on the surface of M cells (Secott et al. 2001, 2004). Another bacterium that translocates through M cells during pathogenesis is *Brucella abortus*, that causes reproductive tract infection and abortion in domestic animals (Poester et al. 2013). *Brucella* adheres to M

cells via the Hsp60 protein, which binds to PrP^c that is specifically expressed on the surface of M cells (Nakato et al. 2012). The possibility of transcytosis through M cells has been reported

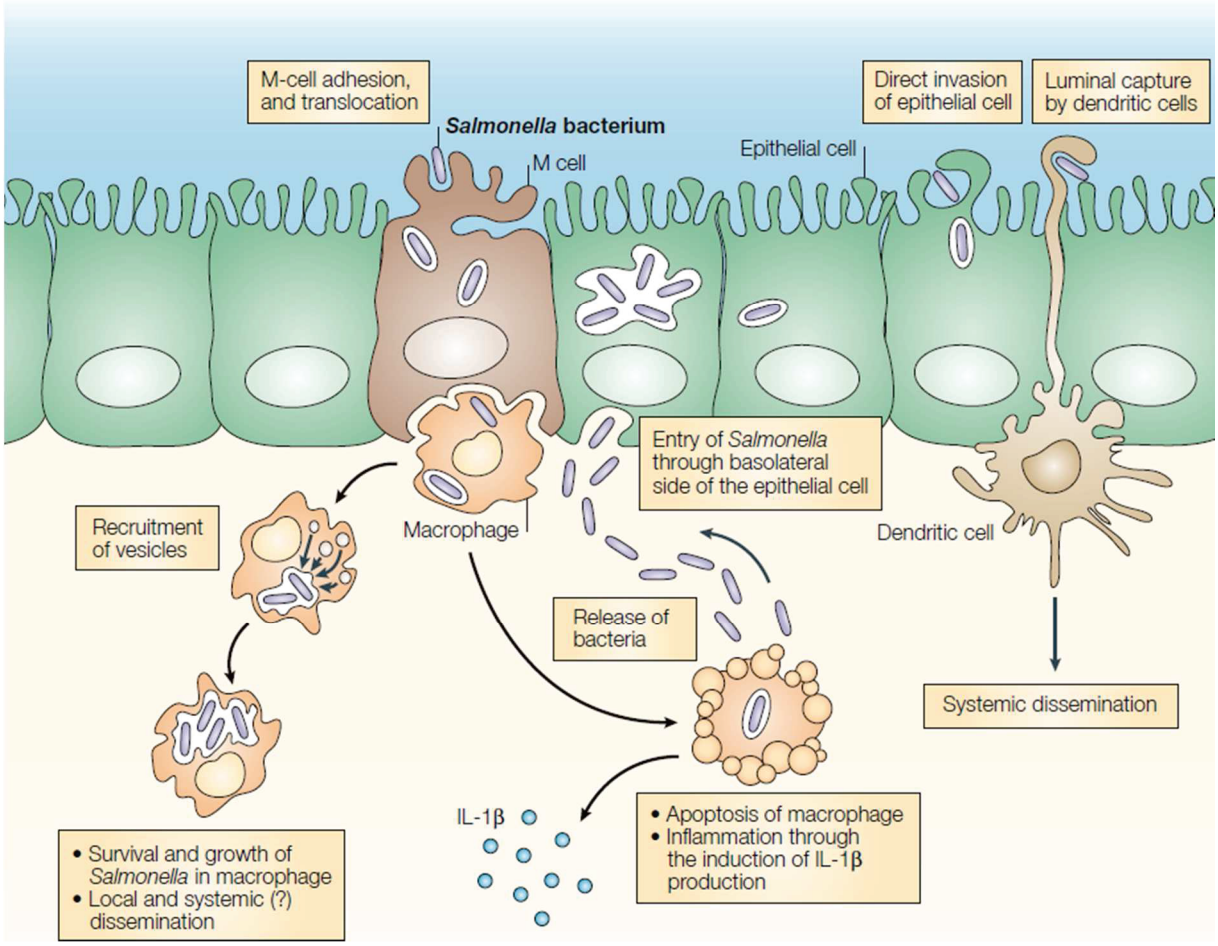

Figure 3. Schematic representation of *Salmonella* interaction with M cells.
This image is taken from Sansonetti (2004) (Copyright License No. 4216980892119). *Salmonella* adheres to M cells through T1F or long polar fimbriae and traverses the intestinal barrier through M cells in a T3SS-independent manner. After translocation through M cells, *Salmonella* encounters phagocytes in the subepithelial dome. *Salmonella* is capable of surviving and multiplying in phagocytes, which serve as vehicles for systemic spread or establishment of carrier state depending on the *Salmonella* serovar and host immune status. *Salmonella* can also invade enterocytes from the basolateral side.

in the case of *Vibrio cholearae*, *Listeria monocytogenes* and *Campylocabter jejuni*, but little is known about the molecular mechanisms of transcytosis or its contribution to virulence of aforementioned bacteria (Owen et al. 1986; Walker et al. 1988; Jensen et al. 1998).

1.3. Pancreatic secretory granule membrane major glycoprotein GP2 (GP2)

1.3.1. Tamm–Horsfall protein (THP) - a GP2 homologue with similar function(-s).

It is important to explain about THP in a separate chapter, before proceeding to GP2, because it will be easier to explain why GP2 is of particular interest. THP (uromodulin) shares 53% identity and 85 % similarity with GP2 (Hoops and Rindler 1991). Therefore, many ideas about the physiological function of GP2 were deduced from studies conducted with uromodulin.

THP is an 85-kDa glycoprotein with an N-terminal signal peptide, three EGF-like domains, a zona pellucida (ZP) domain and a C-terminal glycosylphosphatidylinositol (GPI)-anchor. THP is expressed exclusively by epithelial cells of the thick ascending limb (TAL) of the loop of Henle, and secreted in the urine at a rate of around 30-50 mg/day (Iorember and Vehaskari 2014). The proposed role of THP in TAL is to improve water impermeability and by that increase the efficiency of ion reabsorption (Vyletal et al. 2010). Mutations in the UMOD gene are associated with three chronic tubulointerstitial diseases (Lens et al. 2005). Because of its ability to aggregate, the involvement of uromodulin in renal cast formation was shown before (Fletcher et al. 1970). The formation of aggregates is due to the ZP domain in THP, which causes protein aggregation in various conditions.

The role of THP secreted in urine was the subject of many studies. The immunomodulatory properties of uromodulin have been proposed and shown to be involved in some diseases like acute kidney injury or inflammation (El-Achkar et al. 2008). Moreover, suppressive activities against peripheral blood mononuclear cells were also demonstrated (Hong et al. 2015). Interestingly, an immunostimulatory role has been shown for THP in the renal interstitium (Darisipudi et al. 2012). Another function of secreted THP is in binding to T1F+ uropathogenic *E. coli* (UPEC) and preventing adhesion to the urinary bladder umbrella cells. UPEC targets uroplakin expressed on the surface of umbrella cells during pathogenesis. Secreted uromodulin competes with uroplakin for binding UPEC, resulting in a lower load of bacteria binding to umbrella cells. THP knock-out mice are more susceptible to UPEC infection than wild-type mice (Bates et al. 2004).

1.3.2. GP2 as a part of the secretory granule membrane

GP2 is a glycoprotein that contains an N-terminal signal peptide, an EGF-like domain, a ZP domain and C-terminal GPI-anchor (Jovine et al. 2005). GP2 was first found as the most abundant protein in pancreatic secretory granule membrane (Ronzio et al. 1978). During secretion of granules from the pancreas, GP2 is cleaved from the secretory granule membrane and can be found in the intestines. GP2 sequence orthologues can be found among mammals, but there is no information about GP2 in other classes of vertebrates. Depending on the animal, various numbers of alternative splice variants are expressed (Fukuoka 2000). Four isoforms have been predicted for human, two in cattle and pig and one for mouse according to NCBI Gene (Maglott et al. 2005). The influence of GP2 splice variation on the protein's physiological role has not been investigated so far. GP2 is a N-glycoprotein, with glycosylation profiles of GP2 varying between species. Rat, pig and bovine GP2 glycans are tri- or tetraantennary-type oligosaccharides, but their reactivity with selected lectins is species specific (Havinga et al. 1983; Tsujii-Hayashi et al. 2002).

The physiological role of GP2 in the pancreas is not known. It has been proposed that GP2 plays an important role in the sorting of proteins to the zymogen granule or regulating membrane recycling after secretion from the membrane (Fritz et al. 2002). GP2 knock-out mice did not have any alterations in their pancreas, zymogen granule secretion and were healthy (Yu et al. 2004). As GP2 has high homology to uromodulin and as it has been shown that uromodulin is involved in renal cast formation, a study analysing the involvement of GP2 in intraductal plug formation in chronic pancreatitis was conducted (Freedman et al. 1993). The formation of aggregates by both proteins is due to the ZP domain, which determines protein aggregation at certain pH values and in the presence of divalent cations. Yet another possible function of GP2 - immunomodulation – can be deduced from studies with uromodulin. It has been shown that GP2 modulates innate and adaptive immune response (Werner et al. 2012). The role of GP2 in mucosal immunity might also be extrapolated from the fact that antibodies against GP2 can be found in sera of Crohn's disease patients (Roggenbuck et al. 2009). The possible role of GP2 as a molecular decoy preventing binding of bacteria to intestinal cells has also been investigated. First, because of high homology to uromodulin, the ability of T1F of *E. coli* to bind to GP2 was studied. Indeed, T1F expressed by *E. coli* could bind to recombinant human GP2 (Yu and Lowe 2009). GP2 was found bound to bacteria in proteomic studies analysing microbial communities from healthy donors and patients with Crohn's (Juste et al. 2014).

1.3.3. GP2 and M cells

M cells have been the subject of many studies since their discovery. In order to investigate the unique properties of M cells, gene expression profiles of M cells were analysed. Comparison of gene expression between intestinal epithelial cells revealed that GP2 was specifically expressed on the surface of M cells, but not in any other intestinal cell type (Terahara et al. 2008). This finding changed the view about the role of the GP2 protein. It was proposed that GP2 is expressed on the surface of M cells as a receptor for T1F+ bacteria. Next, the role of GP2 in M cell transcytosis of *Salmonella* was investigated. *Salmonella* translocates through M cells in a GP2-dependent manner and *fimH* gene-deficient *Salmonella* or GP2 knock-out mice show a large decrease in M cell transcytosis of *Salmonella*. Translocation of FimH-positive *Salmonella* through M cells leads to increased amounts of bacteria in the mesenteric lymph nodes and immune response to antigens expressed by these bacteria, which confirms a physiological role for GP2 expressed on the surface of M cells as part of GALT immunosurveillance (Hase et al. 2009).

As shown in chapter 1.2.2, pathogenic bacteria exploit the physiological role of M cells in their pathogenesis. In order to validate this possibility, binding of various bacterial species to recombinant human GP2 isoform 2 was investigated (Schierack et al. 2014). Among tested bacteria, all investigated *E. coli* pathotypes, *Salmonella* and *Klebsiella* bound to GP2. *Proteus, Buttiauxella, Pantoea, Raoultella, Serratia* and methicyllin-resistant *Staphylococcus aureus* (MRSA) were low or not binding to GP2. Next, the role of *E. coli* FimH sequence variation in GP2 binding was investigated and a correlation between FimH amino acid sequence and binding to GP2 was shown.

The high relevance of M cells and GP2 in *Salmonella* pathogenesis was shown in the work of Tahoun et al. (2012). The ability of *S.* Typhimurium to transform FAE into M cells shed light on the convergent evolution of pathogen with its host. *Salmonella* gains on translocating through M cells to such a level that it developed a way to increase the number of M cells. Transdifferentiation from FAE into M cells is mediated by the T3SS effector protein SopB. An increase in the number of M cells in PP leads to an increase in translocation of *Salmonella* and to local or systemic spread. An increase in the number of M cells in PP leads to an increase of GP2-expressing cells in PP.

1.4. Aims of the study

The physiological role of M cells is antigen uptake and transcytosis. *Salmonella* uses M cells as entry site to underlying tissues and spreads locally or systemically. The finding, that GP2 is specifically expressed on the surface of M cells has changed the view about the process of *Salmonella* translocation through M cells. It was proposed that GP2 is expressed on the surface of M cells as receptors for T1F-positive bacteria and that *S.* Typhimurium translocates through M cells in a GP2-dependent manner. T1F are one of the most common adhesive organelles in the family of Enterobacteriaceae and important adhesion factor in *Salmonella* pathogenicity. The FimH protein is located on top of the T1F shaft and directly interacts with glycoprotein-receptors. Several studies have shown that serovars -associated FimH variants from *Salmonella* specialists and generalists can differ significantly in receptor recognition or tropism to different tissue types, and this can lead to a change in the course of infection. It was shown that *S*. Typhimurium can bind to murine and human GP2, but the possible differences of *Salmonella* FimH variants in binding to GP2 isoforms from various hosts have not been investigated. Therefore, the aim of this study was to compare *Salmonella*-GP2 interaction between two hosts to test for species- and serovar-specific interaction. Human and pig – (two important hosts of *Salmonella* infection) were chosen.

The specific aims of this thesis are as follows:

1. Clone and express porcine GP2 isoforms in SF9 insect cells.
2. Determine *fimH* gene sequences and T1F expression levels in 128 *Salmonella* isolates.
3. Create a *Salmonella* isogenic model to study the influence of FimH variation on GP2 adhesion.
4. Investigate adhesion of *Salmonella* to GP2 isoforms of human and pig origin.
5. Create stable cell lines expressing human and pig GP2 isoforms.
6. Investigate adhesion of *Salmonella* to GP2 expressing cell lines.

2. Materials

2.1. Bacterial strains

Table 1. List of bacteria used in this study

Strains	Description	Source
Escherichia coli XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIq ZΔM15 Tn10 (Tetr)]*	Agilent Technologies
Escherichia coli DH10Bac	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 ΔlacX74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ– *rps*L *nup*G/bMON14272/pMON7124	Thermo Scientific
Salmonella enterica subsp. *enterica* serovar Typhimurium	20 strains, isolated from human diarrhoea cases	Mydlak/Thorasch Diagnostic Laboratory, Cottbus
Salmonella enterica subsp. *enterica* serovar Typhimurium	20 strains, isolated from porcine stool samples	BfR, Berlin
Salmonella enterica subsp. *enterica* serovar Enteritidis	20 strains, isolated from human diarrhoea cases	Mydlak/Thorasch Diagnostic Laboratory, Cottbus
Salmonella enterica subsp. *enterica* serovar Enteritidis	14 strains, isolated from chicken stool samples	BfR, Berlin
Salmonella enterica subsp. *enterica* serovar Gallinarum	19 strains, isolated from chicken stool samples	BfR, Berlin
Salmonella enterica subsp. *enterica* serovar Dublin	20 strains, isolated from bovine stool samples	BfR, Berlin
Salmonella enterica subsp. *enterica* serovar Choleraesuis	15 strains, isolated from various sources (porcine stool, wild boars, meat products, reptile)	BfR, Berlin
Salmonella enterica subsp. *enterica* serovar Typhimurium SL1344	+pFPV25.1GFPmut3Kan plasmid	Dr. Karsten Tedin, FU, Berlin

2.2. Plasmid vectors

Table 2. List of plasmid vectors used in this study

Plasmid	Source
pJET1.2	Thermo Scientific
pDONR221	Thermo Scientific
pDEST8	Thermo Scientific
pACYC177	Dr. Karsten Tedin, FU, Berlin
pKD46-Gm	Dr. Benoit Doublet, INRA, France
pCP20-Gm	Dr. Benoit Doublet, INRA, France
pKD3	Dr. Karsten Tedin, FU, Berlin
pKD4	Dr. Karsten Tedin, FU, Berlin
pLVX-IRES-puro	Clontech Laboratories
pFPV25.1GFPmut3Kan	Dr. Karsten Tedin, FU, Berlin

2.3. Primers

All primers with the amplified gene/target sequence name, primer sequence, melting temperature (T_m) and reference are shown in Table 3. Melting temperature was calculated in Primer BLAST with the following assumptions: concentration of monovalent cations- 50.0 mM, concentration of divalent cations- 1.5 mM, concentration of deoxynucleotides (dNTPs) - 0.2 mM, annealing oligonucleotides concentration- 0.2 µM.

Table 3. List of primers used in this study

No.	Primer Name	Gene(-s)/ Target	Primer sequence (5'-3')	Primer Length	T_m	Reference
1.	O1545 GP2SuFor	GP2	ATGGGAAGCATGGCTCCC	18	62.0	This study
2.	O1546 GP2SuRev	GP2	TCAGAACAGCGCAGCCAG	18	63.0	This study
3.	pJET1.2 forward sequencing primer	pJET1.2	CGACTCACTATAGGGAGAGCGGC	23	60.5	Kit manual
4.	pJET1.2 reverse sequencing primer	pJET1.2	AAGAACATCGATTTTCCATGGCAG	24	62.0	Kit manual

5.	O1628 GP2SuForGate	GP2	GGGGACAAGTTTGTACAAAAAAGCAGGCTATACCATGGGAAGCATGGCTCCC	52	62.0	This study
6.	O1739 GP2SuRevGate3	GP2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATGGTGATGGTGATGGTGTCCACTTCCACTTCCCATGACGCCAAGAGACTGG	85	61.0	This study
7.	pUC/M13 Forward	Bacmid	CCCAGTCACGACGTTGTAAAACG	23	64.5	Kit manual
8.	pUC/M13 Reverse	Bacmid	AGCGGATAACAATTTCACACAGG	23	61.0	Kit manual
9.	O1532 fimH_Sal_For	*fimH*	ATCCAGTGGGGAGAGGG	17	60.0	This study
10.	O1533 fimH_Sal_Rev	*fimH*	GAGTTGGCCTGACTCAGC	18	60.5	This study
11.	O1901 fimHdelfwd	*fimH*	ATGAAAATATACTCAGCGCTATTGCTGGCGGGGACCGCGCTCTTTTTCACTGTGTAGGCTGGAGCTGCTTC	71	62.5	Zeiner et al., 2012
12.	O1902 fimHdelrev	*fimH*	TTAATCATAATCGACTCGTAGATAGCCGCGCGCAGTAAACGGCCCTTCCGCATATGAATATCCTCCTTAG	70	52.3	Zeiner et al., 2012
13.	O1534 fimH_Sal_qPCR_for	*fimH*	GCGTGCAGGACTCAAAGC	18	62.2	This study
14.	O1535 fimH_Sal_qPCR_rev	*fimH*	CTTTGCCGCTGTAGCTAATGG	21	62.2	This study
15.	K2	*kanR*	CGGTGCCCTGAATGAACTGC	20	62.5	Datsenko & Wanner, 2000
16.	O1919 FimHpACYCfor	*fimH*	ACATGGATCCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAGGAGGACAGCTATGAAAATATACTCAGCGCTATTG	80	58.5	This study
17.	O1920 FimHpACYCrev	*fimH*	ACATGGATCCTTAATCATAATCGACTCGTAGATAG	35	59.0	This study
18.	O1461 GP2HoEcoRIFor	GP2	GTCACGAATTCATGCCTCACCTTATGGAAAGG	32	60.0	This study

19.	O1462 GP2HoBamHIRev	GP2	CGTGGATCCTCAGAACAGCCAAGCCAGG	28	62.5	This study
20.	O1590 GP2SuForEco	GP2	GTCACGAATTCATGGGAAGCATGGCTCCC	29	62.0	This study
21.	O1591 GP2SuRevBam	GP2	CGTGGATCCTCAGAACAGCGCAGCCAG	27	63.0	This study
22.	O1573 GP2HoqPCR2for	GP2	ATCAACGTGATTCCACCATCC	21	61.0	This study
23.	O1574 GP2HoqPCR2rev	GP2	TTGAGCAAGAAGGCTGGC	18	61.0	This study
24.	O2229 GP2SuForqPCR2	GP2	AACAGCTCGGATGTCAACAG	20	61.0	This study
25.	O2230 GP2SuRevqPCR2	GP2	AGATCCAGTTCCCATCCTGTC	21	61.5	This study
26.	O876 RPLP0_fw	RPLP0	AAATGTTTCATTGTGGGAGC	20	57.0	George et al.,2016
27.	O877 RPLP0_rv	RPLP0	ATATGAGGCAGCAGTTTCTC	20	58.0	George et al.,2016

2.4. Antibodies

Table 4. List of antibodies used in this study

Antibody name	Producer/ Source
Rabbit IgG anti-6-His (Cat. No. A190-114A)	Bethyl Laboratories Inc.
Goat IgG anti-Rabbit IgG HRPO (Cat. No. A-6154)	Sigma
Rabbit IgG anti-GP2Ho#1	Generic Assays
Rabbit IgG anti-GP2Su#1	Pineda Antibody-Service
Rabbit IgG anti-GP2Su#2	Pineda Antibody-Service
Rabbit IgG anti-FimH	A. Orłowska, WUELS, Wrocław
Mouse IgM α tubulin (TU-02): sc-8035	Santa Cruz Biotechnology

Goat IgG anti-mouse IgM HRPO (Cat. No. 115-035-020)	Dianova

2.5. Standard kits

Table 5. List of kits used in this study

Kit name	Producer
RNeasy Mini Kit	Qiagen
Maxima First Strand Synthesis Kit for RT-qPCR	Thermo Scientific
MiniElute Gel Extraction Kit	Qiagen
CloneJET PCR Cloning Kit	Thermo Scientific
JETQuick PCR Purification Spin Kit	Genomed
DNeasy Blood and Tissue Kit	Qiagen
JETSTAR Plasmid Purification Mini Kit	Genomed

2.6. Solutions

2.6.1. Agarose gel electrophoresis

TBE buffer (1x)	Final concentration
TRIS	89 mM
Boric acid	89 mM
Ethylenediaminetetraacetic acid (EDTA)	2mM

TAE buffer (1x)	Final concentration
TRIS	40 mM
Acetic acid	20 mM
EDTA	1mM

2.6.2. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blotting

5xSDS Loading Dye	Final concentration
TRIS-HCl pH=6.8	250mM
SDS	10%
Glycerol	30%
ß-mercaptoethanol	5%
Bromophenol blue	0.2%

Resolving gel (12%)	Volume [ml]
H_2O	1.6
30% Acryl-bisacrylamide mix	2.0
1.5M TRIS pH=8.8	1.3
10% SDS	0.05
10% Ammonium persulfate	0.05
N,N,N′,N′-tetramethylelthylenediamine (TEMED)	0.002

Stacking gel (5%)	Volume [ml]
H_2O	3.4
30% Acryl-bisacrylamide mix	0.83
0.5M TRIS pH=6.8	0.63
10% SDS	0.05
10% Ammonium persulfate	0.05
TEMED	0.005

ECL solution	Volume [ml]
100mM TRIS-HCl pH=8.5	20.0
30% H_2O_2	0.0075
40mM p-coumaric acid (in DMSO)	0.1
250mM luminol (in DMSO)	0.1

RIPA (Radio Immuno-Precipitation Assay) buffer	Final concentration
NaCl	150 mM
NP-40	1.0 %
Sodium deoxycholate	0.5 %
SDS	0.1 %
TRIS-HCl, pH= 8.0	50 mM

2.6.3. Affinity chromatography

GP2 His-tag Binding buffer	Final concentration
Na_2HPO_4	16.2 mM
NaH_2PO4	3.8 mM
NaCl	200 mM
Imidazole	5 mM
Adjust to pH= 7.5	

GP2 His-tag Elution buffer	Final concentration
TRIS-HCl	20 mM
NaCl	200 mM
Imidazole	500 mM
Adjust to pH= 7.5	

2.6.4. Fluorescence in situ hybridization (FISH)

Hybridization buffer	Final concentration
NaCl	0.9 M
TRIS-HCl	20 mM
SDS	0.01 %
Formamide	15.0 %

Hybridization buffer	Final concentration
NaCl	0.9 M
TRIS-HCl	20 mM
SDS	0.01 %

2.7. Nutrition media

2.7.1. Lysogeny broth (LB) Miller

LB

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
dH_2O	Ad 1000ml

LB agar

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Agar	15 g/l
dH_2O	Ad 1000ml

The medium was autoclaved at 121˚C for 15’ at 15 psi.

2.7.2. Super Optimal broth with Catabolite repression medium (SOC)

SOC

Tryptone	20 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l
KCl	0.186 g/l
dH_2O	Ad 950 ml

The medium was autoclaved at 121˚C for 15’ at 15 psi.

$MgCl_2$ (syringe filter sterile)	To final concentration 10 mM
Glucose (syringe filter sterile)	To final concentration 20 mM
Final volume	1000 ml

3. Methods

3.1. Cloning and expression of porcine GP2 isoforms

3.1.1. RNA isolation

Porcine pancreas was obtained from a freshly killed pig at a slaughterhouse. The tissue was preserved in RNAlater solution (Qiagen). Samples were then stored overnight (O/N) at 4˚C and transferred to -20˚C for longer storage. RNA was isolated with use of RNeasy Mini Kit (Qiagen). 25 mg of RNAlater stabilised porcine pancreas was placed in a 2 ml Eppendorf tube with 600 µl of RLT lysis buffer (plus 6 µl of ß-mercaptoethanol added) and one stainless steel bead (5 mm diameter). The tissue was disrupted and homogenised in TissueLyser (Qiagen) for 3 minutes at 30 Hz. The lysate was centrifuged for 3 minutes at 13000xg. Nucleic acids were precipitated with 600 µl of 70% ethanol. The solution was transferred to an RNeasy spin column. On-column bound RNA was washed with 350 µl RW1 buffer. In the next step, DNA was digested with DNase I solution (10 µl of DNase I in 70 µl RDD buffer, Qiagen) for 15 min. After this step, the column was washed with 350 µl RW1 and two times 500 µl RPE buffers. DNA-free RNA was eluted with 40 µl of RNase-free water. RNA quantity and purity was assessed with Colibri Microvolume Spectrometer (Titertek-Berthold).

3.1.2. Reverse transcription (RT)

The RT reaction was performed with Maxima First Strand Synthesis Kit for RT-qPCR (Thermo Scientific). The reaction mix and reaction conditions are shown below. The control "RT-" reaction did not contain Maxima Enzyme Mix.

Reaction Mix	
5x Reaction Mix	4 µl
Maxima Enzyme Mix	2 µl
RNA	1 µg
H_2O	to 20 µl

Reaction conditions	
25˚C	10’
50˚C	30’
85˚C	5’
4˚C	∞

3.1.3. Polymerase Chain Reaction (PCR) amplification of the porcine GP2 isoforms

Primers for amplification of coding sequence (cds) were designed with predicted porcine GP2 isoforms using GenBank sequences as references (Acc. No.: XM005662102 and XM003124571). Touchdown (TD) PCR was performed to amplify porcine GP2 isoforms with O1545 GP2SuFor and O1546 GP2SuRev. The reaction mix and conditions are shown below. The PCR product was run on a 1% agarose gel. Next, the gel was stained with ethidium bromide and the image was taken with BioDocAnalyze (Biometra).

PCR mix	Volume [µl]
H_2O	17.25
HF PCR buffer (5x)	5
dNTP (10mM)	0.5
Primer O1545 GP2SuFor (10 µM)	0.5
Primer O1546 GP2SuRev (10 µM)	0.5
Phusion polymerase (2U/µl)	0.25
cDNA	1

PCR conditions

Temperature	Time	Repeats
98˚C	30’’	
98˚C	5’’	
70˚C -1˚C/cycle	15’’	15x
72˚C	50’’	
98˚C	5’’	
55˚C	15’’	20x
72˚C	50’’	
72˚C	5’	
10˚C	∞	

3.1.4. Preparation of chemocompetent bacteria and transformation of bacteria with heat-shock method

Chemocompetent bacteria were prepared according to the protocol of Dagert and Ehrlich (1979) with minor modifications. 200 mL of LB medium was inoculated with 2 ml of O/N bacterial culture, incubated at 37 °C at 180 rpm until OD_{600} = 0.4 has been reached. Cells were incubated on ice for 10', centrifuged at 5000xg for 10' at 4°C. The pellet was resuspended in 80 ml of ice-cold 50mM $CaCl_2$ and incubated on ice for 20'. Next, cells were centrifuged at 5000xg for 10' at 4°C, resuspended in 4 ml of 50mM $CaCl_2$ and 1 ml of 50% glycerol, aliquoted to 100 µl per tube and stored at -80˚C. 1-10 µl of DNA was used for transformation. DNA was added to chemocompetent cells, incubated on ice for 30', heat-shocked at 42˚C for 45'' and incubated for 2' on ice before addition of SOC. 900 µl of SOC medium was then added and bacteria were incubated at 37 °C with shaking at 180 rpm for 1-2h. In the next step, bacteria were centrifuged, resuspended in 200 µl and spread on two LB agar plates (with an appropriate antibiotics).

3.1.5. Cloning of porcine GP2 isoforms into pJET1.2 plasmid

The TD PCR product was run on a 1% agarose gel. In the next step, the gel was stained with ethidium bromide and appropriate bands were excised and purified with the MiniElute Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's protocol. The purified DNA was cloned with the use of CloneJET PCR Cloning Kit (Thermo Scientific) in line with the manufacturer's protocol with minor modifications. Briefly, 25 ng (short GP2 isoform) and 37.5 ng (long GP2 isoform) were ligated into the vector pJET1.2. The ligation mix and reaction conditions are shown below.

Ligation mix

Reaction buffer (2x)	5 µl
Purified PCR product	25-37.5 ng
pJET1.2 vector	0.5 µl
H_2O	To 9.5 µl
T4 DNA ligase	0.5 µl
Reaction conditions	
22˚C	5'
4˚C	∞

Ten (10) µl of ligation mixture was used for transformation of chemocompetent *E. coli* XL1Blue like in Section 3.1.4. Bacteria were spread on LB Ampicillin (Amp) plates (100µg/ml of ampicillin) and incubated O/N at 37˚C. Colonies were analysed for the presence of inserts within the plasmid using colony PCR with a pJET1.2 forward and reverse sequencing primer pair according to the manufacturer's protocol. The reaction mix and reaction conditions are shown below. The PCR product was run on a 1% agarose gel. Next, the gel was stained with ethidium bromide and the image was taken with BioDocAnalyze (Biometra). Three positive plasmids for each isoform were sent for sequencing.

PCR mix	Volume [µl]
H_2O	16.1
DreamTaq PCR buffer (10x)	2
$MgCl_2$ (50mM)	0.6
dNTP (10mM)	0.4
Primer pJET1.2 forward (10 µM)	0.4
Primer pJET1.2 reverse(10 µM)	0.4
DreamTaq polymerase (5U/µl)	0.1

PCR conditions

Temperature	Time	Repeats
95˚C	3'	
94˚C	30''	
60˚C	30''	25x
72˚C	2'	
10˚C	∞	

3.1.6. Prediction of GPI-anchor binding site in GP2

In order to clone only a secretory form of porcine GP2, GPI modification site prediction was done with the FragAnchor (Poisson et al. 2007) and PredGPI (Pierleoni et al. 2008) software and the predicted GP2 sequence was not cloned by Gateway cloning (next Section).

3.1.7. Gateway vector cloning of porcine GP2 isoforms

In order to clone GP2 isoforms into a pDONR221 vector, PCR was performed with primers O1628 GP2SuForGate and O1739 GP2SuRevGate3. The reaction mix and conditions are shown below. PCR products were analysed on a 1% agarose gel. PCR products were purified with JETQUICK PCR Purification Spin Kit (Genomed), according to the manufacturer's protocol.

PCR mix	Volume [µl]
H_2O	16
HF PCR buffer (5x)	5
$MgCl_2$ (50mM)	1.25
dNTP (10mM)	0.5
Primer O1628 GP2SuForGate (10 µM)	0.5
Primer O1739 GP2SuRevGate3 (10 µM)	0.5
Phusion polymerase (2U/µl)	0.25
plasmid DNA (5ng/ µl)	1

PCR conditions

Temperature	Time	Repeats
98˚C	30''	
98˚C	5''	
59˚C	15''	35x
72˚C	50''	
72˚C	5'	
10˚C	∞	

In the next step, BP reaction with pDONR221 vector and purified PCR products was performed. The reaction mix and conditions are shown below. Ten (10) µl of BP reaction mixture was used for transformation of chemocompetent *E. coli* XL1Blue. Plasmids were isolated from three

colonies for each GP2 isoform. To confirm successful cloning, PCR was performed on the isolated plasmids.

BP reaction mix	
PCR product	100 fmol
pDONR221 vector	150 ng
BP Clonase II mix	2 µl
TE buffer, pH=8.0	To 10 µl
BP reaction condtions	
25˚C	4h
4 ˚C	∞

LR reaction was done with the pDONR221-SuGP2 and pDEST8 plasmids. The reaction mix and conditions are shown below. Ten (10) µl of the LR reaction mixture was used for transformation of chemocompetent *E. coli* XL1Blue. Plasmids were isolated from three colonies for each GP2 isoform. To confirm successful cloning PCR was performed on isolated plasmids. Two plasmids containing GP2 (for each isoform) were sent for sequencing.

LR reaction mix	
pDONR221 vector	150 ng
pDEST8 vector	150 ng
LR Clonase II mix	2 µl
TE buffer, pH=8.0	To 10 µl
LR reaction condtions	
25˚C	O/N
4 ˚C	∞

3.1.8. Preparation of electrocompetent bacteria

Electrocompetent bacteria were prepared according to the protocol of Sambrook and Russell (2006) with minor modifications. 100 mL of LB medium (with appropriate antibiotics, when needed) was inoculated with 1 ml of O/N culture, incubated at 37 °C (or 30 °C) at 180 rpm until OD_{600} = 0.6 has been reached. In some cases, for induction of Lambda red

recombinase expression, L-arabinose was added when bacteria reached OD_{600} = 0.1. After reaching OD_{600} = 0.6, bacteria were incubated on ice for 5', centrifuged 6000xg, 10' at 4°C. Pellet was washed one time with 50 ml of ddH_2O and two times with 25 ml of 10% glycerol in ddH_2O. Finally, cells were resuspended in 0.1 ml of 10% glycerol in ddH_2O and aliquoted to 50 µl per tube. 1-5 µl of DNA was used for electroporation. DNA was added to electro-competent cells, incubated on ice for 1', transferred to a cuvette (4 mm wide) and electroporated at a voltage of 2.5 kV, a capacity of 25µF and resistance of 200Ω. 900 µl of SOC medium was added immediately after transformation, bacteria were transferred to 2-ml Eppendorf tubes and incubated 1-2h at 37 °C (or 30 °C) at 180 rpm. In the next step, bacteria were centrifuged, resuspended in 200 µl of medium and spread on two LB agar plates (with appropriate antibiotics).

3.1.9. Generation of the recombinant bacmids with porcine GP2 isoforms

Electrocompetent *E.coli* DH10Bac were electroporated with 20ng of pDEST8 vectors containing porcine GP2 isoforms. After this, 900 µl of pre-warmed SOC medium was added and bacteria were incubated at 37˚C for 4h with shaking at 180 rpm. In the next step, 100 µl of bacterial suspension was spread on LB agar plate (50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracycline, 100 µg/ml X-Gal, 40 µg/ml IPTG) and incubated for 48h at 37˚C. Colonies were analysed for the presence of inserts within the bacmid using blue/white selection.

3.1.10. Isolation of recombinant bacmid DNA

Three white colonies (for each porcine GP2 isoform) were inoculated into 5 ml of LB broth with antibiotics (50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracycline) O/N cultures for bacmid isolation. 2 ml of O/N cultures were centrifuged at 13000xg for 1 min. The bacterial pellets were resuspended in 0.3 ml of solution I (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). In the next step 0.3 ml of solution II (0.2 N NaOH, 1% SDS; filter-sterilized) was added, tubes were gently mixed and incubated for 5' at room temperature. After this 0.3 ml of 3M potassium acetate (pH=5.5) was added, tubes were gently mixed and the samples were incubated for 10' on ice. Next, the solutions were centrifuged for 10' at 14000xg. The supernatants were transferred to microcentifuge tubes containing 0.8 ml of isopropanol and mixtures were incubated for 10' on ice. Next, the solutions were centrifuged for 10' at 14000xg. After centrifugation, supernatants were discarded and DNA pellets were washed with 70% ethanol. In the next step, pellets were air dried and resuspended in 40 µl of ultrapure water. DNA concentration was measured with Colibri Microvolume Spectrometer

(Titertek-Berthold). Purified bacmid DNA was stored at 4˚C. PCR was performed to confirm the presence of GP2 isoforms in bacmid DNA. The reaction mix and conditions are shown below.

PCR mix	Volume [µl]
H_2O	18.75
DreamTaq PCR buffer (10x)	2.5
$MgCl_2$ (50mM)	1
dNTP (10mM)	0.5
Primer pUC/M13 forward (10 µM)	0.5
Primer pUC/M13 reverse (10 µM)	0.5
DreamTaq polymerase (5U/µl)	0.25
bacmid DNA	1

PCR conditions

Temperature	Time	Repeats
95˚C	3’	
94˚C	30’’	
55˚C	30’’	35x
72˚C	5’	
72˚C	7’	
10˚C	∞	

3.1.11. Cultivation and maintaining of SF9 insect cells

SF9 cells are clonal derivate from the SF21 cell line, which originated from fall armyworm (*Spodoptera frugiperda)* ovary. SF9 cells were cultured in SF-900 II SF medium at 27˚C as an adherent cell line for transfection with bacmid DNA, baculovirus titration and protein expression optimisation. Cells were passaged when they reached 70-90% confluency. For detachment of cells from the flask, a cell scraper was used. SF9 cells were cultured in SF-900 II SF medium at 27˚C with shaking at 150 rpm in suspension cultures for baculovirus

amplification and large-scale protein production. Cells were passaged when they reached $2x10^6$ cells/ml of culture.

3.1.12. Production of recombinant baculovirus

SF9 cells were seeded at a concentration of $8x10^5$ cells per well in 6-well plate. Cells were left for 30' at room temperature to attach. Next, the medium was exchanged for 2.5 ml of Grace's medium. In the meantime, transfection mix was prepared. 100 µl of Grace's medium was pipetted into two microcentrifuge tubes. 8 µl of Cellfectin II reagent (Thermo Scientific) was added to tube I. 2 µg of bacmid DNA was added to tube II. Both tubes were vortexed for 10'' and then both solutions were combined together. The transfection mix was vortexed and incubated 30' at room temperature. In the next step, the transfection mixture was added dropwise onto cells prepared earlier and cells were incubated at 27˚C. After 4h, the transfection medium was replaced with 2 ml of SF-900 II SF medium. The cells were inspected every day for signs of virus production and usually, after four days, the supernatant (P1 virus stock) was harvested by centrifugation at 500xg for 5'. The P1 virus stock was stored in the dark at 4˚C.

3.1.13. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting analysis of the SF9 supernatants

SDS-PAGE was performed similarly as described by Laemmli (1970). 12% SDS-PAGE gels were prepared with the stock solutions mentioned in Section 2.6.2. The samples were prepared by mixing with 5xSDS loading dye and denaturating for 5' at 95˚C. Electrophoresis was run with constant voltage: for first 30'- 80V, later the voltage was increased to 140V. After this, one part of the gel was stained with Coomassie B-250 for 1h and destained with a solution of 10% acetic acid. The second part of the gel was used for semi-dry protein transfer to a PVDF membrane (activated for 10 seconds with methanol) for 60' with a constant current value of 250 mA using the Fastblot Apparatus (Biometra). In the next step, the PVDF membrane was blocked with 5% solution of dried, defatted milk in PBS+0.1% Tween 20 (PBST) buffer for 1h. Next, the membrane was incubated O/N at 4˚C with primary antibody anti-6-His (Bethyl) at a dilution of 1:10,000 in PBST. On the next day, the blot was washed three times 10' with PBST solution and was incubated for 1h at room temperature with anti-rabbit peroxidase- conjugated secondary antibody at a dilution 1:10,000 in PBST. Next, the blot was washed three times 10' with PBST solution and was incubated for 2' in ECL solution for development. The blot was scanned using a Lumi-Imager F1 Workstation (Roche).

3.1.14. Amplification of baculoviral stock

The P1 viral stock was amplified two times in order to obtain a virus stock in a larger volume and concentration. The first amplification was done as follows: 10 ml of SF9 cells at a concentration of $2x10^6$ cells/ml in a T-75 flask was inoculated with 200 µl of P1 viral stock. The cells were grown for 5 days and the P2 viral stock was harvested by centrifugation for 5'at 500xg. The P3 viral stock was generated by inoculation of 50 ml SF9 cells suspension ($2x10^6$ cells/ml) with 1 ml of P2 viral stock. Like with the P2 viral stock, the cells were grown for 5 days and the P3 viral stock was harvested by centrifugation for 5'at 500xg. The P2 and P3 viral stocks were checked by western blotting for the presence of recombinant GP2 (like in Section 3.1.13).

3.1.15. Baculovirus titration

Baculoviral P3 stocks were titrated with BacPAK Baculovirus Rapid Titer Kit (Clontech). The SF9 cells were seeded in a 96-well plate at a concentration $6.5x10^4$ cells/well and incubated for 1h at 27˚C. The medium was exchanged with 25 µl of diluted P3 viral stocks (10^{-4}-10^{-6} dilutions in triplicates) and the cells were incubated for 1 h at room temperature. Next, the viral dilutions were exchanged with 50 µl of Methyl Cellulose Overlay and incubated at 27˚C for 44-47 h. For detection of viral infection, the cells were first fixed with 4% PFA in PBS for 30' at room temperature. Next, cells were washed three times with 200 µl of PBS+0.05% Tween 20. Later, the cells were incubated at 37˚C for 25' with primary antibody (mouse monoclonal gp64 antibody) and subsequently with secondary antibody (goat anti-mouse HRP-conjugated antibody). After each antibody incubation, cells were washed three times with 200 µl of PBS+0.05% Tween 20. In the next step, cells were incubated with Blue Peroxidase substrate for 3h at room temperature. The virus titer was determined by counting stained foci in the highest dilution wells containing a number of foci between 5 and 25. Virus titer was calculated with following formula: "average no. of foci per well" x "dilution factor of baculoviral stock" x 40.

3.1.16. Optimising recombinant GP2 expression in small volume

$6x10^5$ SF9 cells were seeded per well in a 24-well plate and incubated for 1h at 27˚C. The medium was exchanged with media containing different dilutions of baculovirus. Four multiplicity of infection (MOI) values (1, 3, 5, 10) were tested for 5 days and the supernatant medium was harvested each day for each MOI. The supernatants were tested for recombinant GP2 expression by western blotting (like in Section 3.1.13).

3.1.17. Recombinant GP2 expression

Protein expression was done in 500 ml Erlenmeyer flask with 200 ml of SF9 cells ($2x10^6$/ml) in SF-900 II SF medium at 27˚C with shaking at 150 rpm. After infection with baculoviral stock, cells were incubated for an appropriate time and then harvested by centrifugation 3000xg for 15' at 4˚C. The pH of the supernatant was adjusted to 7.5 and was stored at -25˚C till purification.

3.1.18. Recombinant GP2 purification

First, the supernatant containing recombinant GP2 was thawed on ice. Next, the supernatant was centrifuged at 6,000xg for 1h at 4˚C. $NiSO_4$ was added to final concentration of 0.8 mM and the supernatant was filtered through a 0.45 µm syringe filter. GP2 was first purified by affinity chromatography on prepacked Ni Sepharose (HisTrap, 1ml, GE Healthcare) by the use of AKTA Prime System (GE Healthcare). The column was equilibrated with 10 ml and washed with 30 ml of binding buffer (Section 2.6.3). Proteins were released with elution buffer (Section 2.6.3). The eluate was tested for recombinant GP2 by use of Coomassie staining and western blotting (Section 3.1.13). The elution buffer was exchanged for binding buffer with an Amicon Ultra-15 Centrifugal Unit (Merck Millipore). Protein was further purified on self-packed gravity flow Ni agarose column (1ml of HisPur Ni-NTA resin, Thermo Scientific). The column was equilibrated with 10 ml of binding buffer and then eluate from the first purification was added to the resin. In the next step, the column was washed with 30 ml of binding buffer and protein was eluted with a step gradient of imidazole: 25mM, 50mM, 100mM, 250mM and 500mM (5 ml for each step). Eluates were tested for the presence of recombinant GP2 with Coomassie staining and western blotting (like in Section 3.1.13). Fractions containing pure recombinant GP2 were pooled, concentrated and the buffer was exchanged for 20 mM TRIS-HCl pH=7.5 with Amicon Ultra-15 Centrifugal Unit.

3.1.19. Silver staining of purified porcine GP2 isoforms

In order to confirm protein purity, 500 ng of both porcine GP2 isoforms was resolved on SDS-PAGE (like in Section 3.1.13) and silver stained according to the protocol of Blum et al. (1987) with minor modifications. First, the gel was incubated in a solution containing 25 ml of methanol, 6 ml of acetic acid, 250 µl of 4% paraformaldehyde (PFA) and 18.75 ml of ddH_2O. After 1h, the gel was washed for 10' with 50% ethanol and for a further 10' with 30% ethanol. Next, 20 ml of 0.1% $Na_2S_2O_3$ in ddH_2O was prepared and 50 µl of this solution was saved for later use. The rest of the solution was diluted 5 times with water and incubated with the gel for

1'. Next, the gel was washed 3 times for 20'' with ddH_2O and incubated with 50 ml of 0.1% $AgNO_3$ in ddH_2O with 312.5 µl of 4% PFA. In the next step, the gel was washed two times with water for 20'' and incubated with 50 ml of 6% Na_2CO_3 with 312.5 µl of 4% PFA and 20 µl of 0.1% $Na_2S_2O_3$ in ddH_2O. The staining process was stopped by adding 50 ml of 5% acetic acid solution.

3.1.20. Protein deglycosylation

Protein deglycosylation was performed with protein deglycosylation mix (NEB). Denaturing and non-denaturing reaction mixes and conditions are shown in the table below. The negative control contained everything but enzyme mix and was incubated under identical conditions as the rest of the samples. After digestion, proteins (2µg for each aliquot) were resolved on SDS-PAGE and stained with Coomassie brilliant blue (like in Section 3.1.13).

Denaturing reaction	
10x Glycoprotein Denaturing Buffer	1 µl
GP2Su	10 µg
H_2O	Ad 10 µl
100˚C, 10'	
ice, 3'	
10x GlucoBuffer 2	2.5 µl
10% NP-40	2.5 µl
H_2O	9.5 µl
Deglycosylation Enzyme Cocktail	0.5 µl
37˚C, 4h	
Non-denaturing reaction	
GP2Su	10 µg
10x GlucoBuffer 2	5 µl
Deglycosylation Enzyme Cocktail	5 µl
H_2O	Ad 50 µl
37˚C, 16h	

For blotting 200 ng of each protein was resolved on SDS-PAGE, transferred onto a PVDF membrane and probed with anti-6-His antibody (like in Section 3.1.13) and Concanavalin A (ConA). To verify for ConA, the membrane was blocked O/N in TBS+0.1%Tween 20 (TBST) with 2% bovine serum albumin (BSA) at 4˚C. In the morning, the membrane was washed with ConA buffer (10mM HEPES pH=7.5, 150mM NaCl, 0.1mM $MnCl_2$, 1mM $CaCl_2$) for 15' at room temperature and incubated with ConA (5µ/ml ConA in ConA buffer with 0.5% BSA) for 30' at room temperature. Next, the membrane was washed 3 times for 10' with TBST and incubated with Streptavidin conjugated with HRP (Streptavidin-HRP at a 1:10,000 dilution in ConA buffer with 0.5% BSA) for 30' at room temperature. Next, the membrane was washed 3 times for 10' with TBST and was incubated for 2' in ECL solution. The blot was imaged using a Lumi-Imager F1 Workstation (Roche).

3.2. Static adhesion assays

3.2.1. VideoScan Platform

The VideoScan hardware consists of an inverse epifluorescence Olympus microscope with multiple fluorescence channels, a digital camera and a motorised scanning stage. A VideoScan software for the platform was developed by Alexander Böhm and Jörg Nitschke. The VideoScan Platform has been used in past projects for nucleic acid detection or quantification, *E. coli* quantification in cell line adhesion assays, hemolytic activity of *E. coli* on cell lines and automated detection of autoantibodies in cell-based assays (Rödiger et al. 2013).

3.2.2. VideoScan Module

The VideoScan module works with a 20x magnification objective and first focuses in the well on DAPI encoded poly(methyl methacrylate) (PMMA) 2.5 µm beads-PolyAn Blue 20 (PolyAn, Germany). Afterwards, the software takes images of bacteria and counts bacteria detected on the image. Five images are taken per well.

3.2.3. Protein coating

Proteins were diluted in coating buffer (0.1M Na_2CO_3, pH=9.6) and 50 µl of solution per well was pipetted onto 96-well plates (Nunc MaxiSorp, flat-bottom) and incubated O/N at 4˚C. The coating solution was removed from plates and plates were washed once with 100 µl of PBS/ 1% BSA and dried at room temperature for 30'. Plates were stored not longer than 1

month at 4˚C. Anti-FimH antibody was coated at a dilution of 1:250, GP2 isoforms were coated at a concentration of 2.5µg/ml and HRP and RNase B at a concentration of 5 µg/ml (Grzymajlo et al. 2010).

3.2.4. Determination of assay's linear range

For the assay, bacteria were grown in T1F inducing conditions (Old and Duguid 1970). 1.5 ml Eppendorf tubes were filled with 500 µl of LB broth and inoculated with *Salmonella*. Bacteria were grown under static conditions for 48h at 37˚C. Before an assay, bacteria were centrifuged at 6,000xg for 3', washed once with PBS, centrifuged again at 6,000xg for 3' and resuspended in PBS/BSA 1%. After this, the OD_{600} of bacterial solutions was measured and 100 µl of bacteria were applied in a dilution series (starting with $5x10^7$ of bacteria) on plates coated with anti-FimH antibody and human GP2 isoform 1 (GP2Ho#1). Bacteria were incubated for 2h at room temperature and then plates were washed three times with 100 µl of PBS. Attached bacteria were fixed with 50 µl of 4% PFA in PBS for 30' at 4˚C and the plates were washed three times with 100 µl of PBS. Attached bacteria were stained with 50 µl of propidium iodide (10 µg/ml in ddH_2O) for 15' at room temperature, then plates were washed three times with 100 µl of PBS and 75 µl of DAPI beads solution in PBS was applied as a positive control for VideoScan module measurement. Three independent experiments with three repetitions for each dilution were prepared and measured.

3.3. Characterization of *Salmonella* isolates

3.3.1. Sequencing of the *fimH* gene

Genomic DNA was isolated from 128 *Salmonella* isolates using the DNeasy Blood and Tissue Kit (Qiagen). The *fimH* gene with 50 bp overhangs was amplified via PCR with the use of O1532 fimH_Sal_For and O1533 fimH_Sal_Rev primers. The reaction mix and conditions are shown below. PCR products were analysed on a 1% agarose gel. Subsequently, the gel was stained with ethidium bromide and imaged with a BioDocAnalyze (Biometra). 50 µl of the PCR reaction was sent for each strain to LGC Genomics GmbH (Berlin) and was purified and Sanger-sequenced with the use of O1532 fimH_Sal_For and O1533 fimH_Sal_Rev primers.

PCR mix	Volume [µl]
H_2O	31.5
HF PCR buffer (5x)	10
dNTP (10mM)	1

Primer O1532 fimH_Sal_For (10 µM)	2.5
Primer O1533 fimH_Sal_Rev (10 µM)	2.5
Phusion polymerase (2U/µl)	0.5
genomic DNA	2

PCR conditions

Temperature	Time	Repeats
98˚C	30''	
98˚C	5''	
53˚C	15''	40x
72˚C	45''	
72˚C	10'	
10˚C	∞	

3.3.2. Sequence alignment of the *fimH* gene

The sequence reads were assembled into contigs by use of the CAP3 software implemented into a UGENE platform (Huang and Madan 1999; Okonechnikov et al. 2012). Sequences were aligned with ClustalX 2.1 (Larkin et al. 2007).

3.3.3. Anti-FimH static adhesion assay

All *Salmonella* isolates were tested for T1F expression. 1.5 ml Eppendorf tubes were filled with 500 µl of LB broth and inoculated with *Salmonella*. Bacteria were grown under static conditions for 48h at 37˚C (Old and Duguid 1970). A static adhesion assay was performed similarly like in Section 3.2.4 with the following changes: bacteria were not centrifuged and washed before assays, and $5x10^6$ bacteria were used per well. The test was performed three times in duplicates for each strain.

3.3.4. Static adhesion assay with deglycosylated porcine GP2 isoforms

Deglycosylated GP2 isoforms from Section 3.1.20 were coated onto 96-well plates like in Section 3.2.3. Next, static adhesion assays were done with *Salmonella* Typhimurium 5744

WT and it's *fimH* gene deletion mutant like in Section 3.3.3. The test was performed three times in triplicates for each strain.

3.4. Generation of an isogenic *Salmonella* model

3.4.1. Generation of *Salmonella fimH* gene deletion mutant

S. Typhimurium isolate 5744 was selected for generation of isogenic model. This isolate had the highest T1F expression among all *S*. Typhimurium isolates tested in Anti-FimH static adhesion assay. A deletion mutant of the *fimH* gene was generated using the Datsenko-Wanner method (Datsenko and Wanner 2000) with minor modifications. First, antibiotic resistance for kanamycin (Kan), ampicillin (Amp), chloramphenicol (Cam) and gentamycin (Gm) of the *Salmonella* isolate was tested by the agar disk diffusion method (ADD). In the next step, electrocompetent bacteria were prepared with the protocol described in Section 3.1.8 and transformed with a pKD46-Gm plasmid (Doublet et al. 2008). After transformation, bacteria were grown at 30˚C for 2h with shaking at 180 rpm. Next, bacteria were spread on LB agar plated with Gm (Gm- 10 µg/ml) and incubated at 30˚C for 24h. Positive clones were streaked on new agar plates and then used for the preparation of electrocompetent cells according to the protocol described in Section 3.1.8 with one modification. After bacteria reached OD_{600}=0.1, L-arabinose was added to a final concentration of 5 mM. Electrocompetent cells were transformed with PCR products containing resistance cassettes (Kan or Cam) and homologous extensions of the *fimH* gene (Zeiner et al. 2012). The PCR mix and conditions are shown below. PCR products were analysed on a 1% agarose gel. Next, the gel was stained with ethidium bromide and imaged using BioDocAnalyze (Biometra). PCR products for each resistance cassette were purified with the JETQUICK PCR Purification Spin Kit (Genomed) according to the manufacturer's instructions and DNA concentration was measured with a Colibri Microvolume Spectrometer (Titertek-Berthold).

PCR mix	Volume [µl]
H_2O	17.25
HF PCR buffer (5x)	5
dNTP (10mM)	0.5
Primer O1901 fimHdelfwd (10 µM)	0.5
Primer O1902 fimHdelrev (10 µM)	0.5

Phusion polymerase (2U/µl)	0.25
plasmid DNA (5ng/µl)	1

PCR conditions

Temperature	Time	Repeats
98˚C	30''	
98˚C	5''	
62˚C	15''	40x
72˚C	50''	
72˚C	5'	
10˚C	∞	

After transformation, bacteria were grown at 37˚C for 2h at 180 rpm. Next, bacteria were spread on LB agar plates with antibiotics (Kan – 50 µg/ml, Cam- 10 µg/ml) and incubated at 37˚C for 24h. Positive clones were streaked on new agar plates and then colony PCRs were done (like in Section 3.1.5) for confirmation of successful introduction of the resistance cassette into the *fimH* gene locus, using the following primers: O1532 fimH_Sal_For, O1533 fimH_Sal_Rev, O1535 fimH_Sal_qPCR_rev, K2. In parallel bacteria were streaked onto LB agar with Gm (10 µg/ml) and incubated at 30˚C for 24h to check whether the heat-labile plasmids are present in the bacteria. In the case of bacteria growth, they were re-streaked on LB agar and grown at 42 ˚C for 24h. Next, bacteria were checked again for plasmid removal.

In the next step, the Kan resistance cassette was removed. Electrocompetent bacteria were transformed with pCP20-Gm plasmid and grown at 30˚C for 2h at 180 rpm. Next, bacteria were spread on LB agar with Gm (10 µg/ml) and incubated at 30˚C for 24h. Colonies with the pCP20 plasmid were streaked on LB agar and grown at 42 ˚C for 24h. Next, bacteria were checked for Kan resistance cassette removal with colony PCR (like in the previous step). In parallel bacteria were streaked onto LB agar with Gm (10 µg/ml) and incubated at 30˚C for 24h to check whether the heat-labile plasmid is present in bacteria. In the case of bacteria growth, they were re-streaked on LB agar and grown at 42 ˚C for 24h.

3.4.2. Cloning of *fimH* gene alleles into the pACYC177 plasmid

FimH gene sequences were amplified by PCR. The PCR mix and conditions are shown below. PCR products were analysed on a 1% agarose gel. Subsequently, the gel was stained with ethidium bromide and the imaged with BioDocAnalyze (Biometra). PCR products for each *fimH* allele were purified using the JETQUICK PCR Purification Spin Kit (Genomed) according to the manufacturer's instructions and DNA concentration was measured with a Colibri Microvolume Spectrometer (Titertek-Berthold).

PCR mix	Volume [µl]
H_2O	36
HF PCR buffer (5x)	10
dNTP (10mM)	1
Primer O1919 FimHpACYCfor (10 µM)	1
Primer O1920 FimHpACYCrev (10 µM)	1
Phusion polymerase (2U/µl)	0.5
genomic DNA (30-70ng/µl)	0.5

PCR conditions

Temperature	Time	Repeats
98˚C	30''	
98˚C	5''	
58˚C	15''	35x
72˚C	40''	
72˚C	5'	
10˚C	∞	

The pACYC177 plasmid was isolated using the JETSTAR Plasmid Purification Mini Kit (Genomed) and DNA concentration was measured with a Colibri Microvolume Spectrometer (Titertek-Berthold). Purified PCR products and plasmid DNA were digested with Fast Digest

(FD) BamHI enzyme (Thermo Scientific). The digestion mix and reaction conditions are shown below.

Digestion mix	
FD buffer (10x)	3 µl
BamHI	1 µl
DNA	1 µg
FastAP (only for plasmid DNA)	1 µl
H_2O	To 30 µl
Reaction conditions	
37˚C	30’
80˚C	5’
4˚C	∞

The digested pACYC177 plasmid was run on a 1% agarose gel. In the next step, the gel was stained with ethidium bromide and an appropriate target band was excised and purified with the MiniElute Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol. The digested PCR products were purified with the JETQUICK PCR Purification Spin Kit (Genomed) according to the manufacturer’s instructions and DNA concentration was measured using a Colibri Microvolume Spectrometer (Titertek-Berthold).

The purified DNA was ligated with the use of T4 DNA ligase (Thermo Scientific) in line with manufacturer’s protocol with minor modifications. The ligation mix and reaction conditions are shown below.

Ligation mix	
Reaction buffer (10x)	2 µl
Purified PCR product	20-30 ng
pACYC177 vector	25 ng
T4 DNA ligase	1 µl
H_2O	To 20 µl
Reaction conditions	
22˚C	60’
4˚C	∞

Ten (10) µl of the ligation reaction mixture was used for transformation of chemocompetent *E. coli* XL1Blue. Next, bacteria were spread on LB agar plated with Kan (50 µg/ml) and incubated at 37˚C O/N. On the next day, positive colonies were tested with the rapid colony screening protocol (Casali and Preston 2003). Plasmids were isolated from positive colonies using a rapid colony screening protocol. To confirm successful cloning, plasmids were digested with BamHI and Sanger sequenced.

3.4.3. Electroporation of pACYC177 plasmids into *Salmonella* Typhimurium 5744Δ*fimH*

Electrocompetent *Salmonella* Typhimurium 5744Δ*fimH* was prepared as in section 3.1.8 and was electroporated with pACYC177 plasmids carrying various *fimH* gene alleles, with an empty pACYC177 plasmid as a negative control. Next, bacteria were spread on LB agar containing Kan (50 µg/ml) and incubated at 37˚C O/N. Three to four positive colonies were re-streaked on fresh LB agar plates with kanamycin (50 µg/ml) and checked for FimH protein expression levels as in section 3.3.3 with one difference- kanamycin was added to the medium to the final concentration of 50 µg/ml.

3.4.4. Static adhesion assay.

Proteins were coated like in section 3.2.3. The following proteins were used: four human GP2 isoforms expressed in SF9 cells (GA, Berlin, Germany), two porcine GP2 isoforms expressed in SF9 cells (see section 3.1), horseradish peroxidase (HRP) (Sigma Cat No. P6278), RNase B (Sigma Cat No. R7884) and rabbit polyclonal anti-FimH antibody. Bacteria were induced for T1F expression like in section 3.3.3 and the assay was performed like in section 3.3.3. The test was performed three times in triplicates for each strain. For testing of mannose and glucose blocking, bacteria were preincubated 30 minutes in PBS/1% BSA with 0.2M mannose or glucose to confirm the specificity of the binding between T1F and coated proteins (Schierack et al. 2014).

3.5. Generation of GP2-expressing cell lines

3.5.1. Cloning of human and porcine GP2 isoforms

The coding sequences of GP2 isoforms were amplified by PCR. The PCR mix and conditions are shown below. PCR products were analysed on a 1% agarose gel. Subsequently, the gel was stained with ethidium bromide and imaged with BioDocAnalyze (Biometra). PCR

products for each GP2 isoform were purified using the JETQUICK PCR Purification Spin Kit (Genomed) according to the manufacturer's instructions and DNA concentration was measured with a Colibri Microvolume Spectrometer (Titertek-Berthold).

PCR mix	Volume [µl]
H_2O	35.5
HF PCR buffer (5x)	10
dNTP (10mM)	1
Primer O1461 GP2HoEcoRIFor or O1590 GP2SuForEco (10 µM)	1
Primer O1462 GP2HoBamHIRev or O1591 GP2SuRevBam (10 µM)	1
Phusion polymerase (2U/µl)	0.5
plasmid DNA (10-20ng/µl)	1.0

PCR conditions

Temperature	Time	Repeats
98˚C	30''	
98˚C	5''	
59˚C	15''	35x
72˚C	1'	
72˚C	5'	
10˚C	∞	

The pLVX-IRES-puro plasmid was isolated with use of the JETSTAR Plasmid Purification Mini Kit (Genomed) and DNA concentration was measured with a Colibri Microvolume Spectrometer (Titertek-Berthold). Purified PCR products and plasmid DNA were digested with

FD BamHI and EcoRI enzymes (Thermo Scientific). The digestion mix and reaction conditions are shown below.

The digestion mix	
FD buffer (10x)	3 µl
BamHI	1 µl
EcoRI	1 µl
DNA	1 µg
FastAP (only for plasmid DNA)	1 µl
H_2O	To 30 µl
Reaction conditions	
37˚C	30’
80˚C	5’
4˚C	∞

The digested pLVX-IRES-puro plasmid was run on a 1% agarose gel. In the next step, the gel was stained with ethidium bromide and an appropriate band was excised and purified with MiniElute Gel Extraction Kit (Qiagen, Germany) according to the manufacturer’s protocol. The digested PCR products were purified with the JETQUICK PCR Purification Spin Kit (Genomed) according to the manufacturer’s instructions and DNA concentration was measured with a Colibri Microvolume Spectrometer (Titertek-Berthold).

The purified DNA was ligated with the use of T4 DNA ligase (Thermo Scientific) in line with manufacturer’s protocol. The ligation mix and reaction conditions are shown below.

The ligation mix	
Reaction buffer (10x)	2 µl
Purified PCR product	20-30 ng
pLVX-IRES-puro	50 ng
T4 DNA ligase	1 µl
H_2O	To 20 µl
Reaction conditions	
22˚C	60’
4˚C	∞

Ten (10) µl of the ligation reaction mixture was used for transformation of chemocompetent *E. coli* XL1Blue cells (like in section 3.1.4). Next, bacteria were spread on LB agar with Amp (100 µg/ml) and incubated at 37˚C O/N. Four colonies for each GP2 isoform were inoculated and plasmids were isolated with use of the JETSTAR Plasmid Purification Mini Kit (Genomed) and DNA concentration was measured with a Colibri Microvolume Spectrometer (Titertek-Berthold). To confirm successful cloning plasmids were digested using BamHI and EcoRI and Sanger sequenced.

3.5.2. Cell culture

Each cell line was grown in a cell culture incubator at 37˚C with 5% CO_2 and passaged at a confluency of 80-90%. The cell line was washed with PBS and trypsinized with Trypsin/EDTA solution in a cell culture incubator until all cells were detached. Next, trypsin was inactivated with cell culture medium, centrifuged at 100xg for 5' at room temperature, resuspended in cell culture medium and diluted as necessary in a flask. The medium used for IPEC-J2 and HEp-2 cells consisted of D-MEM/Ham's F12 (Millipore) supplemented with 5% bovine serum (Millipore), 2 mM L-glutamine and penicillin/streptomycin (Millipore). The concentration of puromycin for cell line selection and maintenance was 1µg/ml and 0.25 µg/ml, respectively.

3.5.3. MTT assay for assessment of puromycin resistance of the cell lines

In order to determine optimal puromycin selection concentration, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was done with the HEp-2 and IPEC-J2 cell lines (Owczarek et al. 2013). Five 96-well plates with 5,000 cells per well in 21 wells per plate were prepared for each cell line. After O/N incubation of cells, the medium was replaced with 150 µl of medium with different concentrations of puromycin dihydrochloride (Sigma) (0.5, 1.0, 2.5, 5.0, 7.5, 10.0 µg/ml). After 24h, to one plate for each cell line, 50 µl of MTT solution in PBS (5mg/ml) was added and cells were incubated at 37˚C for 2h. Next, the medium was aspirated and the plate was stored in -20˚C until the test was finished for all time points (protected from the light). The procedure was repeated for each cell line for the next four days. Forty-eight (48) hours after addition of puromycin, the medium was changed in three plates. After the test, cell debris was resuspended with 200 µl of solvent buffer (DMF: 37.5 mL; SDS: 11.25 g ddH_2O: ad 80mL) and the absorbance was read at 570 nm wavelength and 650 nm wavelength as reference wavelength using a Tecan reader (Tecan). The

concentration of puromycin that killed all the cells between three and five days was used for the antibiotic selection after cell line transduction.

3.5.4. Generation of lentiviruses

The Lenti-X Lentiviral Expression System (Clontech) was used to produce lentiviruses. First, an 80% confluent Lenti-X 293T cell line (Clontech) in a T-75 flask was co-transfected with six plasmids (one containing a GP2 isoform or "empty" vector, and five packaging plasmids) according to the manufacturer's protocol. After 48-72h, the supernatant was harvested and centrifuged at 1,000xg for 10' at room temperature. Next, the supernatant was filtered through a 0.45 µm syringe filter (Roth) into Amicon Ultra-15 centrifugal filter units (100,000 Nominal Molecular Weight Limit (NMWL)) and concentrated by centrifugation at 2,400xg for 10' at room temperature. The concentrated supernatant was used for fresh transduction of an appropriate cell line.

3.5.5. Transduction of cell lines with lentiviruses

To transduce a cell line, $2x10^4$ cells were suspended in 800 µl of medium and 100 µl of concentrated lentivirus. Next, the cells were centrifuged at 2,400xg for 2.5h at 23°C, the supernatant was discarded, and the pellet of cells was resuspended in 500 µl of medium and transferred onto six-well plates. In parallel negative control was done, with medium instead of lentivirus. After 72h of cell growth, the medium was exchanged for medium with puromycin to start the antibiotic selection. The medium with antibiotic was changed every 48h. The selection was considered successful when all cells in the negative control were dead.

3.5.6. Reverse transcription PCR (RT PCR) and reverse transcription-quantitative PCR (RT-qPCR)

Confirmation of GP2 expression in transduced cell lines was done with RT PCR and RT-qPCR. RNA from transduced cells was isolated with RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. Next, the RT reaction was performed like in section 3.1.2. PCR was performed in an Eppendorf Mastercycler ep Gradient (Eppendorf) and qPCR was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The PCR mix and reaction conditions are shown below. RPLP0 was used as a reference gene (George et al. 2016).

PCR mix	Volume [µl]
H_2O	17.05
PCR Buffer (10x)	2.5
$MgCl_2$ (50mM)	2.0
EvaGreen (qPCR)	1.25
dNTP/dUTP (10mM/20mM)	0.5
Primer O1573 GP2HoqPCR2for or O1630 GP2SuForqPCR or O876 RPLP0_fw (10 µM)	0.5
Primer O1574 GP2HoqPCR2rev or O1631 GP2SuRevqPCR or O877 RPLP0_rv (10 µM)	0.5
Biotherm polymerase (5U/µl)	0.2
cDNA	1.0

PCR conditions

Temperature	Time	Repeats
95˚C	3’	
95˚C	30’’	
61˚C	30’’	40x
72˚C	1’	
65-95 ˚C	5’’	30x
10˚C	∞	

3.5.7. Western blotting

$1x10^6$ of cells were lysed in RIPA buffer and protein concentration was determined with Roti-Quant universal (Roth). A 5x loading dye was added to the lysate and the sample was boiled for 5’. SDS-PAGE and western blotting was performed like in section 3.1.13. In SDS-

PAGE, 40 µg of protein was added per sample. An anti-GP2Ho#1 rabbit polyclonal antibody was used as primary antibody for HEp-2 cell lines and anti-GP2Su#2 rabbit polyclonal antibody was used for IPEC-J2 cell lines. After the first blotting, the membrane was incubated with 30% H_2O_2 for 20' at 37˚C to inactivate peroxidase activity (Sennepin et al. 2009), washed two times with PBST and blocked for 2h with 5% dried, defatted milk solution in PBST. Next, the membrane was incubated with α tubulin antibody (1:1,000 dilution) O/N. The antibody solution was discarded and the membrane was washed three times 10' with PBST. After that, the membrane was incubated with a secondary antibody conjugated to HRP for 1h, washed three times 10' with PBST and was incubated for 2' in ECL solution. The blot was visualised by use of a Lumi-Imager F1 Workstation (Roche).

3.5.8. Indirect immunofluorescence (IIF)

To confirm GP2 expression at the protein level IIF was used. Cells were seeded in 96-well plate at a density of $1x10^4$ cells per well. When cells reached a monolayer, they were washed three times with 150 µl of PBS and fixed with 50 µl of 4% PFA in PBS (pH=7.4) for 30' at 4˚C. Next, cells were washed three times with 100 µl of PBS and blocked with 100 µl of blocking buffer (BB, 0.5% BSA in PBS) for 5' at 4˚C. The blocking buffer was exchanged with 50 µl of primary anti-GP2 antibody (1:250 to 1:1,000 dilutions in BB) and cells were incubated for 1h at 4˚C. Next, the primary antibody solutions were discarded, and the cells were washed three times with 100 µl of BB and incubated with 50 µl of secondary antibody (1:250 dilution in BB, FITC-conjugated) for 1h at 4˚C. In the next step, the secondary antibody solutions were discarded, and the cells were washed three times with 100 µl of PBS and incubated with 50 µl of DAPI (50 µg/ml in ddH_2O) for 30'' at room temperature. Afterwards, the cells were washed 1x with 100µl of PBS and left in PBS solution for fluorescence microscopy evaluation (hardware like in section 3.2.1). The images were taken at 10x and 20x magnification in the DAPI and FITC filter channel.

3.5.9. Flow cytometry

Flow cytometry was done using the following protocol. First, cells in a T-75 flask were trypsinized (like in section 3.5.2) and counted with Neubauer chamber. All centrifugation and incubation steps were performed at 4˚C. In each aliquot, $1x10^6$ of cells were blocked in PBS/BSA 1% for 30'. The cells were centrifuged at 300xg for 5', resuspended in 100 µl of anti-GP2 antibody in PBS/1% BSA and incubated for 45'. Next, the cells were washed three times with 1 ml PBS/1% BSA, resuspended in 100 µl of secondary antibody (FITC-conjugated) and

incubated for 30'. Cells were washed three times in 1 ml PBS/1% BSA and resuspended in 600 µl of PBS. Flow cytometry was performed on BD FACSCanto II flow cytometer (BD Biosciences).

3.6. Cell line infection assays

3.6.1. VideoScan Module

The VideoScan module works with a 20x magnification objective and first focuses in the well on DAPI-stained nuclei. Afterwards, the software takes an image of bacteria (GFP or Atto647-FISH stained) and counts bacteria detected on the image. Forty (40) images per well were taken during read-out.

3.6.2. Cell culture

Cell lines were passaged like in section 3.5.2. For infection assays, cells were seeded in the following densities: IPEC-J2-$0.75x10^4$; HEp-2-$1x10^4$ in 96-well plate for four days before assay. The cell lines were washed three times with 200 µl of PBS and 100 µl of pre-warmed medium without antibiotics was applied before an assay.

3.6.3. Determination of assay's linear range

For the assay, *S.* Typhimurium SL1344, transformed with pFPV25.1GFPmut3Kan were first grown O/N at 37˚C and 180 rpm in 3.5 ml of LB supplemented with Kan (50 µg/ml) in a 15-ml Falcon tube. The O/N culture was diluted to OD_{600}=0.1 in 5 ml of LB with Kan (50 µg/ml) in a 15-ml Falcon tube and grown to OD_{600}=0.95-1.0 at 37˚C and 180 rpm. Before the assay, bacteria were centrifuged at 6,000xg for 3', washed once with PBS, centrifuged again 6,000xg for 3' and resuspended in 800 µl of cell culture medium. After this, OD_{600} of bacterial solution was measured and 50 µl of bacteria were applied in a dilution series ($2x10^4$-$3x10^7$) on plates with a monolayer of IPEC-J2 and HEp-2 cells. Bacteria were incubated for 1h in a cell culture incubator and then plates were washed three times with 150 µl of PBS. The cell line monolayer with bound bacteria was fixed with 50 µl of 4% PFA in PBS for 30' at 4˚C, then plates were washed three times with 100 µl of PBS. Cell nuclei were stained with 50 µl of DAPI (50 µg/ml in H_2O) for 30'' at room temperature, then plates were washed one time with 100 µl of PBS and 100 µl of PBS was applied. Next, the plates were measured using the VideoScan module. Three independent experiments with three repetitions for each dilution were prepared and measured.

3.6.4. Cell line infection assay

Cell lines were seeded in 96-well plates and assays were performed when cells reached a monolayer. Before each assay, cell lines were washed with PBS and 100 µl of medium without antibiotics was added. An isogenic *Salmonella* model was used for infection assays. Bacteria were grown like in section 3.4.4. Bacteria used for infection assays were also checked for T1F expression with the static adhesion assay (like in section 3.4.4.). Before an assay, OD_{600} of bacterial solution was measured, bacteria were diluted to a concentration of $1x10^8$ bacteria/ml and 50 µl of bacteria were applied per well to a monolayer of IPEC-J2 and HEp-2 cells. For the mannose blocking assay, bacteria were first pre-incubated for 30' with 0.2M mannose. Bacteria were incubated for 2h in a cell culture incubator and the plates were washed three times with 150 µl of PBS. Cell lines with bound bacteria were fixed with 50 µl of 4% PFA in PBS for 1h at 4˚C, then plates were washed three times with 100 µl of ddH_2O. Next, the cells were dehydrated with 50 µl of 95% ethanol for 5'', dried and stored at 4°C until FISH staining. Bacteria were FISH-stained with the following protocol. Hybridization and washing buffer were freshly prepared before each assay and pre-warmed to 46°C. FISH probe EUB338 Atto647N (sequence: GCWGCCWCCCGTAGGWGT) was diluted in hybridization buffer to a final concentration of 5 ng/µl and 40 µl of this solution was applied on cell lines. Next, the plates were incubated in a humid chamber for 1h at 46°C and washed once with 100 µl of washing buffer. Later, plates were incubated for 10' at 48°C with 100 µl of washing buffer, nuclei were stained with DAPI (50 µg/ml in ddH_2O) and washed once with 100 µl of ddH_2O. Plates were left to dry at room temperature and then measured with the VideoScan. Three independent experiments with three repetitions for each FimH variant and cell line were prepared and measured.

3.7. Figures and statistical analysis

Statistical analysis was performed using the R software (R Development Core Team, 2017). All figures were prepared with the ggplot2 package implemented in the R software (Wickham 2010). Cluster analysis was done with the use of the Ward method implemented in the R software (Ward 1963).

4. Results

4.1. Cloning and expression of porcine GP2 isoforms

In order to compare binding of different FimH variants to GP2 from various hosts, porcine GP2 isoforms were cloned and expressed in SF9 cells. First, RNA was isolated from porcine pancreas and cDNA synthesis was performed with this RNA. The porcine GP2 coding sequence was amplified via TC PCR. Porcine GP2 isoforms were first cloned into pJET1.2 plasmids and sent for sequencing. The obtained sequences were identical to porcine GP2 isoforms found in GenBank (Acc. No.: XM005662102 and XM003124571) and were submitted to GenBank under the accession numbers: KU665994 and KU665995 (Fig. 4).

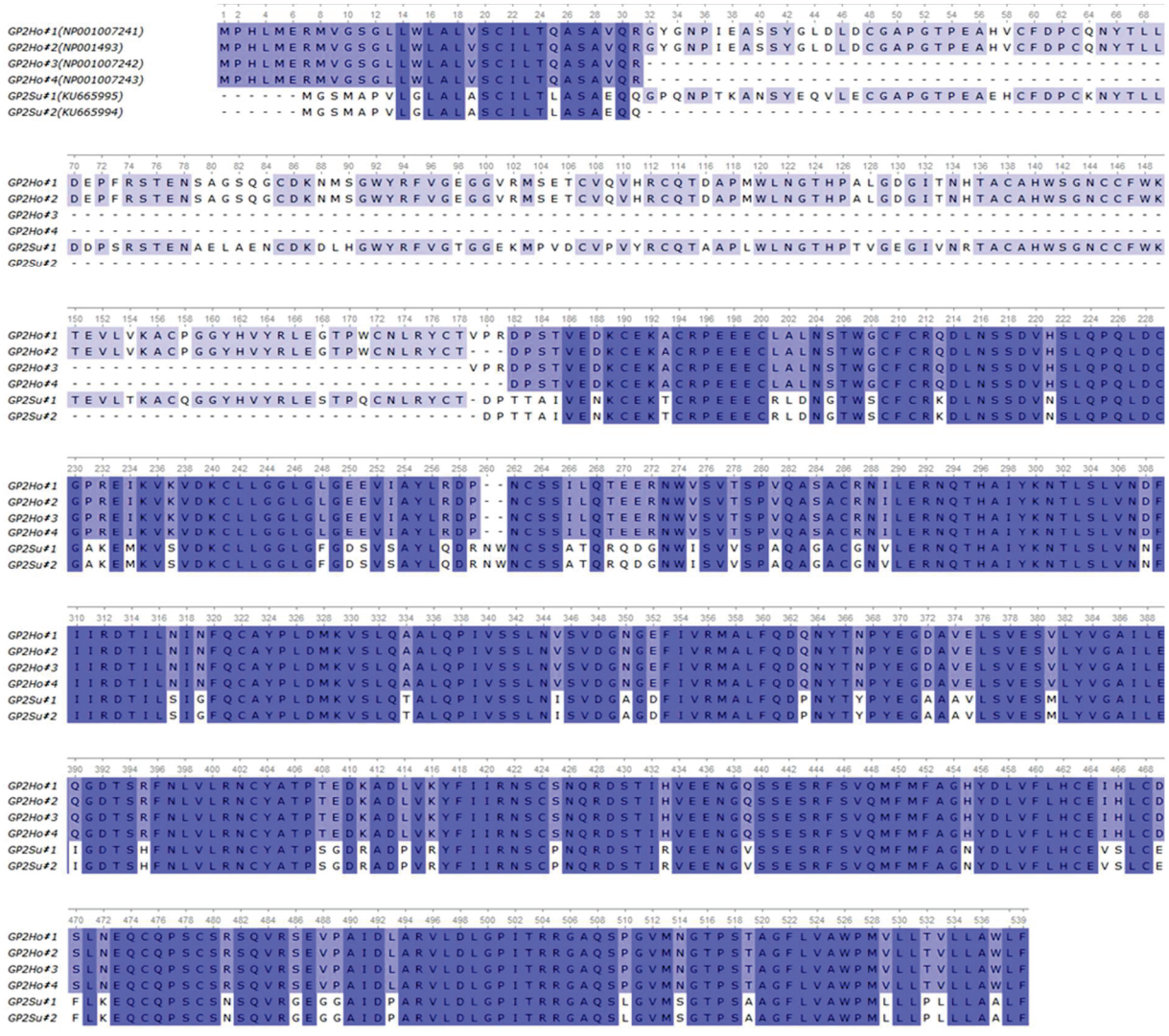

Figure 4. Alignment of amino acid sequences of GP2 isoforms.
Compared are full amino acid sequences of human GP2 isoforms 1 to 4 (GP2Ho#1 to 4) and porcine GP2 isoforms 1 and 2 (GP2Su#1 and 2). The sequence data are available from GenBank under the indicated accession numbers (NP001007241 (GP2Ho#1), NP001007242 (GP2Ho#3), NP001007243 (GP2Ho#4), NP001493 (GP2Ho#2), KU665995 (GP2Su#1), KU665994 (GP2Su#2)).

In order to express GP2 isoforms in SF9 cells, GPI-anchor was identified and excluded so that GPI-anchorless GP2 were cloned into pDONR221 and pDEST8 vectors using the Gateway cloning system. In order to obtain baculovirus, recombinant bacmid was generated and successfully transfected into SF9 cells. Baculoviruses containing recombinant porcine GP2 were amplified and a P3 virus stock was first tested at different multiplicities of infection (MOIs) and time course. Recombinant porcine GP2 isoform 1 and 2 were detected in similar amount after four days in all viral MOIs tested. For protein purification, expression was scaled up and large volumes of SF9 cells in suspension cultures were used. Proteins were purified by affinity chromatography and the purity was checked by silver staining (Fig. 5).

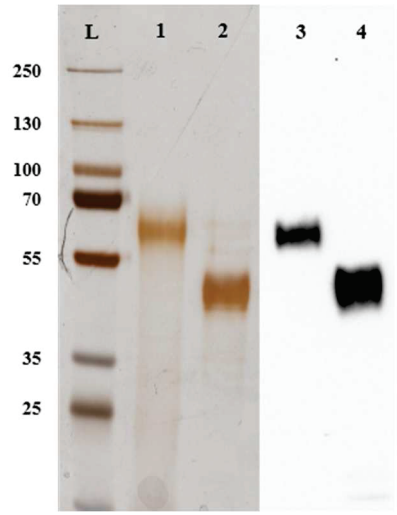

Figure 5. Expression of porcine GP2.
Silver staining and western blotting with anti-6-His antibody of purified porcine GP2 isoforms. Lane L: protein marker (kDa), lanes 1 and 3: porcine GP2 isoform 1 (GP2Su#1), lanes 2 and 4: porcine GP2 isoform 2 (GP2Su#2), lanes 1 and 2: silver staining, lanes 3 and 4: Western Blot.

In order to check whether obtained proteins were glycosylated, protein deglycosylation was performed. After digestion, proteins were resolved on SDS-PAGE and stained with Coomassie brilliant blue (Fig. 6.).

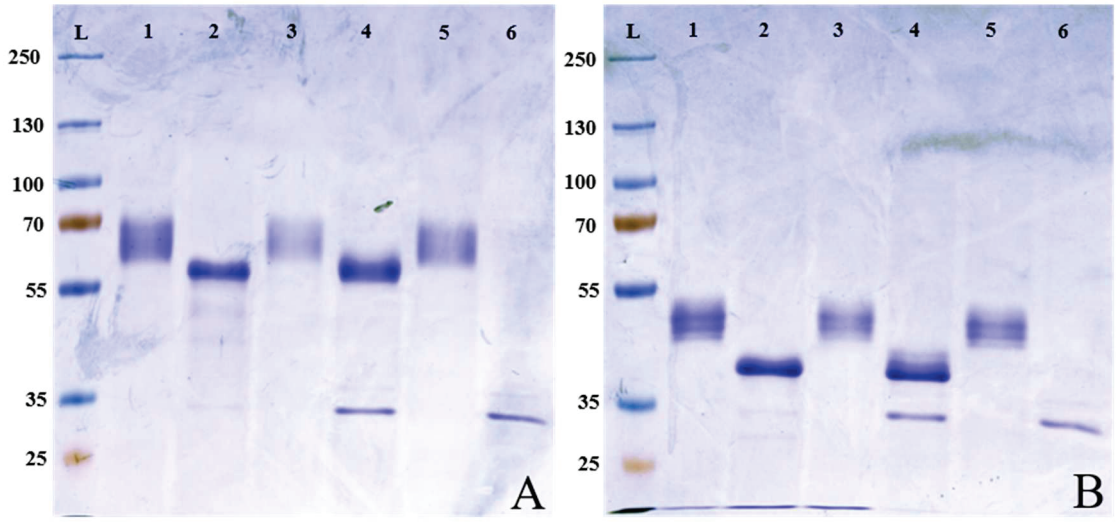

Figure 6. Deglycosylation of GP2 (Coomassie brilliant blue staining).
After deglycosylation of recombinant porcine GP2 isoform 1 (GP2Su#1, gel A) and porcine GP2 isoform 2 (GP2Su#2, gel B) proteins were visualised by Coomassie brilliant blue staining. Lane L: protein ladder (kDa), lane 1: native, non-digested protein, not incubated at 37˚C; lane 2: protein digested under denaturing conditions; lane 3: protein incubated under denaturing conditions without enzymes; lane 4: protein digested under native conditions; lane 5- protein incubated under native conditions without enzymes; lane 6- enzyme mix only.

It is evident that deglycosylation under native conditions was not complete. The state of deglycosylation was further confirmed by staining the blot with ConA. GP2 was completely deglycosylated only when digested under denaturing conditions. ConA is a lectin isolated from *Canavalia ensiformis* which recognises branched alpha-mannosidic structures in high mannose type, hybrid type, and biantennary complex type N-glycans. ConA bound well to non-digested proteins and proteins digested under native conditions (Fig. 7).

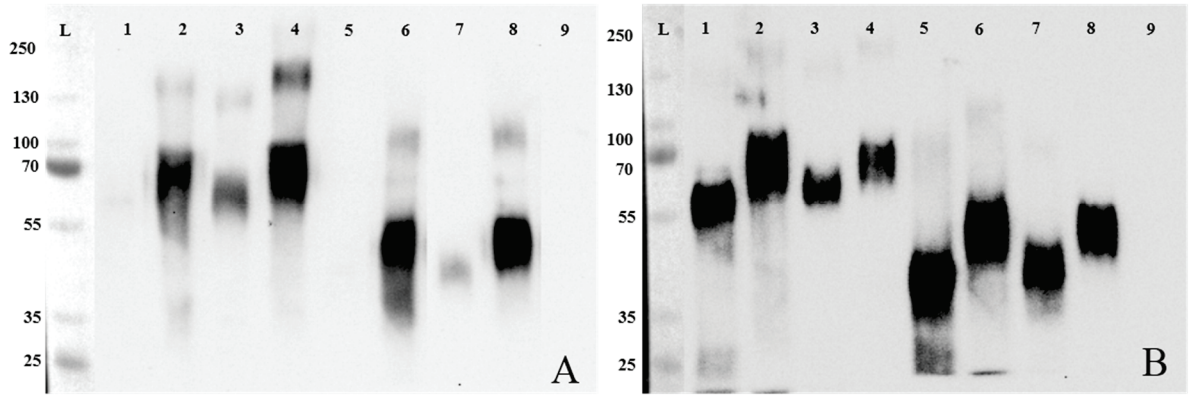

Figure 7. Deglycosylation of GP2 (western blotting).
After deglycosylation of recombinant porcine GP2 isoform 1 (GP2Su#1) and porcine GP2 isoform 2 (GP2Su#2) proteins were visualised by Western Blot using ConA (A) and anti-6-His antibody (B). Lane L: protein ladder (kDa), lane 1: GP2Su#1 digested under denaturing conditions; lane 2: GP2Su#1 incubated under denaturing conditions without enzymes; lane 3: GP2Su#1 digested under native conditions; lane 4: GP2Su#1 incubated under native conditions without enzymes; lane 5: GP2Su#2 digested under denaturing conditions; lane 6: GP2Su#2 incubated under denaturing conditions without enzymes; lane 7: GP2Su#2 digested under native conditions; lane 8: GP2Su#2 incubated under native conditions without enzymes; lane 9: enzyme mix only.

4.2. Static adhesion assays

The possibility to quantify bacteria attached to various immobilised proteins was vital for this work. The VideoScan Platform was utilised to measure adhesion of *Salmonella* isolates to various proteins immobilised on the surface of 96-well plates. A new Videoscan module software was designed by our group, that enabled quantification of bacteria attached to various proteins. After fluorescence staining, bacteria were counted with the epifluorescence microscope-based VideoScan (Fig. 8).

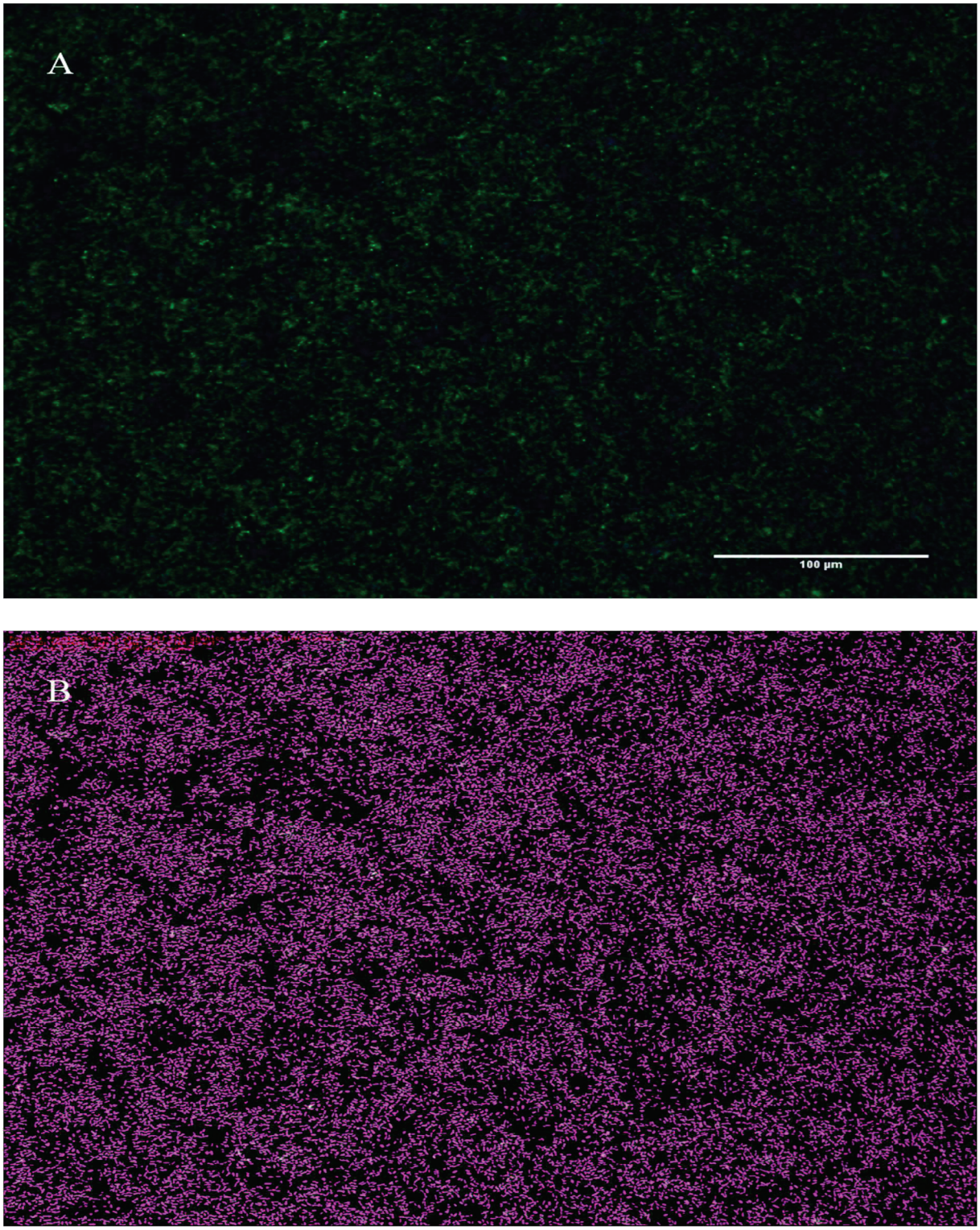

Figure 8. Exemplary image taken by VideoScan during a static adhesion assay.
In this case, GP2 was coated onto 96-well plates. *Salmonella* were incubated and non-adherent bacteria were removed by washing. A) After staining with propidium iodide (PI) images were taken by the VideoScan instrument. Green: PI-stained *Salmonella*; Blue: DAPI-stained focus beads as help for focusing on the bacteria layer. B) Image A was processed with the VideoScan module, and counted bacteria are marked in magenta colour.

First, the module was tested to determine assay's linear range. A dilution series of *Salmonella* Typhimurium 5744 and the *fimH* gene deletion mutant derivate of this strain were used in static adhesion assays with two proteins as an example: human GP2 isoform 1 (GP2Ho#1) and the anti-FimH antibody. Human GP2 isoform was used because at the time of assay development expression of porcine GP2 was in progress. The assay with anti-FimH antibody showed a linear range in all measured points (R^2=0.98), whereas that with GP2Ho#1 showed a linear range from 2.5x10^7 bacteria/well point (R^2=0.97) (Fig. 9). This assay confirmed the possibility of bacteria quantification using the VideoScan in this experimental set-up and allowed for the choosing of the optimal amount of bacteria inoculum for subsequent static adhesion assays performed with the VideoScan.

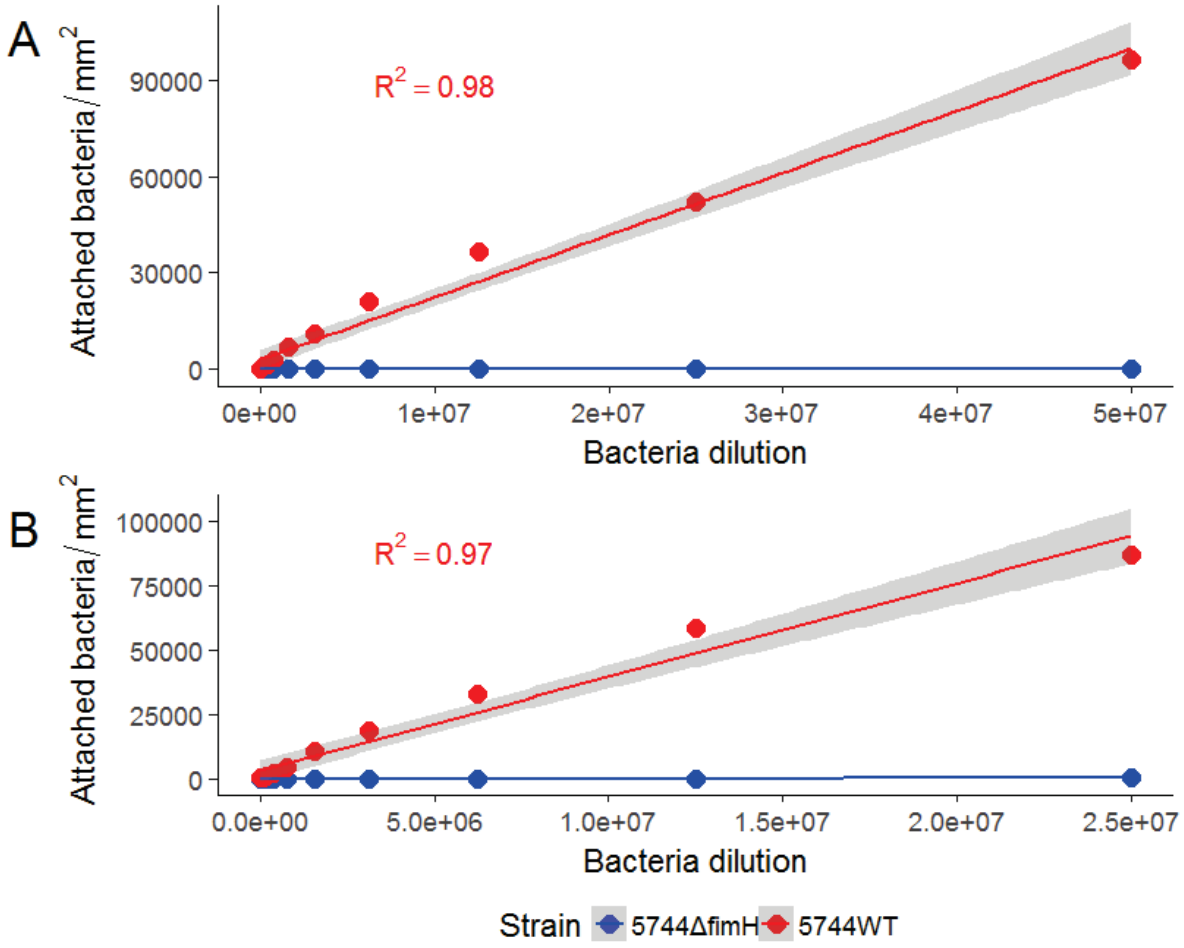

Figure 9. Dilution series of *Salmonella* in static adhesion assays.
This assay was performed to determine the linear range of *S.* Typhimurium 5744 wild type (5744WT, red colour) and it's *fimH* gene deletion mutant (5744ΔfimH, blue colour) in static adhesion assays using human GP2 isoform 1 (A) and an anti-FimH antibody (B) coated in 96-well plates. Each dot represents a median value from three measurements in triplicates. Red and blue lines connecting dots are smoothed trend lines and grey areas around the lines represent the 95% confidence interval of the linear regression.

4.3. Characterization of *Salmonella* isolates

Binding of T1F with receptors (e.g. GP2) is mediated directly by the FimH protein. Therefore, in the first step, different sequences of the *fimH* gene from all isolates (that possessed that gene, n=119) were determined. Analysis of the obtained sequences revealed 12 DNA sequence alleles and 40 variable sites within this group of sequences.

Translation of the *fimH* gene DNA sequences into protein sequences revealed 11 sequence variants with 18 variable sites in this comparison (Table 6). Four *S*. Typhimurium (Tym) alleles translated into four protein variants, in which one of them resulted in translation termination after 79 codons (Tym4). This sequence was found only in one isolate. Among 34 *S*. Enteritidis (Ent) isolates two *fimH* gene alleles were found and the only difference between them was a silent mutation (position 348), therefore only one FimH variant from *S*. Enteritidis was found. In the case of *S*. Choleraesuis (Chol), nine isolates did not possess the *fimH* gene. The rest of the isolates from this serovar had three DNA alleles and protein variants. In all *S*. Dublin (Du) isolates from the collection, one sequence variant was present. In the case of *S*. Gallinarum (Gall) two variants were determined. The first variant, typical for *S*. Gallinarum biovar Pullorum, was obtained from 16 isolates (Gall-Pull, later referred as Pull). The second variant, typical for *S*. Gallinarum biovar Gallinarum, was found in three isolates (Gall-Gall, later referred as Gall).

Table 6. Amino acid variation in *Salmonella* FimH sequence variants.
FimH sequence variants of *S*. Typhimurium (Tym1-4), *S*. Enteritidis (Ent1-2), *S*. Choleraesuis (Chol1-3), *S*. Dublin (Du), *S*. Gallinarum biovar Pullorum (Pull) and *S*. Gallinarum biovar Gallinarum (Gall) discovered in this study were aligned with the FimH sequence of *S*. Typhimurium LT2 (LT2). The FimH sequence of *S*. Typhimurium LT2 was used as a reference in alignment. Only polymorphic positions in the FimH protein are shown. Residues identical to the amino acid sequence of *S*. Typhimurium LT2 are indicated by dots and STOP means stop codon. Numbers in brackets stand for a number of isolates with the particular FimH variant.

Isolate\Position (No. of isolates)	13	50	57	63	78	80	89	101	126	131	137	166	182	222	245	279	285	317
LT2	A	P	P	V	T	R	Q	N	L	Y	K	T	T	A	V	S	T	I
Tym1 (33)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Tym2 (1)	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.
Tym3 (5)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
Tym4 (1)	.	.	.	.	.	STOP	.	.	.	.	.	.	.	.	.	.	.	.
Ent1-2 (34)	.	.	.	.	.	.	.	.	R	S	M	.	.	.	.	.	.	N
Chol1 (1)	.	S	.	.	.	.	.	.	.	S	.	.	.	.	.	G	.	N
Chol2 (1)	.	.	L	G	.	.	R	.	R	S	.	.	.	.	.	.	.	N
Chol3 (4)	.	.	L	.	.	.	R	.	R	S	.	.	.	.	.	.	.	N
Du (20)	.	.	.	.	.	.	.	S	R	S	M	.	.	.	.	.	I	N
Gall- Pull (16)	T	.	.	.	I	.	.	.	R	S	M	.	.	.	.	.	.	N
Gall-Gall (3)	.	.	.	.	I	.	.	.	R	S	M	.	S	V	.	.	.	N

In the next step, expression of T1F among collected strains was checked by use of the static adhesion assay (Fig. 10). To compare the groups- which were made by serovar and isolation source- a general linear model was created and the Tukey contrasts were calculated. The analysis shows that there was no difference between the expression of T1F in human and porcine isolates of Tym. Contrary to Tym, expression of T1F in chicken was higher compared to human isolates of Ent ($p<0.05$). T1F expression in Ent from chicken was higher compared to all other groups ($p<0.005$, Ent human- $p<0.05$). Ent from human has higher expression than isolates of Du ($p<0.05$). No difference in T1F expression was found between Chol, Gall, Tym-human and Tym-pig.

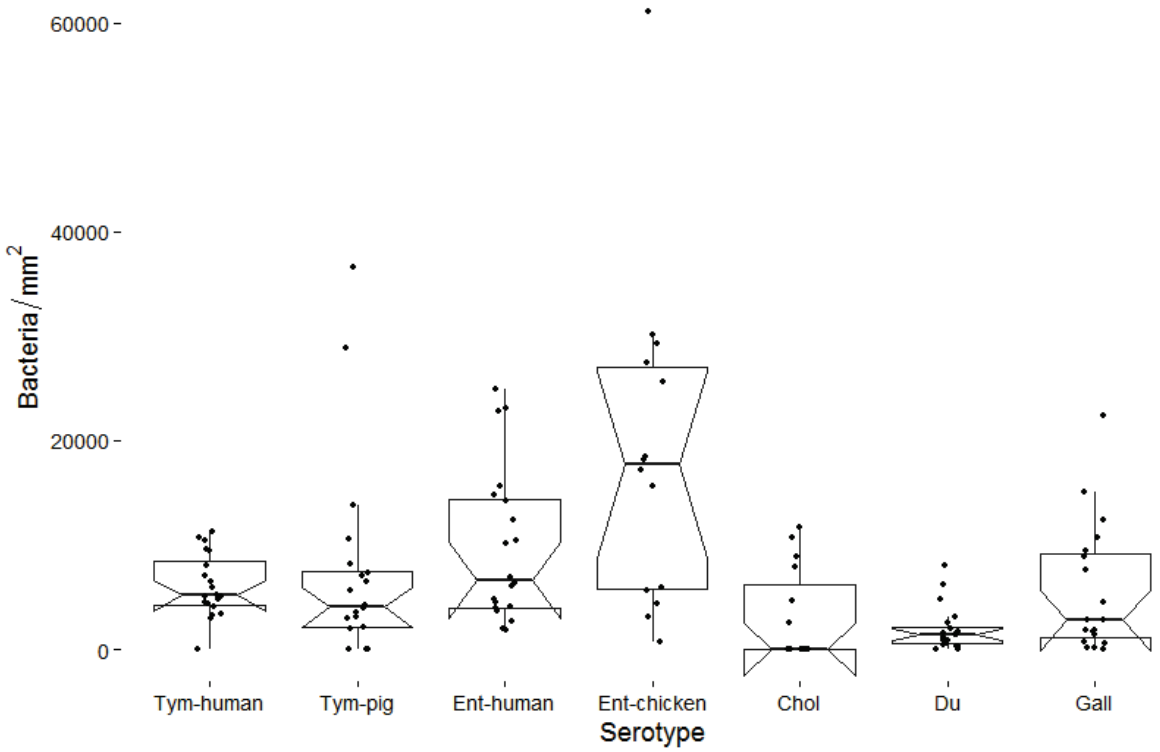

Figure 10. Box-and-whisker plot of T1F expression among *Salmonella* isolates from various hosts. 128 *Salmonella* strains from *S*. Typhimurium of human (Tym-human) and pig (Tym-pig) origin, *S*. Enteritidis of human (Ent-human) and chicken (Ent-chicken) origin, *S*. Choleraesuis (Chol), *S*. Dublin (Du), *S*. Gallinarum (Gall) were compared for T1F expression based on binding to anti-FimH antibodies in static adhesion assays. Each dot represents a median value from three measurements in duplicates for one strain. The bottom and top of each box represent the first (25%) and third (75%) quartiles, and bands inside the boxes are second quartiles (medians). Notches in the boxes show the 95% confidence intervals for medians. Whiskers (vertical lines) extend from the boxes to a maximum of 1.5x of the interquartile range.

Next, by use of cluster analysis, strains were divided by their T1F expression into three groups- high, medium and low T1F expression (Table 7). 86% of *Salmonella* isolates expressed T1F. Ent was the only serovar where all isolates expressed T1F. Because of the tendency of higher T1F expression in strains from host-unrestricted serovars (generalists) and lower T1F expression in host-associated or host-restricted serovars (specialists), all isolates from generalists and specialists were re-grouped together (Fig. 11). From the result of this rearrangement, it is visible, that T1F expression is higher in generalists than in specialists ($p<0.001$, chi-squared test).

Table 7. Cluster analysis for expression of T1F among *Salmonella* isolates.
Results of binding to anti-FimH antibodies in static adhesion assays for *128 Salmonella* strains from *S.* Typhimurium of human (Tym-human) and pig (Tym-pig) origin, *S.* Enteritidis of human (Ent-human) and chicken (Ent-chicken) origin, *S.* Choleraesuis (Chol), *S.* Dublin (Du), *S.* Gallinarum (Gall) were clustered into three groups (low, medium and high T1F expression) with use of the Ward method. The group with low expression also contains strains with no T1F expression (numbers in brackets).

Expression	Tym-human	Tym-pig	Ent-human	Ent-chicken	Generalists	Chol	Du	Gall	Specialists
Low (No Exp.)	11 (1)	11 (4)	8 (0)	3 (0)	33 (5)	11 (9)	18 (2)	12 (2)	41 (13)
Medium	9	7	9	6	31	4	2	6	12
High	0	2	3	5	10	0	0	1	1
Total	20	20	20	14	74	15	20	19	54

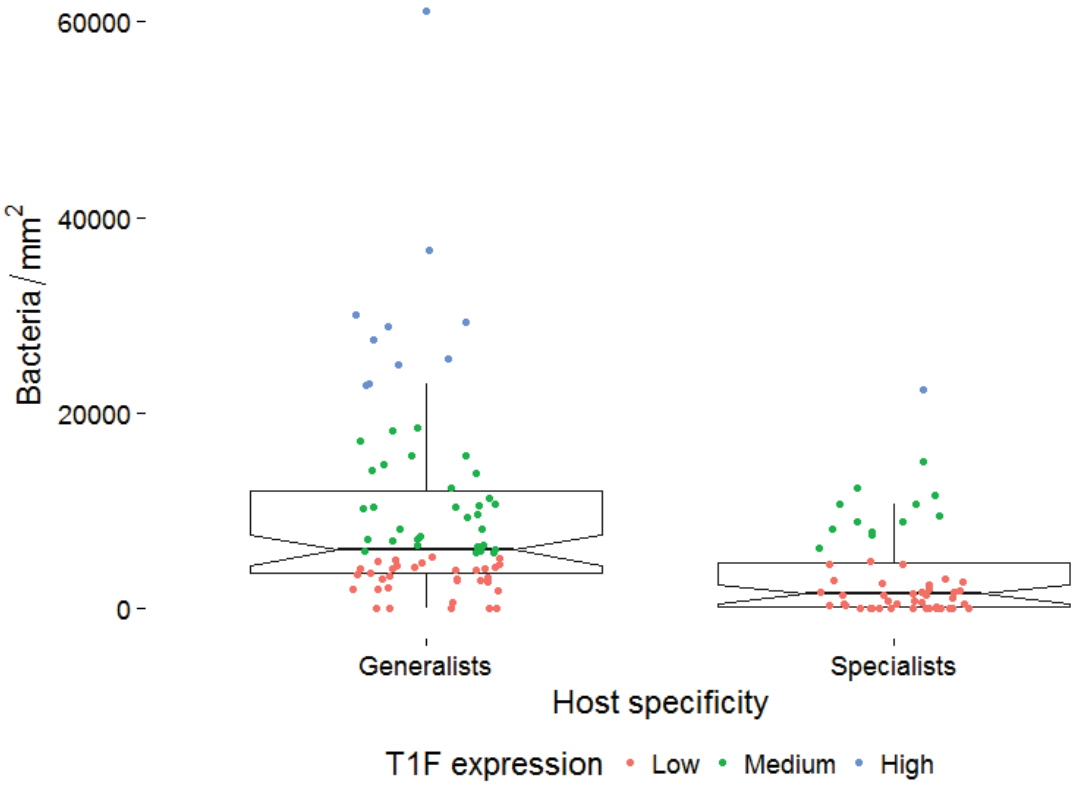

Figure 11. Box-and-whisker plot with T1F expression among *Salmonella* generalists and specialists.
Salmonella strains from host-unrestricted serovars (generalists: *S.* Typhimurium and *S.* Enteritidis) and from host-associated or host-restricted serovars (specialists: *S.* Choleraesuis, *S.* Dublin and *S.* Gallinarum) were compared for T1F expression based on binding to anti-FimH antibodies in a static adhesion assay. Each dot represents a median value from three measurements in duplicates for one strain. The bottom and top of each box represent the first (25%) and third (75%) quartiles, and bands inside the boxes are second quartiles (medians). Notches in the boxes show the 95% confidence intervals for medians. Whiskers (vertical lines) extend from the boxes to a maximum of 1.5x of the interquartile range.

To confirm, that GP2 glycosylation is necessary for *Salmonella* binding, static adhesion assay with deglycosylated porcine GP2 isoforms was performed. As shown above, deglycosylation depended on incubation conditions, and proteins were completely deglycosylated under denaturing conditions. In accordance, *Salmonella* did not bind to GP2 deglycosylated under denaturing conditions and bound lower to GP2 deglycosylated under native conditions (Fig. 12., 41% for GP2Su#1 and 47% GP2Su#2 in comparison to non-digested isoforms). Denaturation of the protein itself lowered binding capacities of GP2 (around 40 times) to bacteria when compared with binding to the native protein. Conclusively, binding of T1F+ *Salmonella* to GP2 depended on glycosylation.

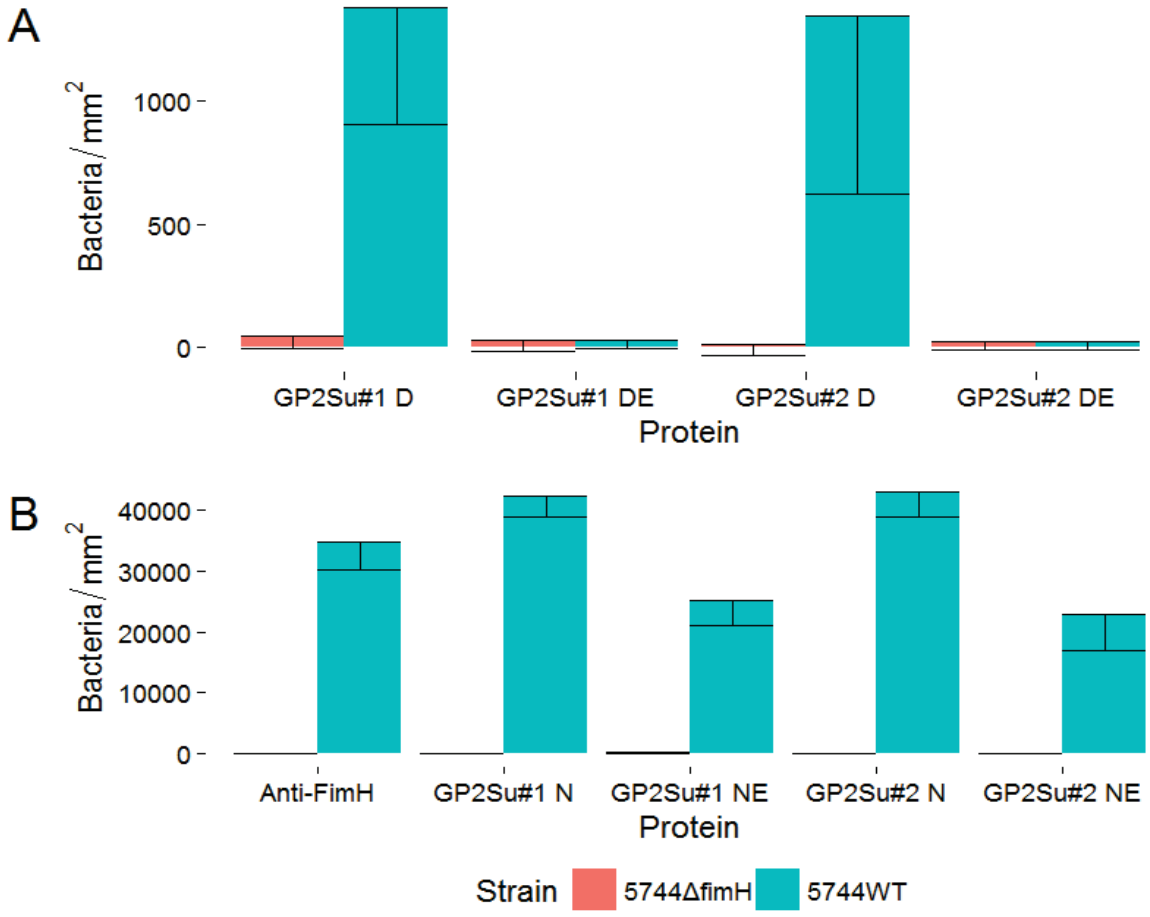

Figure 12. Static adhesion assay with deglycosylated GP2.
S. Typhimurium strain 5744 wild type (5744WT, blue colour) and it's *fimH* gene deletion mutant (5744ΔfimH, red colour) were incubated with A) GP2 deglycosylated under denaturing conditions and B) GP2 partially deglycosylated under native conditions. Expression of T1F was confirmed by binding to an anti-FimH antibody which was included (first two bars in B). X-axis: D: GP2 incubated under denaturing conditions without enzymes; DE: GP2 digested under denaturing conditions; N: GP2 incubated under native conditions without enzymes; NE: GP2 digested under native conditions; GP2Su#1: porcine GP2 isoform 1; GP2Su#2: porcine GP2 isoform 2; Anti-FimH: antibody against FimH.

4.4. Generation of an isogenic *Salmonella* model

In order to investigate the influence of *fimH* gene sequence variation on binding to various GP2 isoforms, an isogenic system was created. The anti-FimH static adhesion assay described earlier allowed for the selection of strains with a high T1F expression for generation of the isogenic model. A deletion mutant of the *fimH* gene in Tym (strain no. 5744) was generated and all *fimH* gene alleles were cloned into a pACYC177 plasmid, and subsequently transformed into Tym 5744Δ*fimH*.

The isogenic model created in the Tym 5744Δ*fimH* strain consisted of ten FimH variants and a strain transformed with the empty pACYC177 plasmid (empty vector control). The isogenic model was used in static adhesion assays with human and porcine GP2 isoforms, standard glycoproteins and anti-FimH antibody (Fig. 13). Adhesion assays using the anti-FimH antibody showed that the expression of FimH protein varied among the FimH variants. Binding to GP2 isoforms and standard proteins was FimH-variant dependent. A high binding phenotype was observed in the case of the variants Tym1, Tym3, Chol3 and Du. A low binding phenotype was observed with the variants Ent, Tym2, and Chol1. The variants Chol2, Gall and Pull did not bind to GP2 (no-binding phenotype). There was no difference in FimH variants binding to GP2 isoforms from the same species: if a variant had a high binding capacity to one GP2 isoform of one host species this variant also had a high binding capacity to the other isoforms of this host. There was also no difference in FimH variant binding between human or porcine GP2 isoforms: if a variant had a high binding capacity to human GP2 this variant also showed high binding to porcine GP2. Additionally, if one variant bound well to GP2 this variant also bound well to the other glycoproteins. The binding to glycoproteins was mannose-dependent (Fig. 13) and glucose independent (data not shown). Conclusively, the *fimH* sequence dictated over GP2 binding, but was not glycoprotein-specific.

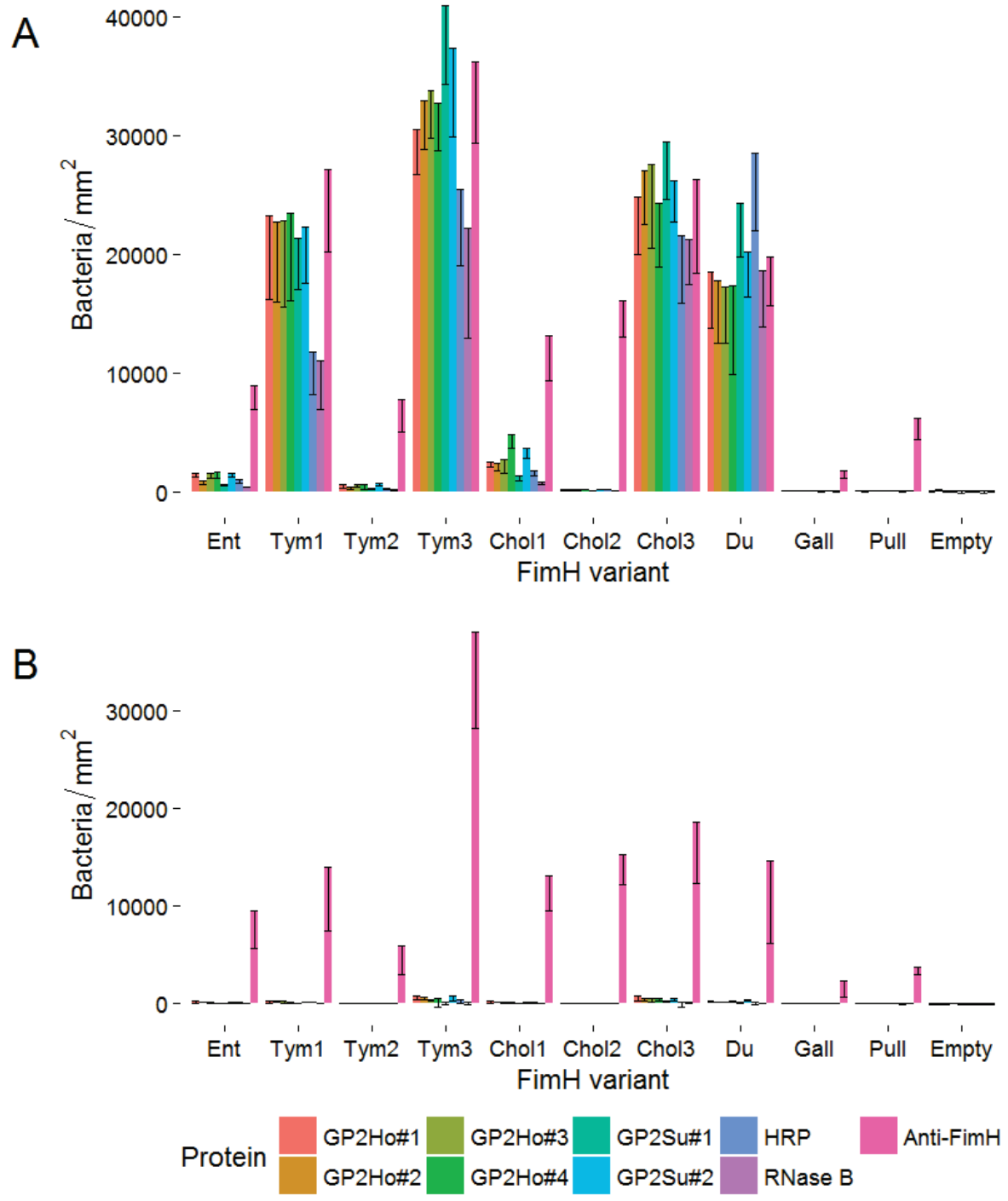

Figure 13. Binding of *Salmonella* to glycoproteins.
The FimH isogenic strains of *S.* Typhimurium strain 5744 with *fimH* gene deletion (Tym5744ΔfimH) were incubated with A) various proteins including human GP2 isoforms 1-4 (GP2Ho#1-4), porcine GP2 isoforms 1-2 (GP2Su#1-2), horseradish peroxidase (HRP), RNase B and anti-FimH antibodies (Anti-FimH); B) identical with A) but with mannose pre-incubation. All isogenic strains expressed FimH as shown by binding to anti-FimH antibodies which were mannose-independent. If one FimH variant bound well to one GP2 isoform then this variant also bound well to other isoforms of the host species. If one FimH variant bound well to human GP2 this variant also bound well to porcine GP2. Binding to all glycoproteins was mannose-dependent (B).

4.5. Generation of GP2-expressing cell lines

In a next step, GP2 was expressed on the surface of epithelial cells: human GP2 isoforms were expressed in the HEp-2 human cell line and porcine GP2 isoforms were expressed in the IPEC-J2 porcine cell line. First, the coding sequences of GP2 isoforms were cloned into plasmids and lentiviruses for each isoform were generated. Before transduction, natural puromycin resistance of cell lines was checked. The results show that optimal puromycin concentration for selection was 1µg/ml for both cell lines. Transduction was done by the spinofection protocol and after successful selection against puromycin, expression of GP2 was first confirmed by indirect immunofluorescence (IIF, Fig. 14). Next, expression of GP2 at the mRNA level was confirmed by RT-PCR (Fig. 15) and RT-qPCR (Fig. 16). All isoforms were expressed at similar levels, as shown in the RT-qPCR results (Fig. 16). Expression of GP2 on protein level was further confirmed by western blotting (Fig. 17) and flow cytometry (Fig. 18). Given the results obtained, it is clear that GP2 is expressed in all cells transduced with various GP2 isoforms in more than 90% of cells for each cell line. No GP2 expression was found in cell lines transduced with “empty” lentiviruses not containing any GP2 sequences.

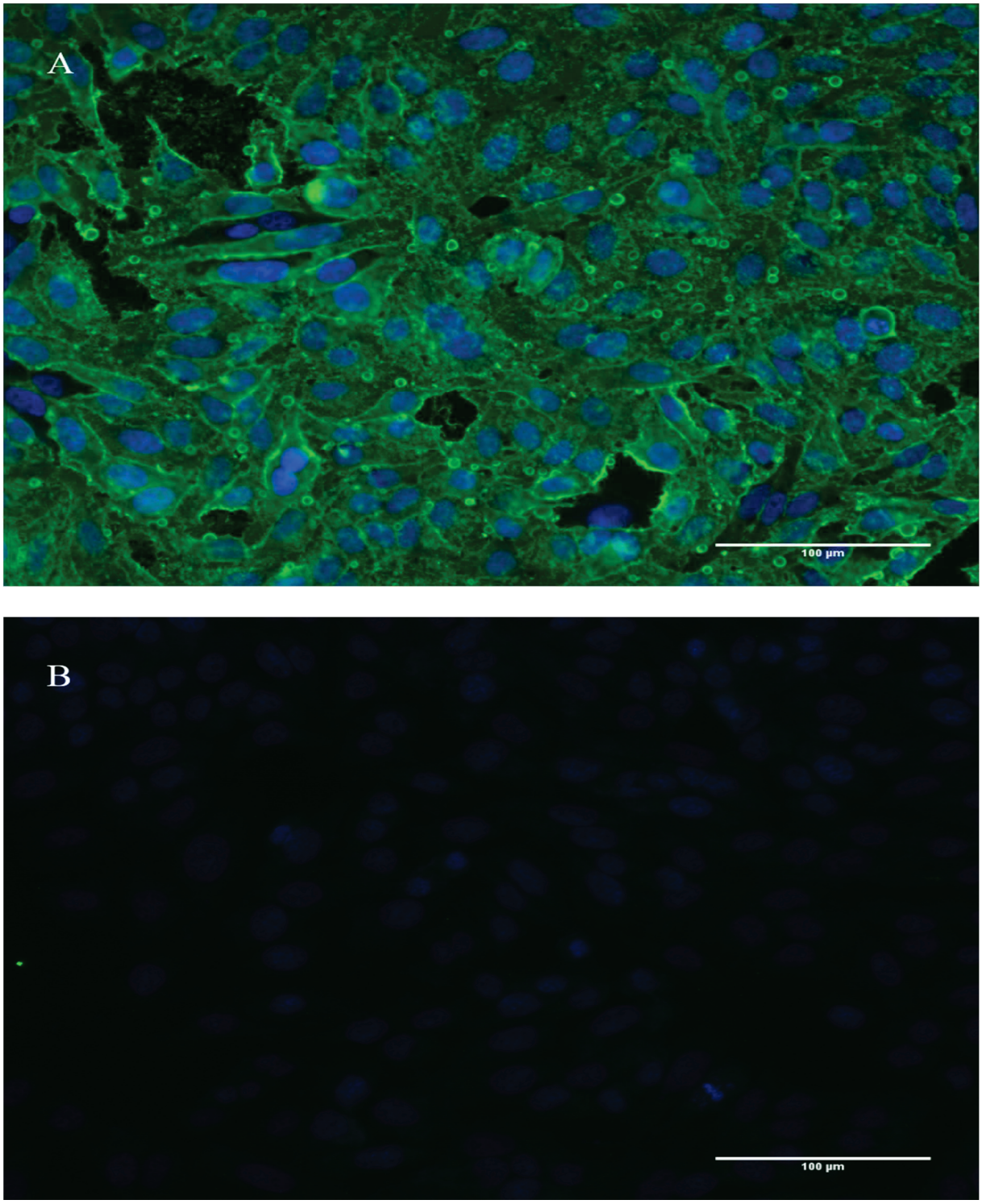

Figure 14. Detection of GP2 expression in epithelial cells by indirect immunofluorescence.

Shown is one example of A) HEp-2 cells expressing human GP2 isoform 2 (HEp-2-GP2Ho#2) and B) HEp-2 cells transduced with an empty vector (HEp-2-pLVX-Empty). GP2 was stained with anti-GP2 antibodies and a secondary antibody conjugated to FITC (green). Cell nuclei were stained with DAPI (blue).

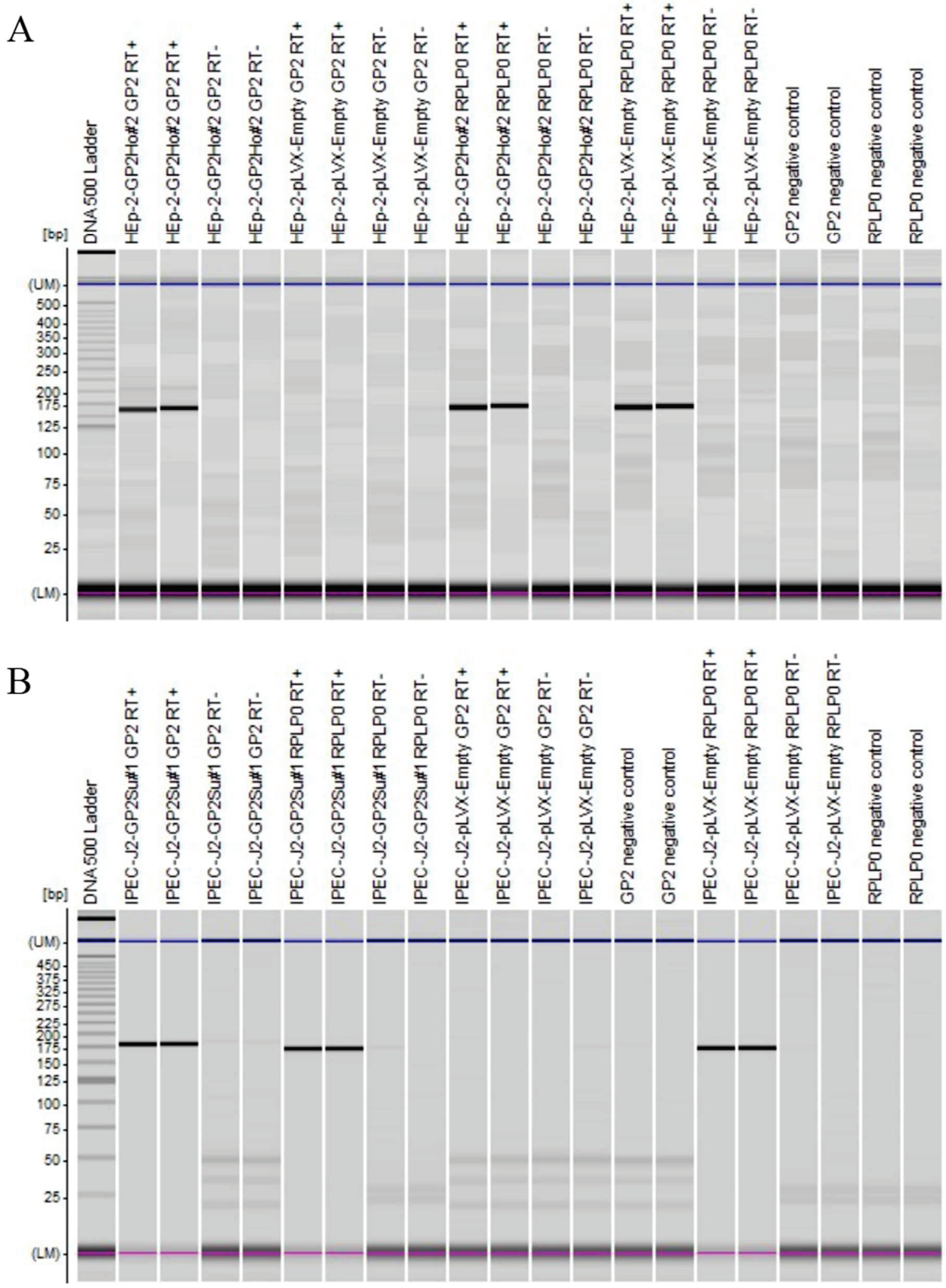

Figure 15. Detection of GP2 gene expression in epithelial cells by RT-PCR.
PCR with primers targeting the GP2 gene (GP2) and the RPLP0 gene (RPLP0) as reference gene with reverse transcriptase positive (RT+) and negative (RT-) reactions as samples was performed. PCR was analysed with the use of a MultiNA Microchip Electrophoresis System. A) lanes 2 and 3: Human GP2 isoform 2 (GP2Ho#2) was expressed in HEp-2 cells, lanes 4-18: controls. B) lanes 2 and 3: porcine GP2 isoform 1 (GP2Su#1) was expressed in IPEC-J2 cells, lanes 4-18: controls.

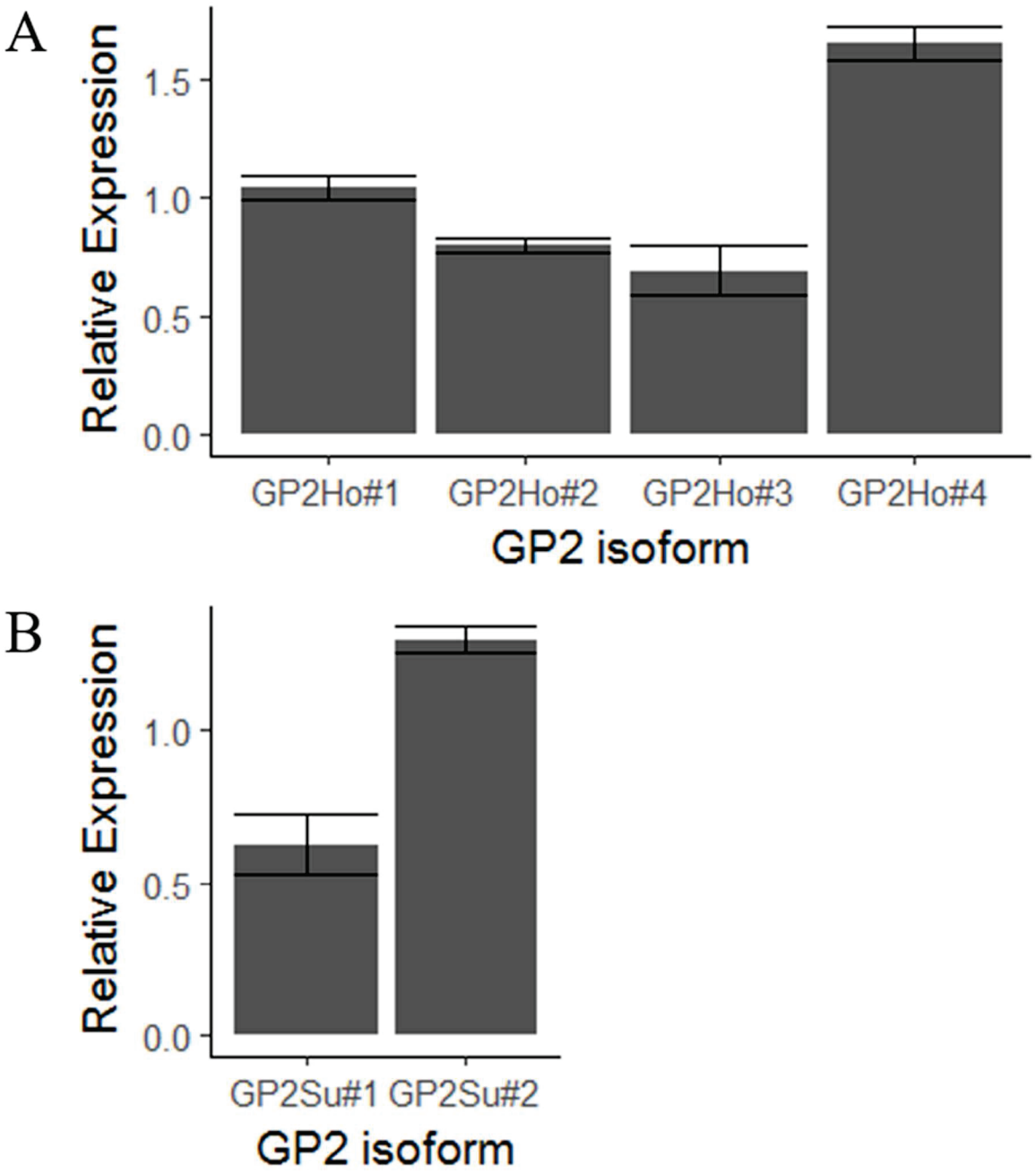

Figure 16. Detection of GP2 gene expression in epithelial cells by RT-qPCR.
RT-qPCR with primers targeting GP2 gene and RPLP0 gene as reference gene was performed. A) Human GP2 isoforms 1-4 (GP2Ho#1-4) were expressed in HEp-2 cells. B) Porcine GP2 isoforms 1 and 2 (GP2Su#1 and 2) were expressed in IPEC-J2 cells. The relative expression was calculated relative to RPLP0 gene expression.

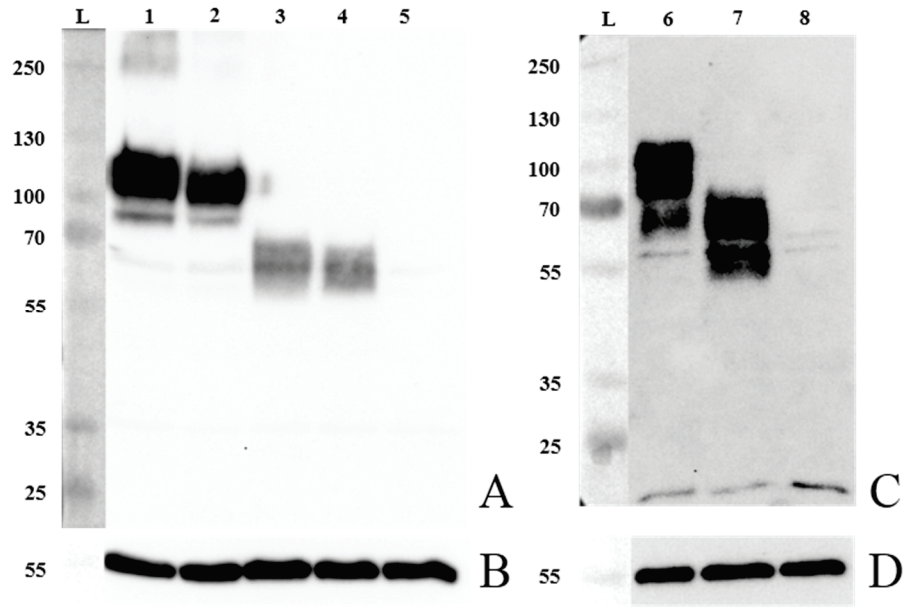

Figure 17. Detection of GP2 expression in epithelial cells by western blotting.
Proteins of cell lysates were separated by SDS-PAGE and GP2 and α tubulin (control) expression was confirmed with A) antibody against human GP2 isoform 1 (anti-GP2Ho#1 antibody) expressed in HEp-2 cells, B, D) antibody against α tubulin and C) antibody against porcine GP2 isoform 2 (anti-GP2Su#2 antibody) expressed in IPEC-J2 cells. Lane L: protein ladder (kDa); lane 1: HEp-2 cells expressing human GP2 isoform 1; lane 2: HEp-2 cells expressing human GP2 isoform 2; lane 3: HEp-2 cells expressing human GP2 isoform 3; lane 4: HEp-2 cells expressing human GP2 isoform 4; lane 5: HEp-2 cells transduced with an empty vector (control); lane 6: IPEC-J2 cells expressing porcine GP2 isoform 1; lane 7: IPEC-J2 cells expressing porcine GP2 isoform 2; lane 8: IPEC-J2 cells transduced with an empty vector (control).

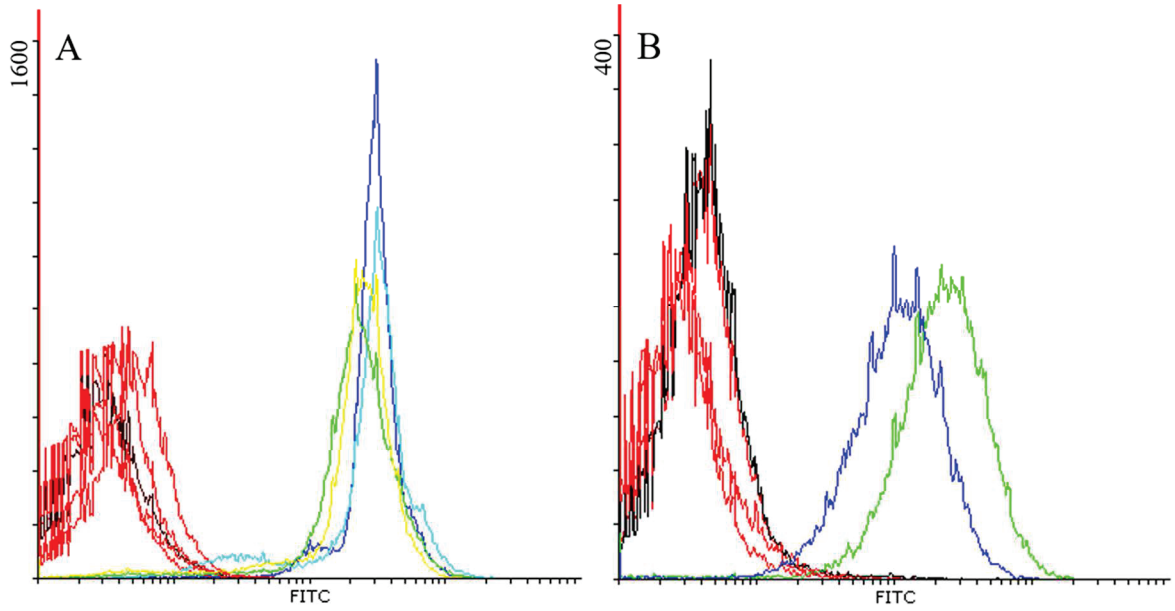

Figure 18. GP2 expressing cells were stained with antibodies and measured by flow cytometry.
A) GP2 expressed in HEp-2 cells and stained with antibody against human GP2 isoform 1 (anti-GP2Ho#1 antibody); blue: HEp-2 cells expressing human GP2 isoform 1; cyan: HEp-2 cells expressing human GP2 isoform 2; green: HEp-2 cells expressing human GP2 isoform 3; yellow: HEp-2 cells expressing human GP2 isoform 4; black: HEp-2 cells transduced with an empty vector; red: control, secondary antibody staining all HEp-2 cell lines. B) GP2 expressed in IPEC-J2 cells and stained with antibodies against porcine GP2 isoforms 1 and 2 (anti-GP2Su#1 and anti-GP2Su#2); green: IPEC-J2 cells expressing porcine GP2 isoform 1; blue: IPEC-J2 cells expressing porcine GP2 isoform 2; black: IPEC-J2 cells transduced with an empty vector; red: control, secondary antibody staining all IPEC-J2 cell lines.

4.6. Cell line adhesion/infection assays

The last step in investigating the interaction of various GP2 isoforms with FimH variants was infection assays with the use of the isogenic model and GP2-transduced cell lines. The VideoScan platform has a built-in module for quantification of bacteria in a cell line infection assay (Fig. 19). The use of an automated microscopy read-out of infection assays for a large number of repeats enabled investigations of all FimH variants on all cell lines expressing GP2 isoforms.

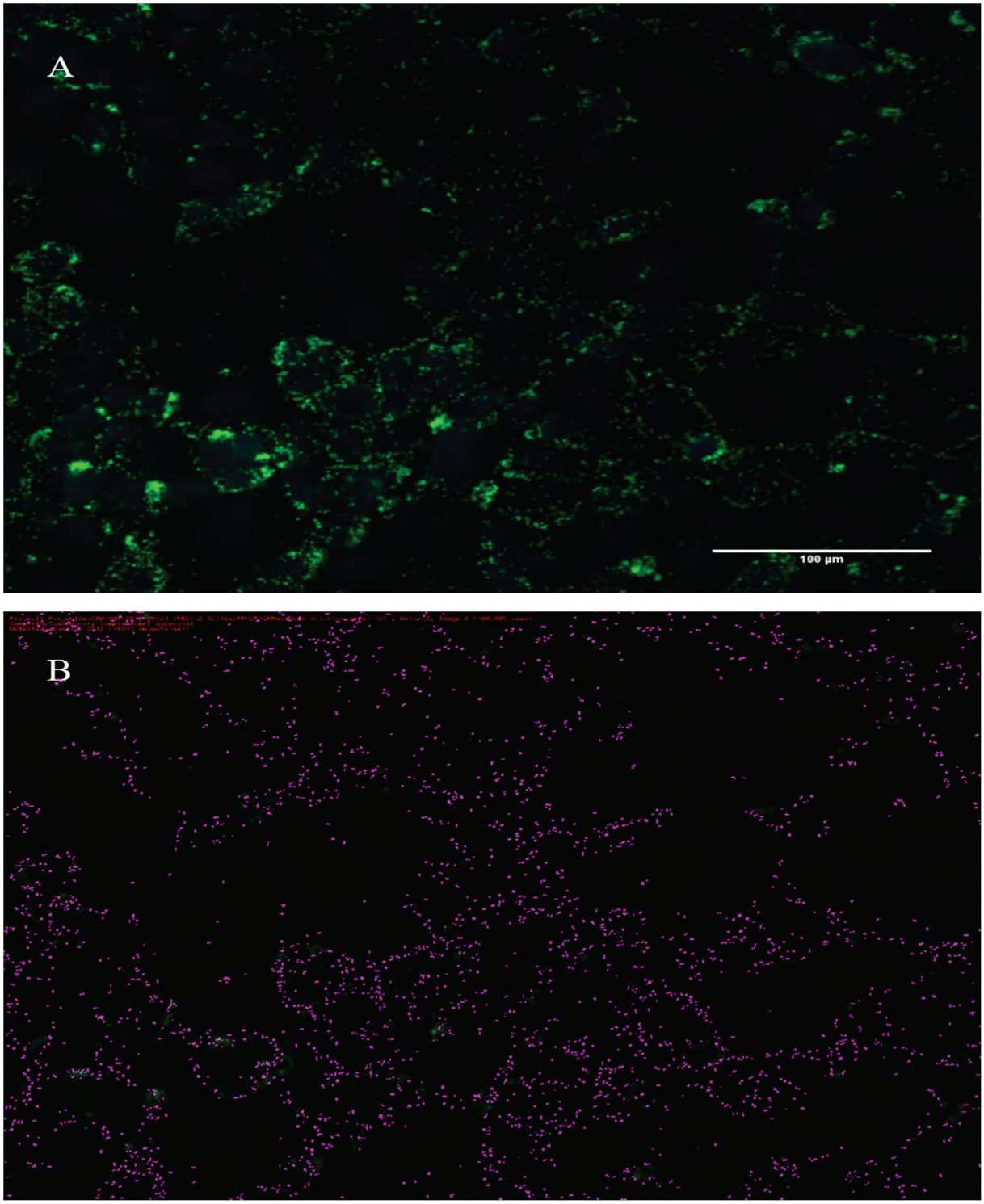

Figure 19. Quantification of *Salmonella* infection by the VideoScan technology.
Exemplary image taken by VideoScan during cell line infection assay. In this case, the IPEC-J2 cell line was seeded in 96-well plates. *Salmonella* Typhimurium SL1344 expressing GFP was incubated with IPEC-J2 cells and non-adherent bacteria were removed by washing. A) After cell nuclei staining with DAPI, images were taken by the VideoScan instrument. Green: GFP-expressing *Salmonella*; blue: DAPI-stained cell nuclei used as guide for focusing on the bacteria. B) Image A processed with the VideoScan module, with counted bacteria marked in magenta colour.

The assay's linear range was investigated in order to confirm the validity of the test. For HEp-2 and IPEC-J2 cell lines, the assay performed well in a wide range of investigated bacterial dilutions (Fig. 20), which makes this assay applicable for *Salmonella* infection assay with cell lines expressing GP2.

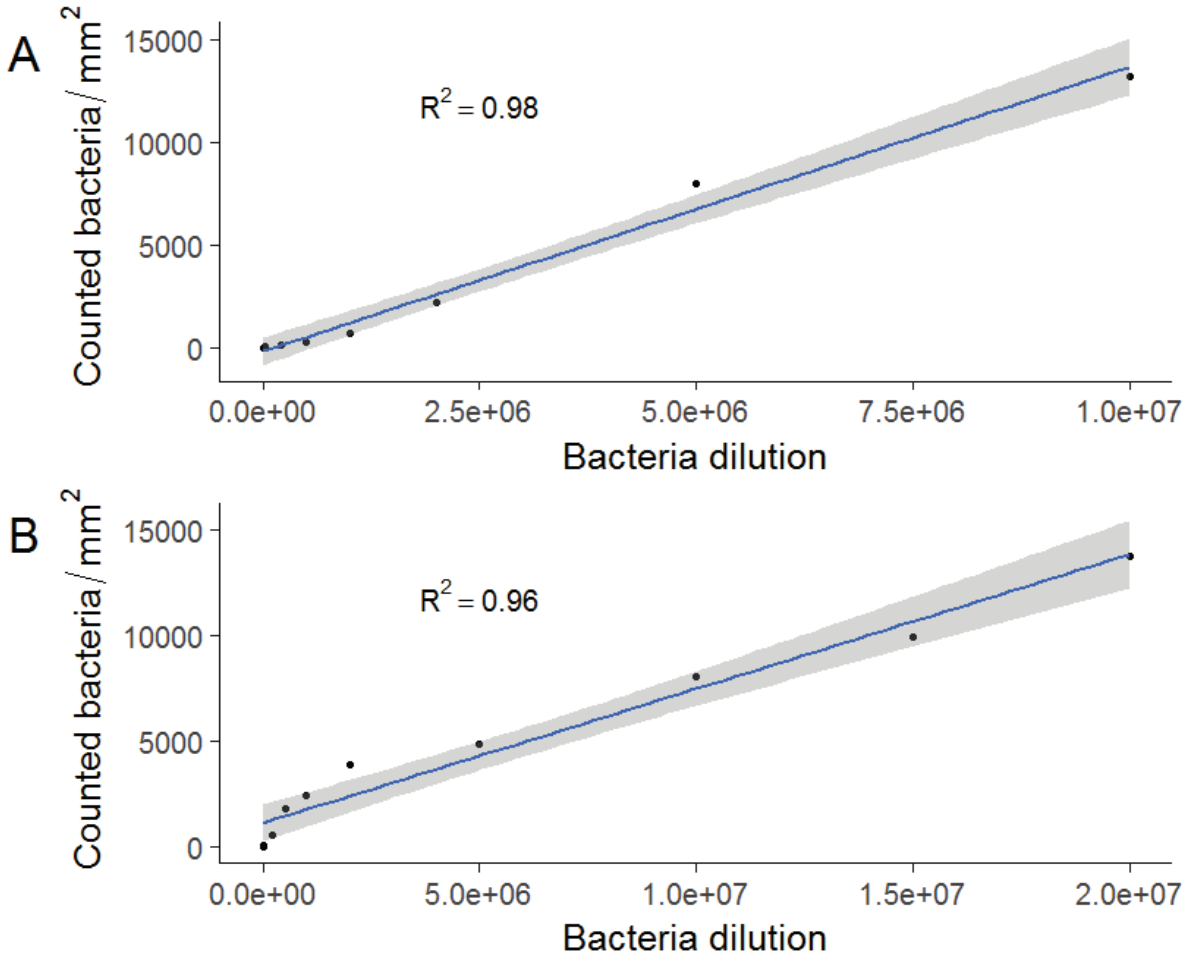

Figure 20. Dilution series of *Salmonella* in cell line infection assays.
This assay was used to determine the linear range of *S*. Typhimurium SL1344 expressing GFP in infection assays with HEp-2 (A) and IPEC-J2 (B) cell lines in a 96-well plate format. Epithelial cells were infected with *S*. Typhimurium SL1344 expressing GFP for 1h in a 96-well format. Images were automatically taken by the VideoScan instrument. Each dot represents a median value from three measurements in triplicates. Blue lines connecting the dots are smoothed trend lines and grey areas around the lines represent the 95% confidence interval of the linear regression.

In the next step, infection assays with cell lines expressing various GP2 isoforms and isogenic *Salmonella* model were carried out. Mannose- sensitive binding was observed in strains carrying Tym3, Chol3, and Du FimH variants (Fig. 21A) to all HEp-2 cell lines and Du FimH variant to IPEC-J2-GP2Su#2 cell line. When compared with cell lines transduced with "empty" lentivirus (negative control) GP2-expression dependent binding was observed only in strains

carrying Tym3, Chol3, and Du FimH variants (Fig. 21B) to HEp-2-GPHo#4 cell line. GP2-expression dependent binding was observed in Chol3 FimH variant to HEp-2-GPHo#2 and HEp-2-GPHo#3 cell line. Du FimH variant bound in a GP2-expression dependent manner to IPEC-J2-GP2Su#2.

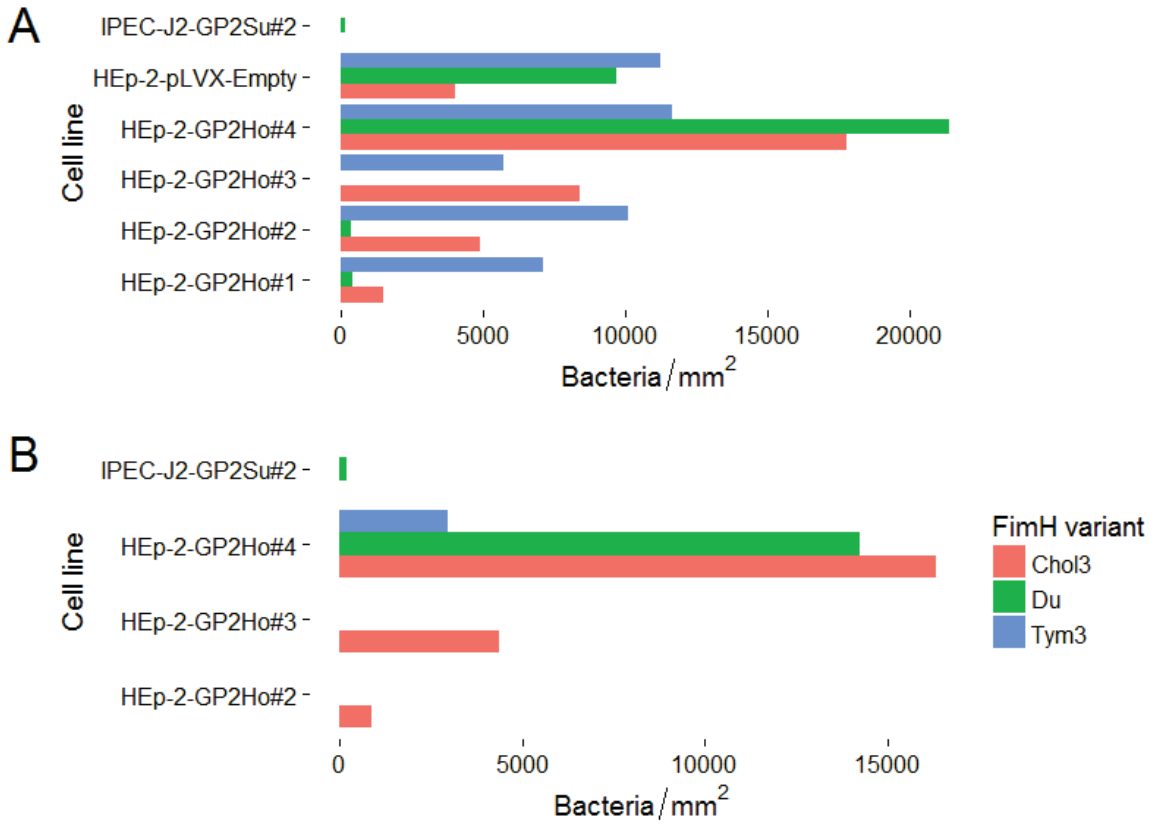

Figure 21. Infection assay with use of Tym 5744ΔfimH isogenic model and cell lines expressing GP2. The FimH isogenic strains of *S*. Typhimurium strain 5744 with *fimH* gene deletion (Tym5744ΔfimH) were incubated with cell lines expressing human GP2 isoforms 1-4 (GP2Ho#1-4) and porcine GP2 isoforms 1-2 (GP2Su#1-2). A) mannose sensitive binding of different FimH variants (see legend: Chol3- *S*. Choleraesuis FimH variant 3; Du- *S*. Dublin FimH variant; Tym3- *S*. Typhimurium FimH variant 3) to cell lines (mannose sensitive binding was calculated by subtracting results of normal assays from results of mannose-blocking assays) B) GP2-dependend binding to different FimH variants (see legend) to cell lines (GP2-dependent binding was calculated by subtracting results from mannose sensitive binding in cell lines with GP2 overexpression from results of mannose sensitive binding in cells lines with no GP2 overexpression).

5. Discussion

The physiological role of GP2 in the pancreas is not elucidated. Due to the high homology of GP2 with the Tham-Horsefall protein (THP, uromodulin), various possible functions of THP were implicated to GP2. According to current findings, GP2 secreted into intestinal lumen serves as an immunomodulatory and physical barrier that sterically hinders binding of bacteria to host cells (Yu and Lowe 2009; Werner et al. 2012). The discovery of GP2 expression on the surface of M cells added a new possible function of GP2- as a transcytotic receptor for T1F-positive bacteria (Ohno and Hase 2010). It was proposed that various FimH variants could have different affinity to GP2 and these differences are associated with host source of GP2. To test this hypothesis, porcine recombinant GP2 isoforms were expressed. Next, the binding properties of different *Salmonella* FimH variants from various serovars to GP2 of human and porcine origin in static adhesion assay were assessed. Furthermore, a cell line model with GP2 expression was established and the role of FimH variation on adhesion to GP2 in a cell line infection assay was tested.

Recombinant human GP2 isoforms are used in routine diagnosis of inflammatory bowel diseases and are easily available. In order to conduct the study, GP2 from at least two hosts was needed and GP2 from pig was chosen. Human GP2 isoforms were expressed in SF9 expression system and were used previously in a study for investigating the binding of various *E. coli* pathotypes to human GP2 (Schierack et al. 2014). Therefore, porcine GP2 isoforms were also expressed in SF9 cells. Up to now, only one porcine GP2 isoform was reported at the protein level, but two isoforms were predicted in GenBank Gene (Maglott et al. 2005). First, expression of both GP2 isoforms in the porcine pancreas was confirmed and both were expressed in the SF9 expression system. It has been shown that FimH of *E. coli* interacts with the glycan part of GP2, therefore it needed to be confirmed that porcine GP2 expressed in SF9 is glycosylated (Yu and Lowe 2009). Experiments with deglycosylated GP2 confirmed that *Salmonella* FimH binds to the glycan part of GP2 similarly to *E. coli* FimH. Many studies show that the requirement for any protein to be considered as a receptor for T1F is glycosylation (Leusch et al. 1991; Kukkonen et al. 1993; Grzymajło et al. 2013). There is only one protein– plasminogen - that *Salmonella* T1F bind in a non-mannose-sensitive manner (Kukkonen et al. 1998).

FimH variation can have a considerable impact on adhesion to receptors. Therefore, the next step was an investigation of *fimH* gene allelic variation in a collection of 128 *Salmonella* strains gathered for this work. In the collection there are serovars with different host ranges and isolated from different hosts i.e. host-restricted Gall, pig-adapted Chol, cattle-adapted Du, host-

unrestricted Ent- isolated from humans and chicken and host-unrestricted Tym- isolated from humans and pigs. From all *fimH* gene sequences found in the analysed strains, only two *fimH* alleles were not previously reported. Most of Tym isolates contained the Tym1 FimH variant. It has been reported that Tym1 with Val in residue 245 of FimH can be more often found in human isolates than in Tym from an animal source, where Ala is more common (Tym3) (Yue et al. 2015). In contrast, in this study, no porcine isolates with Tym3 FimH were found in strains collected for this study, but there were five of human origin with Tym3 FimH. Chol1 and Tym2 FimH variants were not reported previously and these sequences were also not present in GenBank. It has to be mentioned that Chol1 FimH was found in *S*. Choleraesuis var. Decatur and this strain was isolated from a reptile which might explain the different sequence since other isolates belonged to *S*. Choleraesuis sensu stricto isolated from pigs, wild boars or meat products from these animals. In the case of Du, we found only one FimH variant, that has already been previously reported and it looks that FimH in this serovar is conserved, because we did not find any other Du FimH variant in literature or deposited in GenBank. Ent, Gall and Pull FimH variants found in our strain collection represent the most often found FimH sequences for these serovars/biotypes (Kisiela et al. 2005, 2006).

To further characterise the collection of *Salmonella* strains, T1F expression in these strains was measured by binding of strains to anti-FimH antibodies (anti-FimH static adhesion assay). Most studies, which analysed T1F expression so far, rely on semi-quantitative methods. The studies of Duguid et al. (1958, 1966) confirmed fimbriae expression in more than 80% of 1453 isolates from 149 serovars by the red blood cell agglutination assay and/or electron microscopy. Another study analysed the expression of T1F in Chol and only 4 out of 120 strains expressed T1F (confirmed by yeast agglutination assay and electron microscopy) (Lee and Yeh 2016). The study of Kisiela et al. (2012) used radiolabeling and a calibration curve to quantify bacteria adherent to the anti-FimH antibody. In this thesis, a static adhesion assay that uses an anti-FimH antibody was developed for use with the VideoScan technology. The VideoScan static adhesion assay gave reliable results for a wide range of bacteria inoculum. The VideoScan static adhesion assay provides quantitative results (bacteria/mm^2 bound with anti-FimH antibody), is less time-consuming in comparison to previously used methods (i.e. yeast agglutination and electron microscopy), safer (in comparison to radiolabeling) and do not require the addition of a calibration curve each time the assay is performed. The results of the static adhesion assay show that more than 80% of isolates expressed T1F, similar like in the aforementioned study of Duguid et al. (1966). Lee and Yeh (2016) observed that T1F production in Chol is dependent

on the presence of Gly at residue 63. Results of anti-FimH static adhesion assay show that isolates with Gly and Val in residue 63 expressed T1F and nine Chol strains that did not express T1F, have *fimH* gene deletion, which was the reason for the non-fimbriate phenotype. As mentioned before, the big advantage of T1F expression measurement with VideoScan is the quantification of expression. The results clearly show that T1F expression is serovar-specific and host-range-specific. The higher expression of T1F in *Salmonella* host generalists than in *Salmonella* host specialists could be explained in two ways. Both of them are associated with the different lifestyles of host specialists and generalists. It was shown in many studies that T1F mediate binding to various epithelial cell lines and enterocytes (Aslanzadeh and Paulissen 1990; Ernst et al. 1990; Ewen et al. 1997; Boddicker et al. 2002). Therefore, expression of T1F would benefit host generalists which limit infection to intestinal lumen during pathogenesis and adhesion is an important factor for intestinal colonisation. On the other hand, lower expression of T1F could benefit host specialists in spreading to systemic organs but not for intestinal colonisation. Another explanation is associated with recognition by the immune system. Two studies with Ent and Tym in mice infection models showed that T1F-non expressing mutants of these serovars are more virulent than the wild type strains. The authors of one study conclude that the reticuloendothelial system recognises T1F+ bacteria and leads to sequestration of these bacteria in the liver and spleen (Lockman and Curtiss 1992). The authors of the second study with Ent conclude that T1F+ bacteria adhere to intestinal lumen better than T1F- bacteria and then T1F+ Ent is recognised by the immune system, which leads to local inflammatory response and limits spread outside the intestinal tract (Kuźmińska-Bajor et al. 2015). The virulence of T1F+ and T1F- Chol or Du was not investigated, but lower expression of T1F in host specialists like Chol and Du can lead to the conclusion that lower T1F expression gives an advantage in systemic spread by avoiding recognition by the immune system.

To examine functional properties of FimH adhesin variants, binding of isogenic *Salmonella* models to GP2 in static adhesion assay was measured. First, it was found that Gall and Pull FimH variants do not bind to GP2. This finding is in line with previous works, which identified that Gall and Pull FimHs did not bind to glycoproteins and that this property is mediated by single amino acid substitution in the FimH sequence (Kisiela et al. 2005). Three Chol FimH variants presented no binding (Chol2), low binding (Chol1) and high binding (Chol3) phenotypes in static adhesion assays with GP2. The presence of non-binding phenotype (Chol2) and high binding phenotype (Chol3) in the case of Chol FimH adhesins was also reported previously (Kisiela et al. 2012). Surprisingly, no low binding phenotype of FimH was

previously reported for Chol. The strain with low binding phenotype Chol1 FimH belongs to a different biotype of Chol than the rest of isolates according to the White-Kauffmann-Le Minor scheme and was isolated from reptile (Issenhuth-Jeanjean et al. 2014). Therefore, multilocus sequence typing or next generation sequencing of strains bearing Chol FimH variants could help establish genetic relatedness between these strains and explain whether isolate bearing a Chol1 variant should be considered as *S*. Choleraesuis var. Decatur or *S*. Decatur (Achtman et al. 2012). It was mentioned in the previous paragraph, that it has been proposed that T1F production in Chol is dependent on the presence of Gly in residue 63 (like Chol2), but the results of GP2 static adhesion assay show that both Gly and Val in residue 63 resulted in fimbriated phenotype also in the Tym isogenic model (Lee and Yeh 2016). The binding properties of Tym1, Du and Ent FimH to glycoproteins, that we observed are in agreement with previous results (Grzymajlo et al. 2010). Two FimH variants found in Tym- Tym2 and Tym3, represented low and high binding phenotypes in GP2 static adhesion assay, respectively. Both binding phenotypes were found in the past in Tym, but they were associated with different amino acid substitutions in different loci. This indicates that evolutionary adaption in Tym selects for mutations leading to these two phenotypes in this adhesin (Boddicker et al. 2002).

In next step, FimH-GP2 interaction was investigated in a cell line model. Therefore cell lines expressing GP2 isoforms were generated, and a new adhesion assay protocol and a new VideoScan module for bacteria quantification on epithelial cells were developed. The conventional reference assay to determine the number of bacteria infecting cell lines is the colony forming unit (CFU) determination by plating serial dilutions of bacteria suspensions on LB agar (Lee and Falkow 1990). Other methods include staining of bacteria (GFP, antibody) and manual bacteria counting with fluorescence microscopy or enumeration with flow cytometry (Santos et al. 2013; Knodler et al. 2014). The aforementioned methods are not suitable for cell line infection studies with use of a larger amount of bacterial strains and cell lines. Therefore, the VideoScan technology was used for automated enumeration of bacteria infecting cell lines (Rödiger et al. 2013). This allowed testing all isolates from isogenic *Salmonella* models (11) with all cell lines expressing GP2 isoforms and negative controls (8). The biggest issues in this assay were possible interactions of T1F with other receptors and the interaction of other adhesins to the cells lines. These issues were resolved by adding mannose blocking assay as a control for mannose-dependent binding specific for T1F which included testing of adhesion of bacteria to GP2 non-expressing cell lines. The results of this assay show clearly that *Salmonella* can bind in a T1F-dependent manner on cells not expressing GP2 and

in a T1F-nondependent manner to cell lines expressing and not expressing GP2. This "background" binding was a probable reason that T1F-dependend binding of low-binding phenotype FimHs was not detected. As to why the Tym1 FimH variant did not bind to GP2 expressed on cell lines is a question that probably will remain unanswered. There was another factor influencing binding of T1F to GP2 in different cell lines because adhesion of high-binding phenotype FimHs was not observed in all GP2-expressing cell lines. It is possible that cell lines expressing different GP2 isoforms had altered expression of other receptors that were binding *Salmonella*.

The initial idea behind using GP2-expressing cell lines was to imitate a more natural environment, assess whether GPI-anchored protein and mammalian glycosylation influence GP2 recognition by FimH variants. Unfortunately, it turns out that this model is not the best solution for that kind of investigation. Probably, it would have been better to overexpress and purify GP2 isoforms from mammalian cell lines. This would allow to test GP2 with mammalian glycosylation profile in static adhesion assay and give a more conclusive response to the question of the role of glycosylation on FimH binding. Another factor influencing results of cell line infection assay was a non-equivalent expression of T1F in the isogenic *Salmonella* model. This issue was easy to control in static adhesion assays, but in cell line infection assays, it is impossible to predict whether the lower T1F expression in one strain from an isogenic model leads to higher expression of other virulence factors. This possibility makes it even harder to design an experiment with appropriate negative controls. Still, we were able to observe mannose-dependent and GP2-dependent binding to cell lines in the case of three FimH variants with high binding phenotype. The low binding phenotype was not observed in cell line infection assays. The reason might be due to very small amount of bacteria binding to cell lines in a FimH-dependent manner in comparison to negative controls.

One of the goals of this thesis was to check for interactions between FimH and GP2 that could implicate GP2 in host specificity. Unfortunately, there are no clear indications for a role of any *Salmonella* FimH variant in host specificity, because there were no differences in binding to GP2 isoforms from different species in both static adhesion assays and cell line infection assays. For example, the FimH variants Chol3 and Du from host-associated Chol and Du bound well to GP2 irrespective of host source. These results support the assumption that Du and Chol can use GP2 as receptor during pathogenesis, but it is not specific to the host with which they are associated. In the case of Chol, successful infection of pigs without interaction with GP2 should be also possible, because Chol2 FimH variant did not adhere to GP2 isoforms. The role of GP2

in host generalists (Ent and Tym) infection seems to be also dispensable as there are high and low GP2-binding variants. Tym isolates of human and porcine origin did not contain any unique/ host-related FimH variants giving an advantage in adhesion to human or porcine GP2 isoforms. To summarise, it was found that FimH interaction with GP2 is FimH variant-dependent, but not restricted to FimH variant serovar source. It seems that M cell transcytosis mediated by adhesion to GP2 can be used by both *Salmonella* specialists and generalists.

6. Outlook

The work in this thesis was focused on GP2 as a transcytotic receptor for T1F+ *Salmonella*, but the role of GP2 as an opsonin and physical barrier that sterically hinders binding of bacteria to host cells was not investigated. GP2 is secreted to the intestinal lumen with pancreatic juice and was proposed to work in a similar way like THP in urinary bladder i.e. preventing T1F from binding to receptors expressed on the surface of enterocytes. Moreover, it has been shown that GP2 can bind different bacteria, but the nature of the binding is not known (Juste et al. 2014). Preliminary results from our group show that GP2 can be found on the surface of a wide range of bacteria in faeces (e.g. Bacteroides, Ruminococcaceae, Clostridiales, Streptococcus and Prevotella). It might be that GP2 acts similarly like sIgA and prevents bacterial binding in the gastrointestinal tract. GP2 knockout mice were healthy, but it was not checked whether there were any differences in the microbiome of these bacteria and whether the lack of GP2 secreted by pancreas causes higher susceptibility to bacterial infections (Yu et al. 2004). It is important to mention that GP2 is an autoantigen of antibodies in Crohn's disease and primary sclerosing cholangitis (PSC) (Roggenbuck et al. 2009; Jendrek et al. 2017). It has been shown that patients with Crohn's disease have reduced microbiota diversity and expansion of Proteobacteria (Baumgart and Sandborn 2012). It can be only speculated, but increased amount of Proteobacteria can lead to increased inflammatory response against these bacteria and as a side effect, there is a higher chance of getting antibodies against GP2. PSC is a chronic cholestatic liver disease that leads to liver fibrosis and cirrhosis (Sabino et al. 2016). Lower secretion of bile in PSC leads to changes in microbiota diversity and similar to Crohn's disease, dysbiosis can lead to the rise of anti-GP2 antibodies in PSC patients. The etiology of both aforementioned diseases is not clear, but further investigation of GP2's role on microbiota in healthy patients would shed more light on the role of GP2 in the pathogenesis of Crohn's disease and PSC. As GP2 can bind bacteria, it is possible that GP2 can be recognised by phagocytic cells and act as an opsonin. It has been shown that preincubation of monocytes with GP2 increases antigen uptake (Werner et al. 2012). Moreover, it has been shown that GP2 alone can bind to scavenger receptors expressed on endothelial cells I (SREC-I), which is expressed by DC (Hölzl et al. 2011). To elucidate the possible contribution of GP2 during bacterial phagocytosis, experiments with secretory GP2 and T1F expressing-bacteria should be conducted.

7. Conclusions

1. Expression of porcine GP2 isoforms in SF9 cells results in glycosylated and functional proteins, which bind T1F+ *Salmonella.*
2. Expression of T1F is higher in *Salmonella* generalists than in *Salmonella* specialists.
3. Lentiviral transduction of GP2 isoforms allows generating stable cell lines expressing GP2.
4. Binding of *Salmonella* to GP2 isoforms of human and porcine origin is FimH variant dependent, but there are no signs of host-specific binding of FimH variants from various serovars to GP2 isoforms.

Zusammenfassung

Expression und Charakterisierung von pankreatischen sekretorischen Granulamembran Glykoprotein GP2 von tierischen Ursprung.

GP2 wird spezifisch auf der Oberfläche von M-Zellen exprimiert und ist an der Aufnahme von Typ-1-Fimbrien (T1F) -positiven Bakterien und dem Transport zu dem darunter liegenden Mukosagewebe beteiligt. Diesem Prozess folgt die lokale und systemische Verbreitung von Bakterien. T1F zählen zu den häufigsten adhäsiven Organellen in der Familie der Enterobacteriaceae und sind wichtige Adhäsionsfaktoren in der intestinalen Pathogenität von *Salmonella*. Das FimH-Protein befindet sich an der Spitze des T1F-Schafts und interagiert direkt mit Rezeptoren. Mehrere Studien haben gezeigt, dass sich Serotyp-assoziierte FimH-Varianten bei der Rezeptorerkennung erheblich unterscheiden können und dies zu einer Veränderung im Verlauf der Infektion führen kann. Daher war das Ziel dieser Studie, die Rolle der FimH-Sequenzvariation bei der Bindung an GP2-Isoformen von verschiedenen Wirten zu untersuchen. Porcine GP2-Isoformen wurden in SF9-Zellen exprimiert. *FimH*-Gensequenzen aus 128 *Salmonella*-Isolaten von fünf Serovaren von Mensch, Rind, Schwein und Huhn wurden bestimmt. Die Expression des FimH-Proteins in diesen Isolaten wurde mit einem statischen Anti-FimH-Antikörper-Adhäsionstest geprüft. Es wurde ein isogenes System mit einem *Salmonella*-Stamm generiert. Eine *fimH*-Deletionsmutante wurde erzeugt und zehn Plasmide, die *fimH*-Varianten enthielten, wurden in diese Mutante transformiert. Statische Adhäsionsassays mit vier humanen und zwei porcinen GP2-Isoformen, HRP, RNase B und Anti-FimH-Antikörper wurden durchgeführt. HEp-2- und IPEC-J2-Zellen, die humane und porcine GP2-Isoformen exprimieren, wurden unter Verwendung eines lentiviralen Expressionssystems erzeugt. Als nächstes wurden Infektionsassays mit diesen Zelllinien und dem isogenen *Salmonella*-Modell durchgeführt. Der Vergleich von *fimH*-Gensequenzen aus *Salmonella*-Isolaten ergab 11 Sequenzvarianten mit 18 variablen Stellen. Die T1F-Expression war abhängig vom Serotyp und der Isolationsquelle. Eine Cluster-Analyse ergab, dass die T1F-Expression in Stämmen von Nicht-Wirts-beschränkten im Vergleich zu Wirts-assoziierten oder Wirts-beschränkten Serovaren höher ist. Im isogenen *Salmonella*-Modell war die Bindung an GP2-Isoformen und an Standardproteine FimH-Varianten- und mannoseabhängig und glukoseunabhängig. Ein stark bindender Phänotyp wurde bei vier FimH-Varianten beobachtet, ein niedrig bindender bei drei Varianten und keine Bindung bei den anderen drei Varianten. In Zelllinien-Infektionsassays wurde eine Bindung abhängig von der GP2-Expression der Zelllinie im Fall von drei FimH-Varianten des stark bindenden Phänotyps beobachtet. Es wurde keine wirtsspezifische Bindung des FimH-Adhäsins an GP2 in statischen Adhäsionsassays und

Zelllinien-Infektionsassays beobachtet. Die Adhäsion von FimH-positiven Bakterien an GP2 könnte ein zusätzlicher Eintrittsweg für die Invasion von *Salmonella* sein.

Summary

Expression and characterization of pancreatic secretory granule membrane major glycoprotein GP2 of animal origin

GP2 is specifically expressed on the surface of M cells and takes part in the uptake of type 1 fimbriae (T1F)-positive bacteria and transport to underlying mucosal immune tissues. This process continues with the local and systemic dissemination of bacteria. T1F are one of the most common adhesive organelles in the family of Enterobacteriaceae and important adhesion factors in *Salmonella* intestinal pathogenicity. The FimH protein is located on top of the T1F shaft and directly interacts with receptors. Several studies have shown that serovar-associated FimH variants can differ significantly in receptor recognition and this can lead to a change in course of infection. Therefore, the aim of this study was to investigate the role of FimH sequence variation on binding to GP2 isoforms from various hosts. Porcine GP2 isoforms were expressed in SF9 cells. *FimH* gene sequences from 128 *Salmonella* isolates from five serovars of human, cattle, swine and chicken origin were determined. Expression of FimH protein in these isolates was tested with a static anti-FimH antibody adhesion assay. An isogenic system with one *Salmonella* strain was generated. A *fimH* deletion mutant was created and ten plasmids containing *fimH* variants were transformed into this mutant. Static adhesion assays with four human and two porcine GP2 isoforms, HRP, RNase B and anti-FimH antibody were performed. HEp-2 and IPEC-J2 cells expressing human and porcine GP2 isoforms were generated with the use of lentiviral expression system. Next, infection assays with these cell lines and an isogenic *Salmonella* model were carried out. Comparison of *fimH* gene sequences from *Salmonella* isolates revealed 11 sequence variants with 18 variable sites. T1F expression was dependent on serovar and isolation source. Cluster analysis revealed, that T1F expression is higher in strains from host-unrestricted compared to host-associated or host-restricted serovars. In the isogenic *Salmonella* model, binding to GP2 isoforms and standard proteins was FimH-variant and mannose-dependent, and glucose independent. The high binding phenotype was observed in the case of four FimH variants, low in case of three variants and no binding in case of three variants. In cell line infection assays, GP2-cell line expression dependent binding was observed in the case of three FimH variants with high binding phenotype. No host-specific binding of FimH adhesins to GP2 was observed in static adhesion assays and cell line infection assays. Adhesion of FimH-positive bacteria to GP2 might be an additional entry route for *Salmonella* invasion.

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Publications:

1. Durkalec M, **Kolenda R**, Owczarek T, et al (2017) Expression of metallothionein in the liver and kidneys of the red deer (*Cervus elaphus* L.) from an industrial metal smelting area of Poland. Ecotoxicol Environ Saf 137:121–129. doi: 10.1016/j.ecoenv.2016.11.018
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5. **Kolenda R**, Schierack P, Zieba F, et al (2015) First molecular characterization of *Sarcocystis tenella* in Tatra chamois (*Rupicapra rupicapra tatrica*) in Poland. Parasitol Res 114:3885–3892. doi: 10.1007/s00436-015-4619-4
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7. Schierack P, Rödiger S, **Kolenda R**, et al (2014) Species-specific and pathotype-specific binding of bacteria to zymogen granule membrane glycoprotein 2 (GP2). Gut 64:517–519. doi: 10.1136/gutjnl-2014-307854

Poster abstracts and scientific talks:

1. **Kolenda R**, Roggenbuck D, Rödiger S, Reinhold D, Szabo I, Bühling A, Schierack P (2014) Zymogen granule membrane glycoprotein 2 (GP2) binds to specific bacterial species, pathotypes and serotypes. Die Tagung der DVG-Fachgruppe Bakteriologie und Mykologie, Freising (poster)
2. Burdukiewicz M, **Kolenda R**, Schierack P (2014) Prevalence of Shiga toxin-producing, enterohemorrhagic and enteropathogenic *Escherichia coli*- a systematic review. 4th Joint Conference of DGHM & VAAM, Dresden (poster)
3. **Kolenda R**, Burdukiewicz M, Rödiger S, Schierack P (2014) Prevalence of F5 fimbriae in *Escherichia coli* isolated from diarrheic and healthy calves since 1976- a meta-analytical approach. 4th Joint Conference of DGHM & VAAM, Dresden (poster)
4. **Kolenda R**, Burdukiewicz M, Szabo I, Orłowska A, Nitschke J, Böhm A, Schierack P (2016) Influence of *Salmonella* FimH sequence variation on binding to pancreatic secretory granule membrane major glycoprotein GP2 of human and porcine origin. 5th ASM Conference on *Salmonella*, Potsdam (poster)
5. Burdukiewicz M, Mikołajczyk A, Szabo I, Nitschke J, Böhm A, Schierack P, **Kolenda R** (2016) Automated enumeration of *Salmonella* spp. in cell line infection assays and screening of cell line infectivity among various *Salmonella* isolates. 5th ASM Conference on *Salmonella*, Potsdam (poster)

6. **Kolenda R**, Burdukiewicz M, Schiebel J, Szabo I, Orłowska A, Nitschke J, Böhm A, Schierack P (2016) Adhesion of *Salmonella* to pancreatic secretory granule membrane major glycoprotein GP2 depends on FimH sequence variation. 5th Joint Conference of DGHM & VAAM, Würzburg (talk)
7. Burdukiewicz M, Mikołajczyk A, Szabo I, Nitschke J, Böhm A, Schierack P, **Kolenda R** (2017) Screening of cell line infectivity among various *Salmonella* isolates of human, cattle, swine and chicken origin. 5th Joint Conference of DGHM & VAAM, Würzburg (poster)

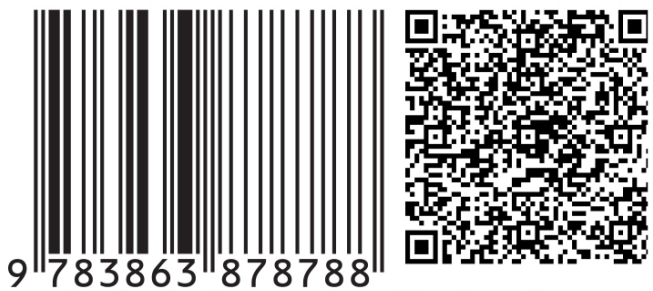
Danksagung

I would like to thank everybody who helped me with my dissertation.

Sebstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 06.02.2018 Rafał Kolenda



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49,90 Euro | ISBN: 978-3-86387-878-8