The zoonotic pathogen *Helicobacter heilmannii* from feline origin: aspects of virulence and gastric colonization

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List of abbreviations

°C	degrees Celcius
µg	microgram
µL	microliter
µm	micrometer
aa	amino acids
Ala	alanine
AlpA	adherence-associated lipoprotein A
AlpB	adherence-associated lipoprotein B
ANI	average nucleotide identity
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BabA	blood group antigen-binding adhesin A
BALB/c	an albino, laboratory-bred strain of the house mouse
BLAST	Basic Local Alignment Search Tool
BLASTP	protein BLAST
bp	base pairs
BP	bipolar
C.	*Campylobacter*
C57BL/6	a black-coated, common inbred strain of laboratory mice
cag PAI	cytotoxin-associated gene pathogenicity island
CagA	cytotoxin-associated protein
cDNA	complementary DNA
Cl^-	chlorine
CLR(s)	C-type lectin receptor(s)
cm	centimeter
CO_2	carbon dioxide
Ct	threshold cycle
DC(s)	dendritic cell(s)
DDH	DNA-DNA hybridization
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay

EMBL	European Molecular Biology Laboratory
FaaA	flagella-associated autotransporter
FBS	fetal bovine serum
FCM	flow cytometry
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FlaA	flagellin subunit A
FlaB	flagellin subunit B
FW	forward
g	gravitational acceleration
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GGT	γ-glutamyltranspeptidase
Glu	glutamic acid
gyrB	gyrase subunit B
h	hours
H&E	haematoxylin/eosin
H.	*Helicobacter*
H^+/K^+ ATPase	hydrogen potassium ATPase
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
Hof	*Helicobacter* OMP family
Hom	*Helicobacter* outer membrane
Hop	*Helicobacter* outer membrane porin
Hor	Hop-related
HPRT	hypoxanthine guanine phosphoribosyltransferase
hrgA	endonuclease replacing gene
HRP	horseradish peroxidase
HSP60	heat-shock protein 60
IceA	induced by contact with epithelium
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Ile	isoleucine
ImaA	immune-modulating autotransporter

Imp/OstA	increased membrane permeability/organic solvent tolerance
iTOL	interactive Tree of Life
Kb	kilo base pairs
kDa	kilodalton
Ki67	a cellular marker for proliferation, also known as MKI67
kV	kilovolt
L	liter
LabA	lacdiNAc-specific adhesin
Le^b	Lewis b blood group antigens
Le^x	sialyl-dimeric-Lewis x glycosphingolipid
log	logarithm
LPS	lipopolysaccharide
LpxR	lipid A deacylase
M	molar
MALT	mucosa-associated lymphoid tissue
Mb	mega base pairs
MFP	membrane fusion protein
mg	milligram
MHC	major histocompatibility complex
min	minutes
mL	milliliter
mM	millimolar
MP	monopolar
MUC	human mucin
Muc	murine mucin
N_2	nitrogen gas
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NapA	neutrophil-activating protein
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHPH	non-*H. pylori Helicobacter*
NLR(s)	NOD-like receptor(s)
nm	nanometer
NOD	nucleotide-binding oligomerization domain

O_2	oxygen
OD	optical density
OipA	outer inflammatory protein A
OMF	outer membrane factor
OMP(s)	outer membrane protein(s)
OMPLA	outer membrane phospholipase
OMR	outer membrane receptor
ORF(s)	open reading frame(s)
PagL	outer membrane-localized lipid A 3-O-deacylase
PAMP(s)	pathogen-associated molecular pattern(s)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	measure of acidity or basicity
Pro	proline
PRR(s)	pattern recognition receptor(s)
RIG-I	retinoic acid-inducible gene-I
RLR(s)	RIG-I-like receptor(s) (RLRs)
R-M system	restriction-modification system
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	real-time PCR
RV	reverse
s	seconds
s.l.	sensu lato
s.s.	sensu stricto
SabA	sialic acid-binding adhesin
SEM	scanning electron microscopy
SfpA	systemic factor protein A
SJL	Swiss Jim Lambert inbred strain of laboratory mice
SPF	specific pathogen-free
SspA	salt-stress induced outer membrane protein
T4SS	type IV secretion system
TEM	transmission electron microscopy

TGF	transforming growth factor
Th	T helper
TlpB	methyl-accepting chemotaxis protein
TLR(s)	Toll-like receptor(s)
TNF	tumor necrosis factor
Treg(s)	regulatory T cell(s)
TTC	triphenyltetrazolium chloride
Tyr	tyrosine
U/L	units per liter
U/ml	units per milliliter
ureAB	urease subunit A/B
v/v	volume/volume
VacA	vacuolating cytotoxin
VEGF	vascular endothelial growth factor
VlpC	VacA-like protein C
vol/vol	volume per volume
wt/vol	weight per volume
λ	wavelength

GENERAL INTRODUCTION

1. *Helicobacter* species that colonize the human stomach

1.1 *Helicobacter pylori*

Helicobacter (H.) pylori is a slightly-curved, Gram-negative and microaerophilic gastric bacterium. It was first cultivated in 1982 by Warren and Marshall (Sokic-Milutinovic et al., 2015; Warren and Marshall, 1983). Large epidemiological studies have shown that this microorganism is undisputedly related with the pathogenesis of gastric and duodenal ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma (Parsonnet et al., 1991; Talley et al., 1991; Wang et al., 2007). So far, *H. pylori* is the only bacterium that is classified as a human carcinogen by the International Agency for Research on Cancer (Talley et al., 1991). *H. pylori* is a bacterium that is adapted to the human host. This agent has only occasionally been described in animals, including cats, dogs, sheep and stripe-faced dunnarts (Dore et al., 1999; Every et al., 2011; Fox, 1995; Handt et al., 1995; Neiger and Simpson, 2000; Thomson et al., 1994). Probably, most of these infections in animals are related to anecdotic anthroponoses. It can also not be excluded that in some of these cases animals were not infected with *H. pylori* itself, but with a *H. pylori*-like organism, especially when the agent was not isolated (Haesebrouck et al., 2009).

1.1.1 Prevalence in humans

H. pylori is highly adapted to the harsh environment of the human stomach and this bacterium has succeeded to colonize more than 50% of the human population worldwide (Keilberg and Ottemann, 2016). The exact transmission route of *H. pylori* from person to person is not completely unraveled yet, but this bacterium is often acquired in childhood most likely via the oral–oral route (Brown, 2000; Keilberg and Ottemann, 2016). Although the prevalence of *H. pylori* infections in childhood is low in developed countries, in developing countries most children are infected by the age of 10 years (Bardhan, 1997; Go, 2002; Queiroz et al., 2013). Once *H. pylori* has colonized the stomach, in the absence of proper antimicrobial treatment, the bacterium usually persists in its host for a lifetime (Cho and Blaser, 2012; Keilberg and Ottemann, 2016). In adults, the prevalence of *H. pylori* ranges from 20 to 50% in developed countries and to more than 80% in developing countries (Kusters et al., 2006; Pounder and Ng, 1995).

1.1.2 Clinical significance in humans

The outcome of an *H. pylori* infection depends not only on the characteristics of the infecting strains, but also on predisposing factors of the host and environmental factors (Sokic-Milutinovic et al., 2015). Most *H. pylori* infections remain asymptomatic or progress to a mild gastritis without complaints (Keilberg and Ottemann, 2016; Suerbaum and Josenhans, 2007). In 10-20% of infected people, the *H. pylori*-induced gastritis evolves to gastric disease, ranging from peptic and duodenal ulcers to MALT lymphoma and gastric adenocarcinoma (Atherton and Blaser, 2009; Suerbaum and Michetti, 2002). The disease outcome is related to the pattern of the *H. pylori*-induced gastritis: a chronic corpus-predominant or a multifocal atrophic gastritis leads to an increased risk of gastric ulcers and cancer, while an antrum-predominant gastritis leads to the development of duodenal ulcers (Correa and Piazuelo, 2012). Gastric cancer is the third most common cancer among males and the fifth most common cancer among females. While the incidence of gastric cancer is declining in developed countries, it is increasing in developing countries where the overall burden of the disease is becoming more and more important (Mbulaiteye et al., 2009; Sokic-Milutinovic et al., 2014). Also in young children, an *H. pylori* infection has more diverse clinical outcomes in developing countries than in developed countries. In these poor resource settings, the infection is more often accompanied by malnutrition and by parasitic and other enteropathogenic infections. Therefore, the clinical outcomes include not only iron deficiency but also growth retardation, diarrheal disease, malabsorption and impaired cognitive function (Queiroz et al., 2013).

1.2 Non-*Helicobacter pylori Helicobacter* species

1.2.1 Overview and nomenclature

Besides *H. pylori*, other spiral-shaped helicobacters, naturally colonizing the stomach of animals, also have been detected in the stomach of humans. These non-*H. pylori* helicobacters were first described in 1987 and were originally referred to as "*Gastrospirillum hominis*" (Dent et al., 1987; McNulty et al., 1989). By sequencing of the 16S rRNA gene, it was shown that these gastric microorganisms belong to the genus *Helicobacter*, and the name was changed into "*Helicobacter heilmannii*" (Solnick et al., 1993). Further analysis of the 16S and the 23S rRNA genes resulted in a sub-classification into "*H. heilmannii*" type 1 and "*H. heilmannii*" type 2, that differ by more than 3% in these nucleotide sequences (Dewhirst et al., 2005). Nowadays, it is known that "*H. heilmannii*" type 1 actually consists of one *Helicobacter* species, *H. suis*, that naturally colonizes the stomach of pigs. This porcine gastric bacterium

was first designated "*Gastrospirillum suis*" (Mendes et al., 1990; Quieroz et al.,1990), but sequencing of the 16S rRNA gene, fluorescent in situ hybridization (FISH) and electron microscopy has shown that this organism belongs to a new taxon of the genus *Helicobacter* (De Groote et al., 1999a; O'Rourke et al., 2004b). *H. suis* has been successfully cultured *in vitro* since 2008 (Baele et al., 2008a; Haesebrouck et al., 2009).

"*H. heilmannii*" type 2 represents a group of several different species originating from the stomach of cats and dogs. At the onset of this thesis, *H. felis, H. bizzozeronii, H. salomonis, H. heilmannii, H. cynogastricus* and *H. baculiformis* were classified within the "*H. heilmannii*" type 2 group (Baele et al., 2008b; Haesebrouck et al., 2009; O'Rourke et al., 2004b; Smet et al., 2012; Van den Bulck et al., 2006). These feline and canine species can be distinguished by sequencing of the HSP60 gene, the urease A and B genes, and the *gyrB* gene (Hannula and Hänninen, 2007; Mikkonen et al., 2004; Neiger et al., 1998; O'Rourke et al., 2004b). To further avoid confusion concerning the nomenclature in literature, the term *H. heilmannii* sensu stricto (s.s.) was introduced to refer to the *H. heilmannii* bacterium identified to the species level. To refer to the whole group of non-*H. pylori Helicobacter* (NHPH) species detected in the stomach of humans or animals, the term *H. heilmannii* sensu lato (s.l.) can be used (Haesebrouck et al., 2011).

Besides canine, feline and porcine *Helicobacter* species, also other animal-associated gastric helicobacters have been described, including *H. acinonychis* from wild felines (Dailidiene et al., 2004; Eaton et al., 1993), *H. cetorum* from dolphin and whales (Harper et al., 2002), *H. mustelae* from ferrets (Fox et al., 1989; Fox et al., 1990) and *Candidatus* H. bovis from cattle. This latter species has not been cultivated *in vitro* so far (De Groote et al., 1999b).

Before the start of this PhD research, 13 gastric *Helicobacter* species had been identified (see **Table 1**). Several of these species are capable of causing disease in humans and thus are of zoonotic importance (Flahou et al., 2016). Remarkably, they all are characterized by an extremely fastidious nature, which to date has resulted in only a low number of *in vitro* isolates available worldwide (Haesebrouck et al., 2009).

Table 1. Gastric *Helicobacter* species and their hosts (adapted from Flahou et al., 2016)

Taxon	Natural Host	Zoonotic potential
'*Candidatus* H. bovis'	Cattle	Yes
'*Candidatus* H. homininae'	Chimpanzee, gorilla	Unknown
H. acinonychis	Cheetah, tiger, lion	Unknown
H. baculiformis	Cat	Unknown
H. bizzozeronii	Cat, dog	Yes
H. cetorum	Whale, dolphin	Unknown
H. cynogastricus	Dog	Unknown
H. felis	Dog, cat, cheetah, New Guinea wild dog, rabbit	Yes
H. heilmannii	Dog, cat, cheetah, bobcat, tiger, lynx, leopard, puma	Yes
H. mustelae	Ferret	Unknown
H. pylori	Human	NA
H. salomonis	Cat, dog, rabbit	Yes
H. suis	Pig, mandrill monkey, rhesus macaque, crab-eating macaque	Yes

1.2.2 Prevalence in humans

Gastric NHPH species have been associated with gastritis, gastric and duodenal ulcers, and low-grade MALT lymphoma in humans (Debongnie et al., 1995; Debongnie et al., 1998; Haesebrouck et al., 2009; Morgner et al., 1995). Generally, these organisms have microscopically been detected in 0.2–6% of gastric biopsy samples from humans with severe gastric complaints. Due to the lack of specific diagnostic tests, this is most probably an underestimation of their true prevalence. It also cannot be excluded that NHPH infections sometimes remain unapparent or only cause mild disease signs which are often not thoroughly examined (Flahou et al., 2016; Haesebrouck et al., 2009).

In Western countries and Japan, different prevalences of *H. heilmannii* s.l. have been described in gastric biopsies from human patients (both adults and children) with gastric complaints, varying from lower than 1% to 2% (Blaecher et al., 2013; Boyanova et al., 2007; Ierardi et al., 2001; Iwanczak et al., 2012; Okiyama et al., 2005). In developing countries, the prevalence is estimated to be higher. This is illustrated in reports from China and Thailand, where prevalences of 2% and 6% are found, respectively (Yali et al., 1998, Yang et al., 1998). A more frequent and closer contact between humans and animals in developing countries might be a possible explanation for the higher prevalence (Meining et al., 1998; Stolte et al., 1994; Svec et al., 2000).

Due to their fastidious nature, isolation of NHPH bacteria from gastric biopsies for routine diagnostic purposes is very difficult. Until now, only *H. bizzozeronii* and *H. felis* have been

successfully cultured from human gastric biopsies (Andersen et al., 1999; Jalava et al., 2001; Kivistö et al.; 2010; Wüppenhorst et al., 2013). Currently, analysis of gastric biopsies by a polymerase chain reaction (PCR)-based technique and histology are the most successful ways to identify infections with *H. heilmannii* s.l. In human gastric biopsy samples with histological evidence of *H. heilmannii* s.l., PCR-based studies have shown that *H. suis* is the most prevalent NHPH species infecting the human stomach, with a prevalence ranging from 14% to 37% in NHPH-infected humans. *H. salomonis* has been detected in gastric biopsy samples of 11%, *H. felis* of 9%, *H. heilmannii* s.s. of 7%, *H. bizzozeronii* of 2% and '*Candidatus* H. bovis' of 0.9 % of human patients infected with NHPH. In 16% of all cases, two or more NHPH species or *H. pylori* and an NHPH species were present in the same gastric biopsy sample (De Groote et al., 2005; Van den Bulck et al., 2005a). So far, there is no information available regarding the presence of *H. acinonychis, H. baculiformis, H. cetorum, H. cynogastricus, H. mustelae* and '*Candidatus* H. homininae' in gastric biopsy samples from humans.

1.2.3 Clinical significance in humans

Similar to *H. pylori*, the outcome of *H. heilmannii* s.l. infections in humans is assumed to depend not only on the characteristics of the infecting strains, but also on predisposing factors of the host and environmental factors. Most infections are also expected to remain asymptomatic or progress to a mild gastritis without complaints (Mazzucchelli et al., 1993). In humans suffering from gastric complaints, *H. heilmannii* s.l. infections are typically characterized by a mild chronic active gastritis in the antrum of the stomach, but with lesions usually less severe than those associated with *H. pylori* (Kaklikkaya et al., 2002; Stolte et al., 1997). Only sporadically, *H. heilmannii* s.l. is accompanied by an acute gastritis (Yoshimura et al., 2002). *H. bizzozeronii, H. felis, H. heilmannii, H. salomonis* and *H. suis* have been associated with peptic and duodenal ulcers and low-grade MALT lymphoma (Boyanova et al., 2007; Iwanczak et al., 2012; Morgner et al., 2000; Okiyama et al., 2005; Stolte et al., 1997; Svec et al., 2000; Yali et al., 1998). The risk of developing MALT lymphoma is considered to be higher after infection with NHPH species than with *H. pylori* (O'Rourke et al., 2004a; Stolte et al., 2002). The contribution of *H. heilmannii* s.l. to the pathogenesis of gastric MALT lymphoma is highlighted in reports in which eradication treatment of the bacteria is followed by symptomatic relief and complete regression of the infection-associated lesions, including the low-grade MALT lymphoma (Goddard et al., 1997; Joo et al., 2007; Morgner et al., 2000).

Histologically, NHPH bacteria are predominantly found in the antrum of the stomach and mainly focally. They can also be seen in the fundus, but only a mild inactive gastritis has been reported in this region. The bacteria are present as single cells or in small groups, located underneath the mucous layer, above the surface cells, and deep in the lumen of the foveolae. By ultrastructural analysis, *H. heilmannii* s.l. can be seen in close contact with the membrane of surface mucous cells, in association with degenerative changes of the cell membrane and partial destruction of the microvilli. NHPH bacteria can also be found inside mucous and parietal cells and inside parietal cell canaliculi in the corpus mucosa (Bento-Miranda and Figueiredo, 2014; Heilmann and Borchard, 1991; Stolte et al., 1997). As mentioned above, the gastric lesions induced by *H. heilmannii* s.l., such as lymphocytic and neutrophilic infiltration, replacement of foveolae by regenerative epithelium and mucus depletion, are generally speaking milder than those induced by *H. pylori*. Moreover, the presence of lymphoid follicles and intestinal metaplasia is usually less common in *H. heilmannii* s.l. gastritis than in *H. pylori* gastritis (Joo et al., 2007; Okiyama et al., 2005; Stolte et al., 1997).

Clinical signs described in patients suffering from an NHPH infection are variable and include acute or chronic epigastric pain, nausea, recurrent dyspepsia, reflux esophagitis, heartburn, vomiting, hematemesis, abdominal pain, irregular defecation frequency and consistency, and dysphagia, often accompanied by a decreased appetite. The infection can also be asymptomatic (Dieterich et al., 1998; Goddard et al., 1997; Heilmann and Borchard, 1991; Mention et al., 1999; Oliva et al., 1993; Seo et al., 2003; Sykora et al., 2003; van Loon et al., 2003; Wegmann et al., 1991; Yang et al., 1998).

1.3 Zoonotic *Helicobacter* species from cats and dogs

1.3.1 Prevalence in cats and dogs

Gastric *Helicobacter* species are highly prevalent in cats and dogs. Prevalences have been described ranging from 67–86 % in clinically healthy dogs, 61–100 % in dogs presenting chronic vomiting, and 41–100 % in healthy cats as well as cats showing chronic vomiting (Amorim et al., 2015; Neiger and Simpson, 2000; Shoajee Tabrizi et al., 2010; Takemura et al., 2009). The prevalence of *Helicobacter* infections in cats and dogs seems to be related to the living conditions of these animals. In most studies examining shelter or colony dogs or cats, higher prevalences have been described than in studies on pet animals (Eaton et al., 1996; Ghil et al., 2009; Neiger and Simpson, 2000; Takemura et al., 2009). Also age possibly plays a role. Young animals may be less often colonized than older animals, although this is still

controversial (Geyer et al., 1993; Happonen et al., 1996; Neiger et al., 1998; Otto et al., 1994). The gastric mucosa of cats and dogs is often naturally infected with multiple gastric *Helicobacter* species (Haesebrouck et al., 2009). As shown in **Table 1**, at the onset of this thesis, 6 different gastric *Helicobacter* species had been isolated from the stomachs of cats and dogs, namely *H. bizzozeronii*, *H. salomonis*, *H. felis* and *H. cynogastricus* from the canine gastric mucosa (Hänninen et al., 1996; Jalava et al., 1997; Jalava et al., 1998; Van den Bulck et al., 2006) and *H. felis*, *H. baculiformis* and *H. heilmannii* from the feline gastric mucosa (Baele et al., 2008b; Lee et al., 1988; Smet et al., 2012). In the canine stomach, *H. bizzozeronii* is the most prevalent species, whereas *H. felis* and *H. heilmannii* are the predominant *Helicobacter* species in the stomach of cats (Priestnall et al. 2004; Svec et al. 2000; Wiinberg et al. 2005). The prevalence of *H. cynogastricus* and *H. baculiformis* in pet animals is so far unknown.

1.3.2 Clinical significance in cats and dogs

Given the fact that gastric *Helicobacter* species are present in both clinically healthy cats and dogs as well as in animals with gastrointestinal problems, the pathogenic significance of these species in cats and dogs remains unclear. It may be related to differences in virulence of the colonizing *Helicobacter* species and strains (Haesebrouck et al., 2009). Chronic vomiting is the main clinical sign that has been described for cats and dogs suffering from a *Helicobacter* infection (Hwang et al., 2002).

In general, feline and canine *Helicobacter* species have been associated with chronic active gastritis (Amorim et al., 2015; Contreras et al., 2009; Diker et al., 2002). In cats, *Helicobacter* infection has also been related to the presence of gastric lymphoma (Bridgeford et al., 2008). Gastric and duodenal ulcers have been rarely reported in cats and dogs, and no clear association has been made with *Helicobacter* infections (Brown et al., 2007).

In both cats and dogs, *Helicobacte*r species are able to colonize all stomach regions. In some studies, the colonization levels were reported to be higher in fundus and corpus of the stomach than in cardia, antrum and pylorus (Diker et al., 2002; Gombač et al., 2010; Happonen et al., 1996; Simpson et al., 1999).

Histologically, in the stomach of cats naturally infected with *H. heilmannii*, changes can be seen in the lamina propria, including a mild mononuclear inflammatory infiltration, the presence of lymphoid follicles, fibrosis and glandular degeneration (Takemura et al., 2009). Also in dogs, *Helicobacter* infections have been described to be accompanied by a mild to moderate intraepithelial lymphocyte infiltration and a mild to moderate gastric epithelial injury (Amorim et al., 2015). Several experimental infection studies in cats and dogs have been

performed to further characterize the histological changes related with the pathogenesis of infections with feline and canine *Helicobacter* species. For instance, experimental *H. felis* infection in young gnotobiotic dogs has been associated with a lymphoid hyperplasia in the fundus and corpus of the stomach (Diker et al., 2002). In specific pathogen-free (SPF) cats, experimental *H. felis* infection was accompanied by a mononuclear infiltration of the gastric mucosa, with a follicular organization of the inflammatory cells (Scanziani et al., 2001). Gastric helicobacters can be located in the superficial and basal portions of the fundic glands and can be found not only free in the cytoplasm but also within lysosomes of parietal cells (Lanzoni et al., 2011).

1.3.3 Zoonotic potential and transmission to humans

Only few data are available on the transmission of canine and feline NHPH species. Among the animals themselves, transmission via oral-oral (saliva) or gastric-oral (emesis) contact has been suggested. For instance, transmission of *H. salomonis* from a dam to her puppies, as well as between infected and non-infected pups, has been described. In this case, the puppies were in very close contact with each other and with the nursing dam, and they ate vomited material (Hänninen et al., 1998).

Evidence is accumulating that cats and dogs constitute reservoir hosts for gastric *Helicobacter* species with zoonotic potential (Haesebrouck et al., 2009). The zoonotic potential of canine and feline NHPH species has been suggested by a series of case studies. In these studies, the transmission of *H. heilmannii* s.l. from dogs to their owners (De Bock et al., 2007; Jalava et al., 2001; Thomson et al., 1994) and from cats to their owners (Dieterich et al., 1998; Hiroshi et al., 2008; Lavelle et al., 1994; Stolte et al., 1994; van Loon et al., 2003) is expected to occur by direct contact. Moreover, a higher prevalence of *H. heilmannii* s.l. infections have been described for people living in rural areas who often have contact with cats and dogs (Meining et al., 1998; Stolte et al., 1994; Svec et al., 2000).

In addition to being present in the stomach, *H. heilmannii* s.l. species have also been reported in the saliva of domestic cats and dogs, indicating that the oral cavity of these animals may act as source of NHPH infection for humans (Ekman et al., 2013; Recordati et al., 2007; Shojaee Tabrizi et al., 2010). Fecal-oral transmission is another possible route for infection, since *H. heilmannii* s.l. DNA also has been detected in fecal samples from cats and dogs (Berlamont et al., 2016; Ghil et al., 2009).

Besides direct contact with animals, other routes of transmission of NHPH should be taken into account. For instance, it has been shown that *H. felis* is able to survive in water for several days, highlighting the possible role of water in the transmission of this species (Azevedo et al., 2008).

2. Virulence-associated factors of gastric *Helicobacter* species

2.1 Motility and chemotaxis

Once in the lumen of the stomach, gastric helicobacters use their motility and chemotaxis signaling system to sense the environment and to reach the gastric epithelium for colonization, where they are able to attach to the epithelial surface (Keilberg and Ottemann, 2016). A first obstacle they encounter, is the very acidic pH (2) in the lumen of the stomach. Gastric helicobacters cannot survive in pH 2 for a long time and therefore, they have developed multiple mechanisms to avoid this exposure (Keilberg and Ottemann, 2016; Marshall et al., 1990). Firstly, all gastric helicobacters possess urease enzymes that hydrolyze host-derived urea to ammonia and carbon dioxide. The released ammonia reacts with and neutralizes the HCl in the stomach, creating a neutral pH environment directly around the bacterium. Urease is mainly located in the cytoplasm of the bacteria, but it can also be found at the cell surface of viable bacteria, after autolysis of surrounding ones (Haesebrouck et al., 2009; Krishnamurthy et al., 1998; Marcus and Scott, 2001; Phadnis et al., 1996). Additionally, gastric helicobacters are able to actively move away from low pH regions, toward the gastric mucosa, by using their flagella. They possess monopolar, bipolar or peritrichous bundles of 2 to 23 flagella, and each flagellum consists of a body, a hook and a flagellar filament. The filament is composed of 2 flagellin subunits, FlaA and FlaB. It works as a propeller and is covered by a sheath, which possibly plays a role in acid protection, masking of antigens and adhesion (Haesebrouck et al., 2009; Jones et al., 1997). The hook of the flagellum links the filament with the basal body. This basal body is embedded in the bacterial cell wall and contains proteins required for rotation and chemotaxis (Haesebrouck et al., 2009; O'Toole et al., 1994). In combination with their flagellar motility, a chemotaxis system enables the gastric helicobacters to swim away from the low pH conditions. The chemotaxis receptor TlpB is required for this pH taxis (Croxen et al., 2006). Thanks to the chemotactic motility, it is possible for the helicobacters to rapidly enter the mucus layer and to escape from the acidic stomach lumen (Lowenthal et al., 2009; Terry et al., 2005). In addition to pH as a repellant, the chemotaxis system also detects urea, amino acids and metals as attractants (Huang et al., 2015; Mizote et al., 1997; Sanders et al., 2013). These compounds likely diffuse from the host blood through the epithelial layer, therefore presumably acting as a

way for gastric helicobacters to localize near the host epithelium (Keilberg and Ottemann, 2016). Furthermore, the helicobacters use chemotaxis to colonize injured epithelium, which may possess particularly high amounts of favorable nutrients (Aihara et al., 2014).

2.2 Outer membrane proteins that function as adhesins

Once the gastric helicobacters have escaped the pH stress, their motility decreases. This might be the response to the neutral pH environment in the mucus layer, and it possibly optimizes their attachment to gastric epithelial cells (Croxen et al., 2006). While the bacteria can persist deep in the mucus layer, they are mainly found attached tightly to the gastric epithelial cells (Odenbreit, 2005). This initial pathogen-host interaction is crucial in order to be able to colonize the stomach (Galdiero et al., 2012).

For *H. pylori*, many bacterial outer membrane proteins (OMPs) have been described that mediate adhesion to the gastric epithelium. In total, 64 well-annotated OMPs are present in *H. pylori*, encoded by 4 % of its genome (Alm et al., 2000). This large set of OMPs probably reflects their importance for this bacterium and possibly originates as a response to the challenging environment of the stomach. They can be divided into 5 paralogous families. Family 1 is the largest family and consists of the Hop (***H****elicobacter* **o**uter membrane **p**orin, 21 members) and Hor (**Ho**p-**r**elated, 12 members) proteins. Family 2 comprises the Hof (***H****elicobacter* **O**MP **f**amily, 8 members) and family 3 the Hom (***H****elicobacter* **o**uter **m**embrane, 4 members) proteins. Family 4 is composed of iron-regulated OMPs (6 members) and family 5 of efflux pump OMPs (3 members). The remaining 10 OMPs are not classified into one of these families and their function remains unknown (Alm et al., 2000; Oleastro and Ménard, 2013). Most of the *H. pylori* OMPs belong to the Hop family and function as porins or as adhesins that promote binding to the gastric epithelium (Exner et al., 1995; Ilver et al., 1998; Mahdavi et al., 2002; Oleastro and Ménard, 2013). Using these OMPs as adhesins might be the result of an adaptation of *H. pylori* to the gastric acidic environment, where any polymeric pilus structure, which is commonly used for adhesion by other Gram-negative bacteria, would likely depolymerize (Alm et al., 2000). The best characterized OMPs that have been shown to play a role in the colonization of *H. pylori*, are the Hop adhesins BabA (HopS), SabA (HopP), AlpA (HopC) and AlpB (HopB), OipA (HopH) and HopZ; the Hor protein HorB and the Hom protein HomB (Backert et al., 2011; Oleastro et al., 2008; Snelling et al., 2007).

The first identified adhesin in *H. pylori* is the blood group antigen-binding adhesin A, named **BabA** or HopS (Borén et al., 1993; Moore et al., 2011; Yamaoka, 2008b). This adhesin mediates the binding of *H. pylori* to fucosylated Lewis b blood group antigens (Le^b) on blood

group O (H antigens), A and B antigens, that are expressed on mucins and gastric epithelial cells (Aspholm-Hurtig et al., 2004; Borén et al., 1993; Ilver et al., 1998). Two closely related paralogs of BabA have been identified, namely BabB (HopT) and BabC (HopU), of which the function has not yet been determined. They present extensive 5' and 3' homology, suggesting that the middle variable region of BabA most likely encodes the adhesin function (Pride and Blaser, 2002). Both *in vitro* and *in vivo*, it has been shown that BabA-mediated adherence to Le^b on the epithelial surface triggers the transcription of genes that enhance inflammation and the development of intestinal metaplasia and precancerous transformation (Ishijima et al., 2011). Moreover, BabA binding to Le^b can induce DNA double-strand breaks and DNA damage in host cells (Toller et al., 2011).

Another well-characterized *H. pylori* adhesin is the sialic acid-binding adhesin, named **SabA** or HopP. SabA mediates the binding of this bacterium to sialyl-dimeric-Lewis x glycosphingolipid (Le^x), which is upregulated on the inflamed gastric tissue after *H. pylori* infection (Mahdavi et al., 2002; Yamaoka, 2008a). To a lesser extent, SabA also participates with BabA in the binding of *H. pylori* to the salivary mucin MUC5B (Walz et al., 2005; Walz et al., 2009). Also SabA has 2 closely related paralogs, SabB (HopO) and HopQ. The 5' and 3' ends of their encoding genes share the highest nucleotide identity (Alm et al., 2000; Talarico et al., 2012). The functions of SabB and HopQ have not yet been determined, but they may also be involved in *H. pylori* adherence (Loh et al., 2008; Yamaoka et al., 2002b). The amount of SabA on the bacterial surface is regulated by gene conversion, and this mechanism thus influences the adherence of *H. pylori* to gastric tissue (Yamaoka et al., 2006).

The adherence-associated lipoproteins **AlpA** (HopC) and **AlpB** (HopB), encoded by the highly similar adjacent genes *alp*A and *alp*B, are also involved in gastric colonization (Alm et al., 1999; Alm et al., 2000; Odenbreit et al., 1997). Both lipoproteins have been shown to contribute to host laminin binding and to influence gastric inflammation (Senkovich et al., 2011). AlpA and AlpB proteins are strongly coproduced and seem to be expressed by all clinical *H. pylori* isolates, indicating an essential function for these OMPs (Odenbreit et al., 2009).

The outer inflammatory protein A, named **OipA** or HopH, is another *H. pylori* OMP that has been shown to be involved in bacterial adherence to gastric epithelial cells *in vitro* (Dossumbekova et al., 2006). OipA was initially identified as a surface protein that promotes IL-8 secretion of epithelial cells (Yamaoka et al., 2000; Yamaoka et al., 2002a). In contrast to other adhesins, the encoding *oipA* or *hopH* gene does not have highly similar paralogs and it is quite distant from other *hop* genes. In *H. pylori*-related pathology, the expression of OipA has been significantly associated with more severe gastric disease, high *H. pylori* density and severe

neutrophil infiltration (Armitano et al., 2013; Franco et al., 2008; Markovska et al., 2011; Yamaoka et al., 2006). Thus, most probably, OipA leads to an increased bacterial adherence and a higher colonization capacity (Dussombekova et al., 2006).

The OMP **HopZ** has also been demonstrated to play a role in adhesion to gastric epithelial cells *in vitro* (Yamaoka et al., 2002b). *H. pylori* typically starts to express HopZ in the early stage of infection, suggesting that this OMP is important during early colonization (Kennemann et al., 2011). In mice, HopZ expression influences both *H. pylori* density and colonization ability (Yamaoka et al., 2002b).

HomB, belonging to the small Hom family of OMPs, is another contributor to *H. pylori* adherence. It has been shown to be associated with IL-8 secretion *in vitro* (Oleastro et al., 2008). The *homB* gene shares 90% sequence identity at the 5' and 3' ends with its closely related paralog, the *homA* gene (Alm et al., 2000), but only the *homB* gene has been related to gastric inflammation and atrophy and thus can be seen as a marker for more virulent *H. pylori* strains (Jung et al., 2009).

Finally, **HorB**, belonging to the Hor family of OMPs, plays a role in the adhesion process of *H. pylori*. *In vitro*, disruption of the *horB* gene reduces *H. pylori* adhesion to gastric epithelial cells by more than twofold, suggesting an important role for this adhesin (Snelling et al., 2007).

Before the onset of this PhD research, little was known about the OMPs from gastric NHPH species and more specifically, it was unknown which OMPs are involved in adhesion to and colonization of the gastric mucosa. The genomes of *H. bizzozeronii* (Schott et al., 2011), *H. felis* (Arnold et al., 2011), *H. heilmannii* (Smet et al., 2013), *H. suis* (Vermoote et al., 2011), *H. acinonychis* (Eppinger et al., 2006), *H. cetorum* (Kersulyte et al., 2013) and *H. mustelae* (O'Toole et al., 2010) have recently been published. Similar to *H. pylori*, they also harbor a large repertoire of OMP-encoding genes. Remarkably, all canine, feline and porcine helicobacters seem to lack the well-characterized and important *H. pylori* Hop and Hom adhesins, indicating that other OMPs are used by these species for adhesion to the gastric mucosa. Recently, two *H. heilmannii* Hof proteins, HofE and HofF, have been shown to function as adhesins (Liu et al., 2016).

2.3 Other factors of gastric *Helicobacter* species involved in pathogenesis

Besides OMPs that mediate adhesion, gastric *Helicobacter* species harbor several other important virulence-associated factors. Most studies concerning *Helicobacter* virulence factors are focused on *H. pylori*, whereas very little information is available about the virulence

mechanisms of gastric NHPH. An overview of the most important *H. pylori* virulence factors is given below. The presence or absence of homologous genes in other gastric helicobacters is mentioned as well. Since the genomes of different *H. pylori* strains are highly diverse, it should be noted that strain-specific genes might play a role in the virulence properties of this species, and that not all virulence factors are present in all *H. pylori* strains (Fischer et al., 2010).

Two well-studied virulence factors of *H. pylori* are the vacuolating cytotoxin (VacA) and the cytotoxin-associated gene pathogenicity island (*cag* PAI). A schematic overview of their mode of action on host epithelial cells is presented in **Figure 1**.

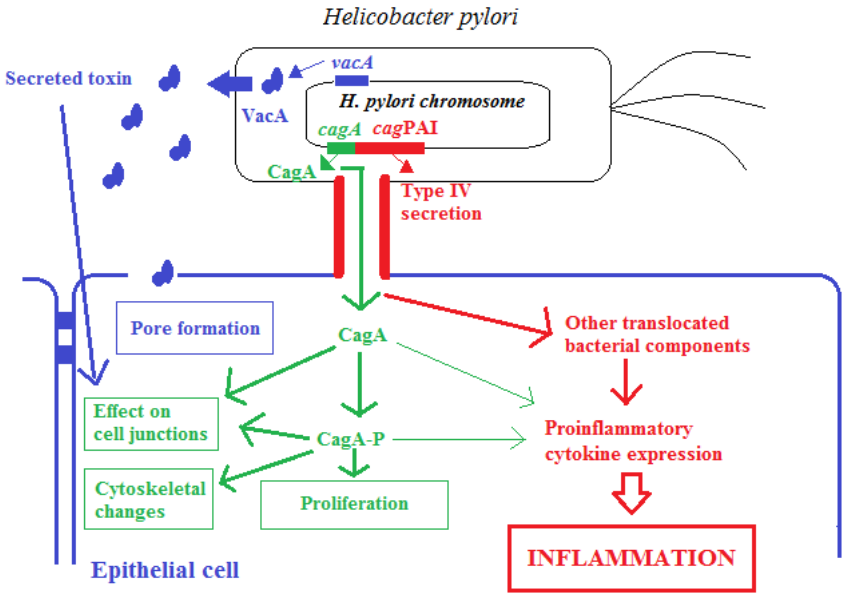

Figure 1. A schematic overview of the well-characterized *H. pylori* virulence factors VacA, *cag*PAI and CagA and their effects on host epithelial cells. The *cag*PAI (red) encodes a type IV secretion system (T4SS) which is a major stimulus for epithelial cell pro-inflammatory cytokine production and thus inflammation. CagA (green) is translocated through the T4SS and also contributes to inflammation. VacA (blue) induces pore formation in gastric epithelial cells and significantly increases the disease risk (adapted from Robinson et al., 2007).

The pore-forming outer membrane toxin **VacA** is the most important secreted virulence factor of *H. pylori* that triggers host cell apoptosis (Cover et al., 2003). After interaction with the host cell, VacA is internalized into the host cell membrane and forms anion selective channels. These initiate a Cl^- influx, leading to a volume increase of endosomes and to the development of vacuoles in the host cells (Szabò et al., 1999). VacA also targets mitochondria and causes loss of the mitochondrial membrane potential, formation of pores in the inner mitochondrial membrane and release of cytochrome c and other mitochondrial proteins into the cytosol. The

disruption of the mitochondrial function leads to a decrease of cellular ATP levels and to host cell apoptosis (Rassow, 2011; Rassow and Meinecke, 2012). Moreover, the apoptosis of epithelial cells has two distinct effects on the *H. pylori* population in the stomach: it can increase its nutrients in the surrounding environment on the one hand, and it can help to diminish the immune response on the other hand, stabilizing the chronic infection (Boncristiano et al., 2003; Gebert et al., 2003).

Structurally, the VacA protein consists of a signal peptide, a central region that forms the functional toxin and a C-terminal domain that is required for transport into the host cell. Its active form is an 88 kDa protein, generated from a 140 kDa precursor that undergoes N- and C-terminal cleavage during the secretion process. The 88 kDa VacA toxin is further processed into its active (A) subunit of 33 kDa, which is required for cell internalization and vacuolation, and its binding (B) subunit of 55 kDa, which is essential for binding to the target epithelial cells (Boquet and Ricci, 2012; Gupta et al., 2008; Sewald et al., 2008). The VacA toxin shows strain-specific differences. This protein is highly variable at the sequence level, which is associated with variations in its functional activity *in vitro*. The encoding *vacA* gene possesses several polymorphic sites, namely the signal (s) region, the midregion (m) and the intermediate region (i). It has been shown that *H. pylori* strains with *vacA* types s1/m1 are associated with duodenal and gastric ulceration and gastric adenocarcinoma (Rhead et al., 2007). In most gastric NHPH species, the VacA protein is lacking. Only in *H. cetorum*, an intact homolog of *H. pylori vacA* has been found, and in *H. acinonychis*, *vacA* sequences are also present but only as fragmented pseudogenes (Dailidiene et al., 2004; Eppinger et al., 2006; Kersulyte et al., 2013).

In addition to VacA, the majority of *H. pylori* isolates (50-70%) carry the *cagA* gene and the *cag* PAI (Peek and Crabtree, 2006). The ***cag* PAI** encodes a type IV secretion system (T4SS), which forms a syringe-like structure that is capable of penetrating gastric epithelial cells and delivering **CagA** into the host cells. Once delivered inside the cell, CagA undergoes a phosphorylation on tyrosine residues, resulting in morphological epithelial cell changes such as disruption of cell-to-cell junctions and the loss of cell polarity (Alzahrani et al., 2014; Odenbreit, 2005; Segal et al., 1999). The phosphorylation is mediated by host tyrosine kinases Src and Abl, which phosphorylate CagA on the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif. CagA harbors one to seven of these motifs, varying from strain to strain (Backert et al, 2010; Mueller et al., 2012; Xia et al., 2009). This polymorphism affects the severity of CagA effects and disease outcome. In general, the presence of CagA is one of the highest risk factors for the development of peptic ulcers and gastric cancer (Crabtree and Farmery, 1995). *H. pylori* strains that deliver CagA with more phosphorylation motifs are able to induce more severe cytoskeletal

changes and are most often associated with gastric cancer (Argent et al., 2004; Azuma et al., 2002; Bridge and Merrell, 2013). In contrast to their great importance for the virulence of *H. pylori*, homologs of *cagA* and *cag* PAI are absent in the genomes of all other gastric *Helicobacter* species (Haesebrouck et al., 2009).

Besides *cag* PAI, CagA and VacA, other *H. pylori* determinants have been shown to be inducers of gastric inflammation. The *γ*-glutamyltranspeptidase (**GGT**), constitutively expressed by all *H. pylori* strains, has been shown to be important for the establishment of an infection (Chevalier et al., 1999). GGT is a low molecular weight secreted or membrane-associated enzyme of *H. pylori*. Its main physiological role is to enable *H. pylori* to use extracellular glutamine and glutathione as sources of glutamate. Since *H. pylori* is unable to directly take up extracellular glutamine and glutathione, these substances are hydrolyzed into glutamate by GGT (Ricci et al., 2014; Shibayama et al., 2007). Glutamine and glutathione are also important nutrients for maintaining a healthy gastric tissue, indicating that their depletion by *H. pylori* GGT has a detrimental effect on host gastric epithelial cells. Moreover, it has been shown that *H. pylori* GGT is able to disrupt the Ras signaling pathway, inducing T cell cycle arrest and thus suppressing T cell proliferation (Gerhard et al., 2005; Schmees et al., 2007). In addition, GGT is strongly involved in *H. pylori*-mediated H_2O_2 production and contributes to gastric inflammation via activation of NF-*κ*B and upregulation of IL-8 in primary gastric epithelial cells (Gong et al., 2010). The glutamine deprivation induced by GGT has also been suggested to be responsible for the induction of gastric inflammation and to increase the risk of developing gastric cancer (Rimbara et al., 2013). Furthermore, GGT leads to a loss of the apoptosis inhibitor survivin, resulting in increased apoptosis of gastric epithelial cells (Valenzuela et al., 2013). GGT is also produced by gastric NHPH species and has been shown to be important for their virulence (Haesebrouck et al., 2009). For instance, for *H. suis* it has been demonstrated that GGT-mediated degradation of glutathione and the resulting formation of glutathione degradation products are involved in the induction of gastric epithelial cell death (Flahou et al., 2011).

The ***iceA*** gene (‘induced by contact with epithelium’) is upregulated by the contact of *H. pylori* with gastric epithelial cells. Two allelic variants of this gene, *iceA1* and *iceA2*, have been described (Peek et al., 1998; Van Doorn et al., 1998; Xu et al., 2002). The *iceA1* gene encodes a homolog of a CTAG-specific restriction endonuclease (*nla*IIIR) of *Neisseria lactamica* (Peek et al., 1998; Xu et al., 2002), but the exact role of the *iceA* gene product during human infection remains unclear. However, the presence of the *iceA1* allele has been related to peptic ulcer disease (Shiota et al., 2012; Van Doorn et al., 1998) and to enhanced mucosal IL-8 expression

and acute antral inflammation (Peek et al., 1998). Before the onset of this PhD research, no information was available concerning the presence of an *iceA* gene in other gastric *Helicobacter* species, except for *H. bizzozeronii* (Schott et al., 2011).
Besides the above described proteins and genes, also other, less characterized virulence factors may play a role in the pathogenesis of gastric *Helicobacter* infections. Further research is necessary, in particular to identify potential virulence factors of gastric NHPH species.

3. Host immune responses to gastric *Helicobacter* infections and their involvement in pathogenesis

Gastric *Helicobacter* species are active stimulators of both innate and acquired immune responses. The immune response towards an *H. pylori* infection has been extensively explored, but only limited data are available for NHPH species.
For *H. pylori*, a local innate recognition by epithelial cells is thought to be an important disease determinant. This agent also elicits strong local and systemic antibody and cell-mediated immune responses. However, the induced immune response does not result in its eradication and *H. pylori* is able to establish a chronic life-long infection. Thus, it is clear that *H. pylori* has evolved several ways to evade the human host immune response. Nevertheless, the inflammatory response towards an *H. pylori* infection is an important part of its pathogenesis (Robinson et al., 2007). The main characteristics of both the innate and adaptive immune response, elicited by an *H. pylori* infection, are described below. Where possible, a comparison with NHPH bacteria is made.

3.1 Innate immune response

During its colonization of the gastric mucosa, *H. pylori* triggers innate host defense mechanisms and stimulates the expression of pro-inflammatory and anti-bacterial factors by gastric epithelial cells, resulting in gastritis. The secretion of pro-inflammatory cytokines by gastric epithelial cells leads to the recruitment of innate immune cells, including neutrophils, macrophages and dendritic cells, that infiltrate the gastric mucosa (George et al., 2003; Gobert et al., 2004; **Figure 2**). The colonization density of *H. pylori*, the level of gastric inflammation and the induction of the adaptive immune response are all influenced by the innate immune response (Trinchieri, 2003; Yamaoka et al., 1997). Therefore, this first-line reaction is an important determinant of the disease severity and is considered to be a major mediator in the process of gastric carcinogenesis (Robinson et al., 2007). The major *H. pylori*-induced pro-

inflammatory cytokines that play a crucial role in gastric inflammation and carcinogenesis, are summarized in **Table 2**.

Table 2. Overview of the major *H. pylori*-induced pro-inflammatory cytokines and their role in gastric inflammation and cancer development (adapted from Robinson et al., 2007)

Pro-inflammatory cytokine	Role in inflammation	Role in cancer development
IL-1β	- Activation of macrophages and polymorphonuclear leukocytes - Stimulation of IL-6 release	- Stimulates hypergastrinaemia, leading to the proliferation of epithelial cells - Pro-angiogenic activity - Induces matrix metalloproteinase secretion and activation
IL-6	- Activation and differentiation of macrophages - Increases the phagocytic activity of neutrophils - B-cell differentiation	- Increases angiogenesis via expression of VEGF
IL-8	- Recruitment of lymphocytes and neutrophils	- Potentiates gastrin release, leading to the proliferation of epithelial cells - Pro-angiogenic activity
TNF-α	- Activation and differentiation of macrophages - Apoptosis of epithelial cells and disruption of the epithelial barrier - Inhibition of microvascular epithelial cell proliferation and wound healing	- Stimulates hypergastrinaemia, leading to the proliferation of epithelial cells

For most NHPH infections, it remains to be investigated which cytokines play a key role in the innate immune response. Experimental *H. suis* infection has been shown to be accompanied by an upregulation of IL-6 in BALB/c and C57BL/6 mice. In the latter mouse strain, also IL-1β was upregulated (Flahou et al., 2012). Recently, *in vitro* studies demonstrated IL-8-production by human gastric epithelial cells as a response to an *H. heilmannii* infection. The expression of this cytokine can be induced by IL-1β and TNF-α, but the exact signaling pathways are currently unknown (Liu et al., 2016).

3.1.1 *Helicobacter* recognition by Toll-like receptors

The innate immune response is largely dependent on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Nurnberger et al., 2004).

The best studied PRRs are the Toll-like receptors (TLRs), a family of 10 proteins expressed on cells surfaces. Each TLR has a different binding specificity for PAMPs (Netea et al., 2004; O'Neill et al., 2013).

Adhesion to **gastric epithelial cells** represents the first point of contact between *H. pylori* and the host, and the expression of TLRs has been shown on the cell surface of many gastric epithelial cell lines (Smith, 2014). TLR2, TLR4, TLR5 and TLR9 have been detected on gastric epithelial cells in the human stomach (Schmausser et al., 2004). In addition, increased TLR2 and TLR4 expression have been reported in the gastric mucosa of *H. pylori*-infected patients (Bäckhed et al., 2003; Ishihara et al., 2004; Uno et al., 2007).

Following an *H. pylori* infection, epithelial cells release a variety of cytokines and chemokines leading to the recruitment of **monocytes/macrophages** to the gastric mucosa. This mononuclear cell infiltration in the lamina propria is characteristic of an *H. pylori*-induced acute infection (Pathak et al., 2006). Human monocytes and macrophages express a wide repertoire of PRRs. An *H. pylori* infection activates both TLR2 and TLR4 signaling in these cells, resulting in the secretion of distinct inflammatory cytokines including IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α (Gobert et al., 2004; Maeda et al., 2001; Mandell et al., 2004; **Figure 2**). It has been demonstrated that IL-10 and IL-12 secretion is induced through TLR4 signaling, whereas IL-1β and IL-6 secretion is induced through TLR2 signaling (Obonyo et al., 2007).

Also **dendritic cells** (DCs), the major antigen presenting cells that play a key role in the induction of the adaptive immune response, express a wide range of PRRs (Muzio et al., 2000). DCs are able to capture antigens from the periphery and to activate naïve T cells by antigen presentation, co-stimulation and cytokine secretion. DC signaling during an *H. pylori* infection has been shown to be mediated by TLR2 and to a lesser extent by TLR4 and TLR9, resulting in the production of cytokines IL-1β, IL-6, IL-10, IL-12 and TNF-α (Kim et al., 2013; Rad et al., 2007; Rad et al., 2009; **Figure 2**).

Besides TLRs, also other PRRs are activated during an *Helicobacter* infection, including nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs) (Smith, 2014). The NOD1 protein for instance is expressed on epithelial cells and recognizes *H. pylori* peptidoglycan, which is delivered by the type IV secretion system or through secreted outer membrane vesicles (Viala et al., 2004).

3.1.2 Recognition of distinct *Helicobacter* components by specific TLRs

The contribution of several individual *H. pylori* components as antigens that trigger the TLR-driven innate immune response has been extensively explored. First, *H. pylori* lipopolysaccharide (**LPS**) may be recognized by the classic Gram-negative bacterial LPS receptor TLR4 (Chochi et al., 2008; Ishihara et al., 2004; Kawahara et al., 2001; Su et al., 2003; Windle et al., 2005). However, accumulating evidence indicates a more pronounced role for TLR2 in the innate immune recognition of *H. pylori* LPS (Lepper et al., 2005; Smith et al., 2003; Smith et al., 2011; Triantafilou et al., 2007; Yokota et al., 2007). Nevertheless, it should be noted that *H. pylori* LPS has a lower endotoxicity than many other Gram-negative bacteria (Smith, 2014; Tran et al., 2005). Also for *H. felis* and *H. bizzozeronii*, activation of TLR2 signaling via LPS has been described (Kondadi et al., 2015; Mandell et al., 2004).

Secondly, TLR5 is the receptor for bacterial **flagellin**, the protein subunit of the polymeric flagellar filament (described above) of gastric *Helicobacter* species and other Gram-negative bacteria (Hayashi et al., 2001). TLR5 is expressed on gastric epithelial cells and gastric epithelial cell lines (Bäckhed et al., 2003; Lee et al., 2003; Rad et al., 2009). The *H. pylori* flagellin is a less potent stimulator of TLR signaling than flagellin from other Gram-negative bacteria (Gewirtz et al., 2004; Lee et al., 2003). The low innate immune response to *H. pylori* LPS and flagellin in the stomach *in vivo* may contribute to the ability of *H. pylori* to evade host responses and to promote long term bacterial persistence (Smith, 2014).

In addition, the 60 kDa heat-shock protein (**HSP60**) of *H. pylori* has been shown as a potent immune antigen that stimulates IL-8 induction in gastric epithelial cells through TLR2 signaling. Moreover, HSP60-induced immune responses have been associated with gastric inflammation and the pathogenesis of MALT lymphoma (Takenaka et al., 2004).

H. pylori secretes the peptidyl prolyl *cis*-, *trans*-isomerase **HP0175**, which can induce apoptosis in gastric epithelial cells via binding to TLR4 (Basak et al., 2005; Basu et al., 2008). HP0175 also interacts with TLR4 on macrophages, inducing IL-6 release (Pathak et al., 2006).

The *H. pylori* neutrophil-activating protein (**NapA**) is a 150 kDa oligomeric virulence factor that is chemotactic for neutrophils, in which it stimulates the production of oxygen radicals. It has been shown that *H. pylori* NapA binds to TLR2. In human monocytes and neutrophils, expression of IL-12 is induced by NapA stimulation, which is an important cytokine for the differentiation of naïve T-helper cells into the T-helper 1 (Th1) phenotype. Moreover, NapA stimulates monocytes to produce IL-23 and to differentiate towards mature DCs (Amedei et al., 2006). NapA plays an important role in the protection of *H. pylori* DNA from oxidative stress

damage (Cooksley et al., 2003). The NapA-encoding gene is a core gene that is also present in gastric NHPH (Arnold et al., 2011; Schott et al., 2011; Smet et al., 2013; Vermoote et al., 2011). Finally, TLR9 is able to recognize **un-methylated CpG DNA** in bacteria (Hemmi et al., 2000). For *H. pylori*, TLR9-mediated recognition of DNA has been demonstrated in DCs, followed by the induction of IL-6 and IL-12 secretion (Rad et al., 2009).

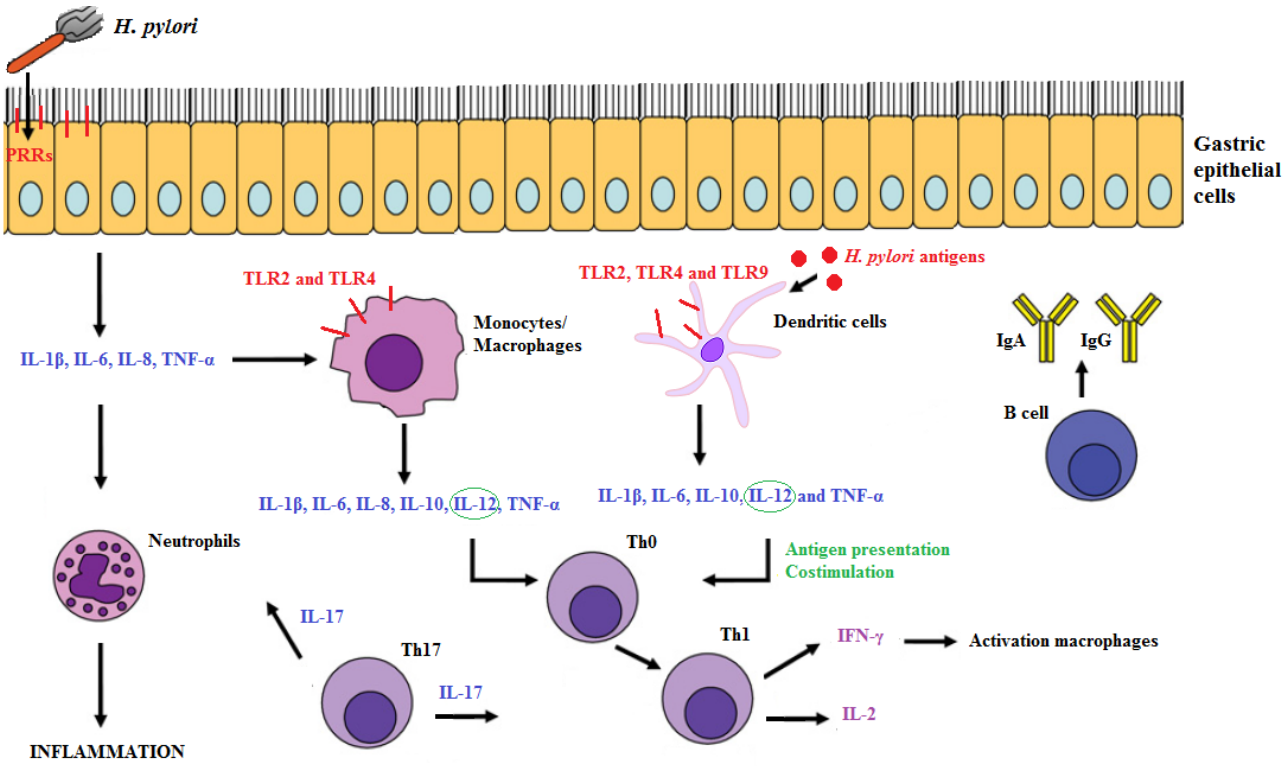

Figure 2. A schematic overview of the innate and the acquired immune responses following an *H. pylori* infection. *H. pylori* pathogen-associated molecular patterns are detected by pattern recognition receptors (PRRs) expressed on gastric epithelial cells. This results in the secretion of pro-inflammatory cytokines, which leads to an infiltration of the gastric mucosa by neutrophils, monocytes/macrophages and dendritic cells that cause gastric inflammation. The macrophages and dendritic cells are further stimulated by *H. pylori* antigens via their Toll-like receptors (TLR), resulting in the secretion of a variety of pro-inflammatory cytokines, and in the recruitment of T-cells. Moreover, via the secretion of IL-12 by macrophages and dendritic cells and via antigen presentation and co-stimulation by dendritic cells, Th0 cells differentiate into Th1 cells, which in their turn produce IFN-γ to activate macrophages. In addition, Th17 cells attract more neutrophils and modulate the Th1 response via the secretion of IL-17. Finally, *H. pylori* also stimulates the production of IgA and IgG antibodies (adapted from Mahdi, 2014).

3.2 Acquired immune response

A *Helicobacter* infection provokes a humoral and cellular immune response in humans, and this acquired immune response contributes to the development of gastric disease (Robinson et al., 2007).

3.2.1 Humoral immunity

H. pylori stimulates the production of mucosal and systemic Immunoglobulin A (IgA) and IgG antibodies (**Figure 2**), but the effect of these antibodies on the bacterial colonization density remains controversial. Protective effects by administration of IgA have been reported, whereas in other studies bacterial colonization is actually promoted by IgA and IgG (Akhiani et al., 2004; Akhiani et al., 2005; Czinn et al., 1993). However, the humoral immune response plays an important role in *H. pylori* pathogenesis through participation in an *H. pylori*-induced autoimmune process. In this, anti-*H. pylori* antibodies, directed to *H. pylori* LPS structures, cross-react with host antigens on gastric epithelial cells, such as Lewis y, Lewis x and H type I blood group structures, and induce local inflammation and damage (Amedei et al., 2003; Appelmelk et al., 1996; D'Elios et al., 2004). Low serum levels of IgG have also been demonstrated in *H. suis*-infected mice, but this antibody response does not result in protective immunity (Flahou et al., 2009).

3.2.2 T-cell response

In humans infected with *H. pylori*, a **Th1 response** dominates in the gastric mucosa. Th1 cells produce IFN-γ that leads to the activation of macrophages and an increase of their bactericidal activity, together with the secretion of pro-inflammatory factors such as TNF-α, IL-12 and IL-18 (Bamford et al., 1998, D'Elios et al., 1997; Fan et al., 1994; Ma et al., 2003; **Figure 2**). It has been shown that the number of IFN-γ-secreting cells in the infected human gastric mucosa correlates with the severity of the gastritis (Lehmann et al., 2002). Moreover, IFN-γ seems to be a key mediator in the induction of pre-cancerous gastric atrophy, metaplasia and dysplasia (Gea, 2003). Several virulence factors are potential promoters of a Th1 response, including the plasticity region locus jhp0947–jhp0949, which is associated with duodenal ulcer disease, and the *H. pylori* neutrophil-activating protein NapA (Amedei et al., 2006; Robinson et al., 2005). More recently, also **Th17 cells** have been shown to be involved in the immune response towards an *H. pylori* infection, and more specifically, in the attraction of neutrophils by the production of IL-17 (Scott Algood et al., 2007; Shiomi et al., 2008; **Figure 2**). In different mouse models

for *H. pylori* infection, a mixed Th17/Th1 response has been demonstrated. In this, Th17 cells precede and modulate the Th1 response by secretion of IL-17 (Shi et al., 2010). Similar to *H. pylori*, an *H. felis* infection is also characterized by a Th1/Th17-polarized response, as has been demonstrated in experimental mouse models (Ding et al., 2013; Hitzler et al., 2012; Stoicov et al., 2009). Experimental *H. suis* infection in mice also leads to a predominant Th17 response, but in contrast with *H. pylori* and *H. felis*, accompanied by a secondary **Th2 response** (Flahou et al., 2012).

3.3 Evasion of the immune response

Although *H. pylori*-infected individuals generate a strong immune response, as described above, they fail to eradicate the bacterium. Emerging evidence suggests an important role for regulatory T-cells (**Tregs**) in the maintenance of an *H. pylori* infection (Smith, 2014). Tregs are important in the prevention of undesirable immune-mediated conditions such as allergy and autoimmunity. Moreover, they are able to down-regulate the immune response to a variety of infections (Coombes et al., 2005; Hawrylowicz and O'Garra, 2005; McGuirk et al., 2002). Tregs usually act via the secretion of suppressive cytokines such as IL-10 and TGF-β (O'Garra et al., 2004; Thompson and Powrie, 2004; Weiner, 2001). Elevated levels of these 2 cytokines have been demonstrated in the human gastric mucosa infected with *H. pylori* (Hida et al., 1999; Lindholm et al., 1998). By eliciting a Treg response, *H. pylori* succeeds to suppress gastric immune and inflammatory responses and is able to maintain a chronic colonization (Lundgren et al., 2003; Strömberg et al., 2005).

In addition, *in vivo* analyses in mice have shown that the **TLR2**-mediated immune response is important to promote survival of the *H. pylori* bacteria. *H. pylori* possibly mediates an immune tolerance through TLR2-derived signals, inhibiting the Th1-response and in this way evading the host defense (Sun et al., 2013).

Finally, other virulence factors of *H. pylori* have been suggested to play a role in evading the host immune response. *H. pylori* **CagA** and a **proliferation-inhibiting protein** are able to induce lymphocyte cell cycle arrest (Knipp et al., 1996; Paziak-Dománska et al., 2000). *H. pylori* **VacA** has been shown to inhibit T-cell responses by blocking MHC class II-mediated antigen presentation and T-cell activation (Boncristiano et al., 2003; Gebert et al., 2003; Molinari et al., 1998). The phase-variable expression of sialyl-Lewis X antigen on *H. pylori* **LPS** contributes to the evasion of the immune response upon initial infection, by molecular mimicry of host glycosylated structures (Monteiro et al., 2000; Moran et al., 2009).

4. *In vivo* models for studying canine and feline *Helicobacter*-related pathogenesis

4.1 Experimental infection studies in cats and dogs

To study the pathogenesis of canine and feline NHPH infections, experimental infection studies with *H. felis* have been performed in their natural hosts, cats and dogs. Experimental infection of specific-pathogen-free (SPF) cats with *H. felis* was accompanied with a mononuclear infiltration throughout the gastric mucosa, with a follicular organization of the inflammatory cells (Scanziani et al., 2001). Moreover, lymphoid follicular hyperplasia, atrophy and fibrosis, mainly in the antrum of the stomach, have also been associated with *H. felis* infection in SPF cats (Simpson et al., 2000). In young gnotobiotic dogs, experimental *H. felis* infection caused a lymphoid hyperplasia in the fundus and the body of the stomach (Diker et al., 2002). In contrast, in another study, a similar degree of inflammation was found in both *H. felis*-infected SPF dogs and uninfected control dogs (Simpson et al., 1999). This might be explained by a lower virulence of the infecting *H. felis* strains.

4.2 Experimental infection studies in rodents

Besides SPF cats and dogs, rodent models have been shown to be useful to study the interplay between gastric helicobacters and their host and in particular, to study the pathogenesis of gastric NHPH infections in humans. To date, in many reports, the pathogenesis of canine and feline *Helicobacter* infections has been studied in rodent models. Experimental infection studies in mice and in Mongolian gerbils are most frequently used (Flahou et al., 2016).

4.2.1 Mouse models

Mice are the most commonly used laboratory animals for *Helicobacter* research. Many different mouse strains are available to study specific characteristics of the *Helicobacter*-related pathogenesis.

H. felis is the first NHPH species for which a high and reproducible colonization ability was shown in mice (Dick et al., 1989, Lee et al., 1990). *H. felis*-infected mice have been widely used as a model for *H. pylori* infection in humans. Similar to *H. pylori*, *H. felis* causes a predominant Th1/Th17 immune response in mice (Ding et al., 2013; Hitzler et al., 2012; Stoicov et al., 2009). However, this species lacks several important *H. pylori* virulence factors, and therefore, an *H. felis* infection might have different disease outcomes than an *H. pylori* infection (Flahou et al., 2016).

Depending on the mouse strains used, different outcomes have been described for an experimental *H. felis* infection with regard to colonization density, inflammatory response and severity of the gastritis. In a study using Swiss Webster outbred mice, *H. felis* bacteria were mainly located in the mucus layer and deep in the gastric pits. An acute inflammatory response with eosinophils and neutrophils was seen at 2 weeks after inoculation and was even more pronounced at 3 weeks after inoculation, with an increasing number of lymphocytes and the formation of micro-abscesses. After 8 weeks post inoculation, multiple micro-abscesses were still present in the pyloric mucosa and more remarkably, large lymphoid follicles were present in the submucosa (Lee et al., 1990). In addition, long-term *H. felis* infection in Swiss outbred mice has been associated with glandular and lymphoid tissue lesions at 13 months post inoculation (Ferrero et al., 2000). In contrast, in a study using *H. felis*-infected BALB/c mice, only limited inflammation was seen in the stomach up to 19 months post inoculation. After 22 months post inoculation, however, large lymphoid aggregates in the mucosa and submucosa were found (Enno et al., 1995). These results were confirmed in another study, in which BALB/c mice presented only a very mild antral gastritis in response to *H. felis* colonization, at 6 months after inoculation (Sakagami et al., 1996). In that same study, a more severe gastritis was found in the fundus of the stomach of SJL and C57BL/6 mice. In these mice, an inverse relation between the degree of *H. felis* colonization and the severity of the gastritis was noted. Based on the above described studies, it is clear that the host plays an important role in the disease outcome following an *H. felis* infection.

Experimental *H. felis* infections in mouse models have also been frequently used to study gastric carcinogenesis. For instance, the effect of gastrin during an *H. felis* infection has been monitored, using hypergastrinemic, gastrin-deficient, and wild-type C57BL/6 mice. Gastrin is produced by G cells mainly in the antrum of the stomach in response to food intake, and it stimulates the secretion of gastric acid by parietal cells. In this study, only the *H. felis*-infected hypergastrinemic mice developed severe corpus dysplasia with mild gastric atrophy, whereas only a mild to moderate antral dysplasia was seen in the gastrin-deficient and the wild-type mice. Thus, gastrin seems to be an essential cofactor for the development of severe gastric dysplasia in *H. felis*-infected C57BL/6 mice (Takaishi et al., 2009). Also, the role of *H. felis* with regard to iron deficiency has been studied in hypergastrinemic C57BL/6 mice. Following an *H. felis* infection, decreased gastric iron concentrations and a reduced number of parietal cells were noted (Thomson et al., 2012). Finally, MALT-lymphoma like lesions have been described in BALB/c mice chronically infected with *H. felis*, indicating that this mouse model is suitable to study gastric MALT lymphoma (Stolte et al., 2002).

In contrast to *H. felis*, less experimental infection studies have been performed using the other gastric canine and feline NHPH species. The pathological changes in the mouse stomach in response to an infection with *H. salomonis* or *H. bizzozeronii* have been evaluated and compared to an *H. felis* infection. *H. salomonis* was not able to colonize the murine stomach and only moderate pathological changes were detected after *H. bizzozeronii* infection, whereas *H. felis* induced more severe inflammatory changes (De Bock et al., 2005). Besides this *Helicobacter* species-related variation in colonization and inflammation level, differences in virulence can also be seen between different strains of the same species, as has been shown for *H. pylori*, *H. felis* and *H. suis* infection in several mouse models (De Bock et al., 2005; Flahou et al., 2012; Van Doorn et al., 1999).

Before the onset of this PhD research, no *in vitro* isolates of *H. heilmannii* s.s. were available. This hampered experimental infection studies with this agent. Only one infection study in BALB/c mice with 10 different homogenates from "*H. heilmannii*"-like infected gastric tissue from humans and animals, had been performed so far. In this study, bacteria were detected in large numbers in both fundus and antrum of the stomach, and the mice developed gastric MALT lymphoma after 18 months of infection (O'Rourke et al., 2004a). However, since homogenized gastric tissue was used as inoculum, it cannot be excluded that other microorganisms were inoculated together with "*H. heilmannii*", which might influence the disease progression. Moreover, the *Helicobacter* bacteria in the homogenates in fact belonged not only to the *H. heilmannii* species, but also to the porcine *H. suis* (Flahou et al., 2010). Thus, to obtain better insights into the pathogenesis of human gastric disease associated with *H. heilmannii* s.s., experimental infection studies with pure cultures of this microorganism are essential. Recently, such cultures have become available (Smet et al., 2012).

4.2.2 Mongolian gerbil models

Besides mice, also Mongolian gerbils have been frequently used to study *Helicobacter*-related gastric pathology. The Mongolian gerbil model has been shown to be suitable for studying hypergastrinemia and hypochlorhydria induced in response to *H. pylori* infection (Takashima et al., 2001). Moreover, Mongolian gerbils have been shown to respond more aggressively to an *H. pylori* infection than mice and these animals rapidly develop severe lesions, including intestinal metaplasia and gastric ulcers. This is an advantage for studying the development of gastric carcinoma (Court et al., 2002; Matsumoto et al., 1997; Zheng et al., 2004).

With regard to canine and feline helicobacters, the effects of infections with *H. felis*, *H. bizzozeronii* and *H. salomonis* have been examined in Mongolian gerbils. At 4 weeks after oral inoculation of gerbils with *H. felis*, an antral-dominant gastritis, together with an increased antral epithelial cell proliferation is seen (Court et al., 2002). Intragastrically *H. felis*-inoculated gerbils show an explicit loss of parietal cells extending from the limiting ridge of the forestomach/stomach transition zone, into the fundus (**Figure 3, B**). In addition, there is a high cell proliferation rate in the mucosal area devoid of parietal cells, and apoptotic cells are present at the transition zone between affected and normal gastric tissue. Moreover, a high number of *H. felis* bacteria are closely associated with the parietal cells (De Bock et al., 2006a; De Bock et al., 2006b). Similar but less severe lesions are found in gerbils infected with *H. bizzozeronii* (**Figure 3, C**). In contrast, *H. salomonis* is not able to colonize the Mongolian gerbil stomach (De Bock et al., 2006a; De Bock et al., 2006b). For *H. heilmannii*, no experimental infection studies had been performed in Mongolian gerbils at the start of this PhD research.

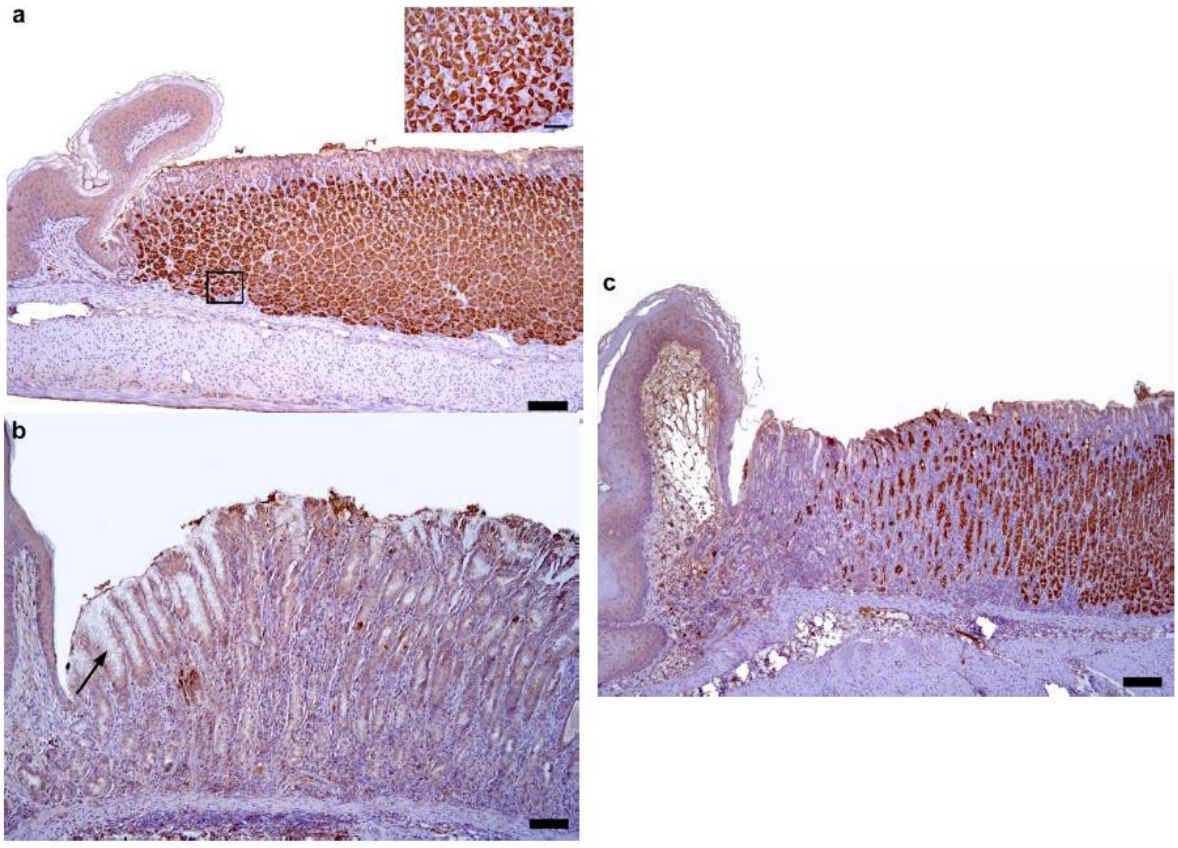

Figure 3. (A) H^+-K^+-ATPase staining of a control gerbil, indicating differential staining between purple chief cells and brown stained parietal cells (Bar insert = 50 µm). (B) Gerbil infected with *H. felis* ATCC 49179, showing a severe loss of parietal cells. (C) Gerbil infected with *H. bizzozeronii* CCUG 35545, showing a mild loss of parietal cells near the limiting ridge. Bars = 100 µm. (adapted from De Bock et al., 2006a).

5. Diagnosis of gastric non-*Helicobacter pylori Helicobacter* infections in cats, dogs and humans

For the detection of *H. pylori* infections in humans, multiple non-invasive tests have been optimized. These include serology (antibody detection), the detection of bacterial antigens and DNA in stool samples, and the detection of urease activity in breath (urea breath test) (Amorim et al., 2015; Neiger et al., 2000). Serology by enzyme-linked immunosorbent assay (ELISA) is frequently used as a diagnostic tool in medical hospitals. Several ELISA test kits are available that measure circulating IgG in serum (Neiger et al., 2000). In contrast, specific non-invasive tests for serological or fecal detection of *H. heilmannii* s.l. do not exist at the moment (Baele et al., 2009; Bento-Miranda and Figueiredo, 2014). Also the urea breath test, used to diagnose an infection with *H. pylori*, is often negative in patients infected with *H. heilmannii* s.l. and is therefore of limited diagnostic value for these species (Matsumoto et al., 2014). This can be explained by the fact that infections with NHPH bacteria are, in contrast to *H. pylori* infections, more often focal and predominantly found in the antrum of the stomach (Stolte et al., 1997).

Invasive tests are currently the only option for diagnosis of NHPH infections in humans. During endoscopy, a gastric biopsy sample is collected and used for histology, bacterial culture, urease testing or PCR (Bento-Miranda and Figueiredo, 2014). The diagnosis of *H. heilmannii* s.l. in general can be based on histological detection using silver staining-based techniques, such as the Steiner and the Whartin-Starry stains (Waring and Shilkin, 1989). However, it is not recommended to identify the different *Helicobacter* species using morphological characteristics such as size, number of spirals and tightness of coils, because different species may be morphologically very similar, and variation in morphology within a single species may also occur (Haesebrouck et al., 2009).

The use of *in vitro* culture as a diagnostic test is also not feasible due to the very fastidious nature of NHPH bacteria. So far, only *H. bizzozeronii* and *H. felis* have been isolated from the human gastric mucosa (Andersen et al., 1996; Jalava et al., 2001; Kivisto et al., 2010; Wüppenhorst et al., 2013).

Currently, the most accurate method available for conclusive species identification of canine and feline *Helicobacter* species is the use of PCR, targeting species-specific genes, followed by sequencing of the amplified DNA-fragments. The urease A and B (*ureA*, *ureB*) genes, the heat shock protein 60 (*hsp60*) gene and the gyrase subunit B (*gyrB*) gene are considered to be appropriate candidate genes to distinguish between canine and feline *H. heilmannii* s.l. (Baele et al., 2004; Hannula and Hänninen, 2007; Mikkonen et al., 2004; Priestnall et al., 2004).

It should be noted that accurate identification of gastric helicobacters to the species level is essential in order to determine the prevalence and clinical significance of all taxa.

Also in cats and dogs, non-invasive diagnosis of gastric NHPH infections remains a challenge. Since these animals often harbor several *Helicobacter* species, it would be difficult to correctly identify the infecting species via serodiagnosis (Neiger et al., 2000). Routine stool tests for detection of NHPH bacteria to the species level are also not available. Although *H. heilmannii* s.l. DNA has been detected in fecal samples and saliva of domestic cats and dogs (Berlamont et al., 2016; Ekman et al., 2013; Ghil et al., 2009; Recordati et al., 2007; Shojaee Tabrizi et al., 2010), it remains to be investigated whether or not infected animals continuously shed a detectable amount of bacterial DNA, before reliable diagnostic stool- or saliva tests can be developed.

For the invasive detection of NHPH species in cats and dogs, a gastric biopsy is required, which is obtained through endoscopy under anesthesia or through necropsy. Similar as in humans, the biopsy sample can be used for bacterial culture, histopathology, urease testing or DNA extraction and PCR (Amorim et al., 2015).

6. Treatment of gastric non-*Helicobacter pylori Helicobacter* infections in cats, dogs and humans

H. heilmannii s.l. eradication treatment is indicated for human patients with severe clinical signs and pathology. The treatment strategies that are used nowadays for *H. heilmannii* s.l. are the same as the triple therapy regimens that are used for *H. pylori* eradication. Triple therapy comprises a proton pump inhibitor such as omeprazole in combination with two antimicrobial agents, namely clarithromycin or tetracycline together with metronidazole or amoxicillin. In general, treatment is continued for two weeks (Bento-Miranda and Figueiredo, 2014; Goddard et al., 1997; Kaklikkaya et al., 2002; Sykora et al., 2003). However, the standard *H. pylori* triple therapy is not always effective to treat NHPH infections and no randomized trials have been performed to evaluate the most suitable treatment for these species. Only few studies have been performed to clarify the intrinsic antimicrobial susceptibility and the presence of acquired resistance in feline and canine NHPH strains. An *in vitro* antimicrobial susceptibility study of *H. bizzozeronii*, *H. felis*, and *H. salomonis* isolates obtained from cats and dogs showed that they were sensitive to ampicillin, clarithromycin and tetracycline, but acquired resistance to metronidazole was observed for some *H. bizzozeronii* and *H. felis* isolates (Van den Bulck et al., 2005b). In a Finnish patient infected with *H. bizzozeronii*, a triple therapy with tetracycline,

metronidazole and lansoprazole for one week could suppress the symptoms, but the patient continued to suffer from a mild nausea and the infection was not cleared. After isolation of *H. bizzozeronii* from the patient's stomach, acquired resistance against tetracycline and metronidazole was shown for this strain (Schott et al., 2012). Furthermore, it was demonstrated that the acquired resistance to metronidazole in *H. bizzozeronii* was due to the contingency nature of an oxygen-insensitive NAD(P)H-nitroreductase. This phenomenon was also described for *H. heilmannii* (Kondadi et al., 2013).

Further research is necessary to obtain better insights in antimicrobial susceptibility of all feline and canine NHPH species, in order to be able to start the most appropriate treatment in patients suffering from *H. heilmannii* s.l infection from feline and canine origin.

Whether antimicrobial therapy should be instituted in domestic pets with gastritis is currently debatable. The lack of knowledge regarding the pathogenicity of the different *Helicobacter* species in cats and dogs makes treatment decisions difficult. They should be based on a biopsy-confirmed gastritis and presence of *Helicobacter* bacteria, in combination with appropriate clinical signs and/or gastric lesions. The current recommended treatment regimens for cats and dogs include amoxicillin or tetracycline, metronidazole or clarithromycin, together with bismuth subsalicylate, and with or without a proton pump inhibitor such as omeprazole or a histamine H_2-receptor blocker such as famotidine, for a duration of two to three weeks (Leib et al., 2007; Neiger and Simpson, 2000). However, only few controlled, randomized, blind therapeutic studies in cats and dogs have been published. In addition, several studies indicate the difficulty of eradicating *Helicobacter* infections in cats and dogs. Although the frequency of vomiting and gastric lesions improve in the treated animals, in most cases, no long-term eradication of the *Helicobacter* infection can be achieved. Whether the antibiotic failure is due to reinfection or recrudescence remains unclear (Cornetta et al., 1998; Leib et al., 2007; Neiger and Simpson, 2000; Perkins et al., 1997; Stoschus et al., 1996).

Given the increasing problem of antimicrobial resistance of bacteria in general, other alternative approaches would be valuable for controlling gastric *Helicobacter* infections and disease (Selgrad and Malfertheiner, 2008). A first alternative approach is the use of probiotics such as *Bifidobacterium* and *Lactobacillus*. Protective effects of these probiotics on *H. pylori* colonization have been described in several studies (Fijan, 2014; Navarro-Rodriguez et al., 2013; Zheng et al., 2013), but large scale studies have not been performed so far. Moreover, the effect of probiotics on gastric NHPH infections remains to investigated. To date, probiotics are only used to increase the efficacy of antibiotic therapy to eradicate *H. pylori*, or to protect the

human microflora from antibiotic-associated side-effects (Cremonini et al., 2002; Servin, 2004; Talebi, 2016).

Prophylactic and therapeutic vaccination are the most promising alternative approaches for prevention and treatment of gastric *Helicobacter* infections. Although vaccination against *H. pylori* has been extensively studied, there is no efficient vaccine in clinical practice yet. *H. pylori* vaccines based on whole cell lysates as well as subunit vaccines using purified or recombinant proteins have been tested and are able to induce protection, but an ideal vaccine formulation with a suitable adjuvant has not yet been found. The complicated host immune response and the high genetic diversity of *H. pylori* also contribute to the difficulty of vaccine development (Talebi, 2016).

With regard to NHPH, only a few preliminary immunization studies against *H. felis* and *H. suis* have been performed in mouse models. In these studies, a partial or complete protection against *H. felis* or *H. suis* challenge was obtained, but further optimization of vaccine formulations is necessary (Corthésy et al., 2005; Flahou et al., 2009; Vermoote et al., 2012; Vermoote et al., 2013; Yamaguchi et al., 2003). To date, there are no vaccines available in clinical practice for prophylactic nor therapeutic treatment of NHPH infections in humans and animals.

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SCIENTIFIC AIMS

Besides *H. pylori*, other non-*H. pylori Helicobacter* (NHPH) species, also referred to as *H. heilmannii* sensu lato (s.l.) have been associated with gastric disease in humans. *H. heilmannii* s.l. represents a group of closely related helicobacters that colonize the stomach of different animal species, including *H. suis* in pigs and *H. bizzozeronii*, *H. felis*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and *H. heilmannii* sensu stricto (s.s.) in cats and dogs. These NHPH species are characterized by an extremely fastidious nature, which so far has resulted in only a limited number of *in vitro* isolates available worldwide. Before the onset of this PhD research, the pathogenesis of *H. suis*, *H. bizzozeronii*, *H. felis* and *H. salomonis* infections had been studied in experimental rodent models by using pure *in vitro* isolates. In contrast, no infection studies had been performed using pure cultures of *H. heilmannii* s.s. In our laboratory, 9 different isolates, classified as *H. heilmannii* s.s. on the basis of their *ureAB* and 16S rRNA gene sequences, were cultured from the gastric mucosae of stray cats.

The **first aim** of this PhD research was to study the bacterium-host interactions of these 9 *H. heilmannii* strains in a Mongolian gerbil model, in order to obtain better insights into the pathogenesis of human gastric disease associated with this NHPH species.

Clear differences in colonization capacity and virulence properties between the 9 feline *H. heilmannii* s.s. strains were detected. They were divided into a highly virulent and a low virulent group. The **second aim** of this thesis was to determine if these differences in pathogenicity might be related to the presence or absence of specific virulence-associated genes. Therefore, the genomes of the 9 isolates were sequenced. In addition, several *in vitro* binding assays were carried out to study the possible differences in their adhesion capacity to the gastric mucosa. Results from this second study showed phylogenetic differences between low and highly virulent strains and led to the reclassification of the low virulent strains into a closely related, but novel feline gastric *Helicobacter* species, for which we proposed the name *H. ailurogastricus*.

The mechanisms used by *H. heilmannii* and *H. ailurogastricus* to colonize the gastric mucosa remain largely unknown. *H. heilmannii*, *H. ailurogastricus* and most other NHPH lack the well-characterized and important *H. pylori* Hop and Hom adhesins. This indicates that other outer membrane proteins (OMPs) are used for colonization of the gastric mucosa by these pathogens. Therefore, the **third aim** of this PhD research was to characterize the OMP repertoire of *H. heilmannii* and *H. ailurogastricus* and to compare this with the other gastric *Helicobacter* species by using phylogenetic analyses. We also aimed to identify the OMP families that are possibly involved in colonization and virulence properties of these micro-organisms.

EXPERIMENTAL STUDIES

Chapter 1

Diversity in bacterium-host interactions within the species *Helicobacter heilmannii* sensu stricto

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Abstract

Helicobacter (H.) heilmannii sensu stricto (s.s.) is a zoonotic bacterium that naturally colonizes the stomach of cats and dogs. In humans, this microorganism has been associated with gastritis, peptic ulcer disease and mucosa associated lymphoid tissue (MALT) lymphoma. Little information is available about the pathogenesis of *H. heilmannii* s.s. infections in humans and it is unknown whether differences in virulence exist within this species. Therefore, a Mongolian gerbil model was used to study bacterium-host interactions of 9 *H. heilmannii* s.s. strains. The colonization ability of the strains, the intensity of gastritis and gene expression of various inflammatory cytokines in the stomach were determined at 9 weeks after experimental infection. The induction of an antrum-dominant chronic active gastritis with formation of lymphocytic aggregates was shown for 7 strains. High-level antral colonization was seen for 4 strains, while colonization of 4 other strains was more restricted and one strain was not detected in the stomach at 9 weeks post infection. All strains inducing a chronic active gastritis caused an up-regulation of the pro-inflammatory cytokine IL-1β in the antrum. A reduced antral expression of H^+/K^+ ATPase was seen in the stomach after infection with 3 highly colonizing strains and 2 highly colonizing strains caused an increased gastrin expression in the fundus. In none of the *H. heilmannii* s.s.-infected groups, IFN-γ expression was up-regulated. This study demonstrates diversity in bacterium-host interactions within the species *H. heilmannii* s.s. and that the pathogenesis of gastric infections with this microorganism is not identical to that of an *H. pylori* infection.

Introduction

Helicobacter (H.) pylori is the most prevalent *Helicobacter* species colonizing the gastric mucosa of humans and has been associated with gastritis, peptic ulcer disease and gastric cancer (Kusters et al., 2006; Parsonnet et al., 1991; Stolte and Eidt, 1993). Besides *H. pylori*, other morphologically distinct non-*H. pylori Helicobacter* (NHPH) species, also referred to as *H. heilmannii* sensu lato (s.l.) (Haesebrouck et al., 2011) have been associated with gastric disease in humans (De Groote et al., 2005; Haesebrouck et al., 2009; Neiger et al., 1998; O'Rourke et al., 2004b; Trebesius et al., 2001; Van den Bulck et al., 2005). NHPH represents a group of closely related but distinct bacterial species, mainly found in different animal species, such as *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. heilmannii* sensu stricto (s.s.), *H. cynogastricus* and *H. baculiformis* in cats and dogs and *H. suis* in pigs (Haesebrouck et al., 2009; Hermanns et al., 1995; Hwang et al., 2002; Neiger et al., 1998; Priestnall et al., 2004; Strauss-Ayali et al., 2001; Van den Bulck et al., 2005; Wiinberg et al., 2005). These microorganisms are characterized by their extremely fastidious nature, which so far has resulted in a limited number of *in vitro* isolates available worldwide. *H. heilmannii* s.s. has only recently been isolated and cultured *in vitro* (Smet et al., 2012). It has been detected in wild feline and in human gastric biopsies, but it is most commonly found in the gastric mucosa of cats and dogs with a prevalence ranging from 20 to 100% (Haesebrouck et al., 2009; Hwang et al., 2002; Neiger et al., 1998; O'Rourke et al., 2004b; Van den Bulck et al., 2005). Although this bacterium has been associated with chronic active gastritis in cats and dogs (Hwang et al., 2002), its pathogenic significance remains enigmatic and is probably strain-dependent. In humans, *H. heilmannii* s.s. has been detected in 8–19% of gastric biopsies with histological evidence of NHPH infection (Haesebrouck et al., 2009; Trebesius et al., 2001; Van den Bulck et al., 2005). Infection with this bacterium in humans has been associated with gastritis, peptic ulcer disease and mucosa associated lymphoid tissue (MALT) lymphoma (Baele et al., 2009; Haesebrouck et al., 2009; O'Rourke et al., 2004a; Trebesius et al., 2001; Van den Bulck et al., 2005). Several infection studies in experimental animal models have been performed to investigate the pathogenesis of *H. pylori* infections in humans. In contrast, little information is available dealing with the pathogenesis of *H. heilmannii* s.s. infections in humans. This bacterium has been propagated in mice for up to 28 months and was able to induce MALT lymphoma in the stomachs of these animals (O'Rourke et al., 2004a). However in this mouse experiment, homogenized gastric tissue was used as inoculum. This implies that other microorganisms were inoculated together with *H. heilmannii* s.s., which might influence the results, as has been described previously

(Flahou et al., 2010). Thus, to obtain better insights into the pathogenesis of human gastric disease associated with *H. heilmannii* s.s., experimental infection studies with pure cultures of this microorganism are essential. Therefore, the aim of the present study was to study bacterium-host interactions of 9 *H. heilmannii* s.s. strains, isolated from the gastric mucosa of different cats. The Mongolian gerbil model has previously been shown to be a useful animal model to study *Helicobacter*-related gastric pathology in humans and was therefore used in the present study (Flahou et al., 2010; O'Rourke and Lee, 2003; Rogers and Fox, 2004).

Material and methods

Bacterial strains

Nine strains of *H. heilmannii* s.s. were obtained from the gastric mucosa of different cats and designated ASB1 (= type strain, DSM 23983, (Smet et al., 2012)), ASB2, ASB3, ASB6, ASB7, ASB9, ASB11, ASB13 and ASB14. Bacteria were cultivated on biphasic *Brucella* agar plates (Oxoid, Basingstoke, UK) supplemented with 20% (v/v) fetal calf serum (HyClone, Logan, UT, USA), 5 mg/L amphothericin B (Fungizone, Brystal-Myers Squibb, New York, USA), Skirrow (Oxoid, contains 10 mg/L vancomycin, 5 mg/L trimethoprim lactate and 2500 U/L polymyxin B), Vitox supplement (Oxoid) and 0.05% HCl (pH 5). After incubation under microaerobic conditions (85% N2, 10% CO2, 5% O2; 37 °C), the bacteria were harvested and the final concentration was adjusted to 7×10^8 viable bacteria/mL, as determined by counting in a Neubauer counting chamber.

Animals, housing and experimental procedure

Specific-pathogen-free (SPF) female five-week-old Mongolian gerbils (Crl:MON (Tum), n = 48) were obtained from Charles River Laboratories (Lille, France). The animals were housed in filter top cages (1500 cm^2) on autoclaved wood shavings and autoclaved hay. They were fed ad libitum an autoclaved commercial diet (TEKLAD 2018S, containing 18% protein; Harlan, The Netherlands) and autoclaved water. For each of the 9 *H. heilmannii* s.s. strains tested, 5 animals were intragastrically inoculated 3 times at 2 days interval with 300 µL of a bacterial suspension. Three animals were inoculated with *Brucella* broth (pH 5, Oxoid) and served as negative controls. Inoculation was performed under brief isoflurane anaesthesia (2.5%), using a ball-tipped gavage needle. At 9 weeks after the first inoculation, the animals were euthanized by cervical dislocation under deep isoflurane anaesthesia (5%). The stomach and the duodenum of each gerbil were resected and samples were taken for histopathological examination and

quantitative real-time (RT)-PCR analysis. The *in vivo* experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2011/090).

Histopathology and immunohistochemistry

A longitudinal section, starting from the end of the forestomach and comprising the antrum and the fundus of the stomach and part of the duodenum, was cut along the greater curvature and fixed in 10% phosphate buffered formalin, processed by standard methods and embedded in paraffin for light microscopy. Three consecutive sections of 5 µm were cut. After deparaffinization and hydration, heat-induced antigen retrieval was performed in citrate buffer (pH 6). To block endogenous peroxidase activity and non-specific reactions, slides were incubated with 3% H_2O_2 in methanol (5 min) and 30% goat serum (30 min), respectively. The first section was stained with haematoxylin/eosin (H&E) to score the intensity of the gastritis according to the Updated Sydney System (Stolte and Meining, 2001) but with some modifications, as described previously (Flahou et al., 2010). On the second section, epithelial cell proliferation was determined by immunohistochemical staining using a mouse monoclonal anti-Ki67 antibody (1/25; Menarini Diagnostics, Zaventem, Belgium). Ki67-positive epithelial cells were counted in 5 randomly chosen High Power Fields at the level of the gastric pits (magnification: 400×), both in antrum and fundus. The average of the positive cell count was calculated for each experimental group in both stomach regions. Parietal cells were identified on the third section by immunohistochemical staining for the hydrogen potassium ATPase using a mouse monoclonal antibody (1/200; Abcam Ltd, Cambridge, UK). Incubation with primary antibodies directed against Ki67 and hydrogen potassium ATPase was followed by incubation with a HRP-labeled secondary antibody (Envision Link Mouse K4007, DakoCytomation, Heverlee, Belgium) for visualization.

DNA extraction and quantification of colonizing *H. heilmannii* s.s. in the stomach and duodenum

From each gerbil, samples from the fundus and the antrum of the stomach and from the duodenum were taken. Tissue samples were stored in 1 mL RNA later (Ambion, Austin, TE, USA) at -70 °C until RNA- and DNA-extraction. Tissue samples were homogenized (MagNALyser, Roche, Mannheim, Germany) and RNA and DNA were separated using TriReagent RT (Molecular Research Center Inc, Cincinnati, USA) according to the manufacturer's instructions. The number of colonizing *H. heilmannii* s.s. per mg gastric tissue was determined in the DNA samples using a *H. heilmannii* s.s.-specific quantitative RT-PCR.

For generation of the standard, part of the ureAB gene cluster (1224 bp) from *H. heilmannii* s.s. ASB1 was amplified using primers U430F and U1735R, as described previously (O'Rourke et al., 2004b). The standard consisted of 10-fold-dilutions starting at 10^8 PCR amplicons for each 10 µL of reaction mixture. One µL of extracted DNA template was suspended in a 10 µL reaction mixture consisting of 0.25 µL of both primers located within the 1224 bp fragment, to yield a 212 bp PCR product (sense primer: HH_SP1: 5'-CTT TCT CCT GGT GAA GTG ATT CTC-3', antisense primer: HH_RVQ: 5'-GCT GTA CCA GAG GCA ATG TCC AAG-3', annealing temperature 58 °C), 3.5 µL HPLC water and 5 µL SensiMix™ SYBR No-ROX (Bioline Reagents Ltd, UK). Both standards and samples were run in duplicate on a CFX96™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad, Hercules CA, USA). The Bio-Rad CFX Manager (version 1.6) software was used for calculation of threshold cycles (Ct)-values and melting curve analysis of amplified DNA. The average values of the duplicates were used for quantification of *H. heilmannii* s.s. DNA in the tissue samples.

RNA preparation and gene expression

Total RNA, from the tissue samples, was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Purity of RNA was demonstrated by measuring the ratio of absorbance at 260 nm and 280 nm with NanoDrop which in all cases was approximately 2. The RNA concentration in each sample was adjusted to 1 µg/µL and cDNA was synthesized immediately after RNA purification using iScript™ cDNA Synthesis Kit (Bio-Rad). Aliquots of cDNA (1/5 dilution) were used as a template for quantitative RT-PCR for measuring gene expression. The mRNA expression levels of different cytokines (IL-1β, IL-5, IL-6, IL-10, IL-12p40, IL-17, IFN-γ and TNF-α), gastrin and H^+/K^+ ATPase were quantified. The housekeeping genes *GAPDH*, *β-actin* and *HPRT* were included as reference genes. Primer sequences are shown in **Table 1** (Chirgwin et al., 2002; Crabtree et al., 2004; Sugimoto et al., 2009; Takenaka et al., 2006; Wiedemann et al., 2009; Yao et al., 2002). For all target genes and reference genes, the primer efficiencies were between 1.9 and 2.1. Reactions were performed in 10 µL volumes containing 1 µL cDNA, 0.05 µL of both primers, 3.9 µL HPLC water and 5 µL SensiMix™ SYBR No-ROX. The experimental protocol for PCR reaction (40 cycles) was performed on a CFX96™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad): denaturation for 15 min at 95 °C, followed by amplification cycles at 95 °C for 20 s, annealing at 60 °C for 30 s and extension at 73 °C for 30 s. Control reactions without the reverse transcriptase step were implemented to exclude DNA contamination of the RNA samples. No-template-control reaction mixtures were included and all samples were run in duplicate. The

Ct-values were normalized to the geometric mean of the Ct-values from the 3 reference genes, after which normalized mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Normality and variance homogeneity of data were analyzed by using Shapiro-Wilk normality test and Levene's test for homogeneity of variances. Gastritis scores, colonization capacity and ATPase and gastrin gene expression were compared between different infected groups and controls using Kruskall-Wallis analysis, followed by a Mann-Whitney *U* test. Cytokine expression and the number of Ki67-positive cells were analyzed by analysis of variance with a Bonferroni post hoc test. Differences were considered statistically significant at $p \leq 0.05$. SPSS Statistics 21 software (IBM) was used for all analyses.

Table 1. Primer pairs. Primer pairs used for measuring the mRNA expression levels of IL-1β, IL-5, IL-6, IL-10, IL-12p40, IL-17, IFN-γ, TNF-α, gastrin and H^+/K^+ ATPase are shown. The housekeeping genes *GAPDH, β-actin* and *HPRT* were included as reference genes.

	Primers	**Sequence (5'→3')**
Cytokines	IL-1β FW (Sugimoto et al., 2009)	GGC AGG TGG TAT CGC TCA TC
	IL-1β RV (Sugimoto et al., 2009)	CAC CTT GGA TTT GAC TTC TA
	IL-5 FW	AGA GAA GTG TGG CGA GGA GAG ACG
	IL-5 RV	ACA GGG CAA TCC CTT CAT CGG
	IL-6 FW	CAA AGC CAG AGC CAT TCA GAG
	IL-6 RV	GCC ATT CCG TCT GTG ACT CCA GTT TCT CC
	IL-10 FW	GGT TGC CAA GCC TTA TCA GA
	IL-10 RV	GCT GCA TTC TGA GGG TCT TC
	IL-12p40 FW	GAC ACG ACC TCC ACC AAA GT
	IL-12p40 RV	CAT TCT GGG ACT GGA CCC TA
	IL-17 FW (Sugimoto et al., 2009)	AGC TCC AGA GGC CCT CGG AC
	IL-17 RV (Sugimoto et al., 2009)	AGG ACC AGG ATC TCT TGC TG
	IFN-γ FW (Crabtree et al., 2004)	CCA TGA ACG CTA CAC ACT GCA TC
	IFN-γ RV (Crabtree et al., 2004)	GAA GTA GAA AGA GAC AAT CTG G
	TNF-α FW (Sugimoto et al., 2009)	GCT CCC CCA GAA GTC GGC G
	TNF-α RV (Sugimoto et al., 2009)	CTT GGT GGT TGG GTA CGA CA
Gastrin	Gastrin FW (Wiedemann et al., 2009)	GCC CTG GAA CCG CAA CA
	Gastrin RV (Wiedemann et al., 2009)	TTC TTG GAC AGG TCT GCT TTG AA
H^+/K^+ ATPase (parietal cells)	ATP4b FW	GGG GGT AAC CTT GAG ACC TGA TG
	ATP4b RV	AAG AAG TAC CTT TCC GAC GTG CAG
Reference genes	GAPDH FW (Takenaka et al., 2006)	AAC GGC ACA GTC AAG GCT GAG AAC G
	GAPDH RV (Takenaka et al., 2006)	CAA CAT ACT CGG CAC CGG CAT CG
	HPRT FW (Chirgwin et al., 2002)	CTC ATG GAC TGA TTA TGG ACA G
	HPRT RV (Chirgwin et al., 2002)	AGC TGA GAG ATC ATC TCC ACC AAT
	β-actin FW (Yao et al., 2002)	CCA AGG CCA ACC GCG AGA TGA C
	β-actin RV (Yao et al., 2002)	AGG GTA CAT GGT GGT GCC GCC AGA C

Results

Infection with virulent *H. heilmannii* s.s. strains induces an antrum-dominant chronic active gastritis

The stomach of all control animals showed a normal histomorphology (**Figure 1a**). Inflammation in the stomach of gerbils infected with *H. heilmannii* s.s. strains ASB1, ASB2, ASB3, ASB6, ASB11, ASB13 and ASB14 was marked by a chronic active gastritis with formation of lymphocytic aggregates in the lamina propria and submucosa of the antrum of the stomach (**Figure 1b**). The mucosal thickness was slightly increased and only few neutrophils were detected. In contrast, *H. heilmannii* s.s. strains ASB7 and ASB9 did not cause explicit antral inflammation and only a mild increase in lymphocytic cell number was observed in the lamina propria of the antrum of the stomach (**Figure 1c**). In all *H. heilmannii* s.s.-infected gerbils, only limited signs of inflammation were detected in the fundus of the stomach (Additional file 1). The antral inflammation scores of each individual animal are shown in **Figure 2d**. A statistically significant difference between inflammation scores for gerbils inoculated with ASB1, ASB2, ASB3, ASB6, ASB11, ASB13 and ASB14 compared with the control group was demonstrated (Mann-Whitney *U* test, $p < 0.05$, **Figure 2d**).

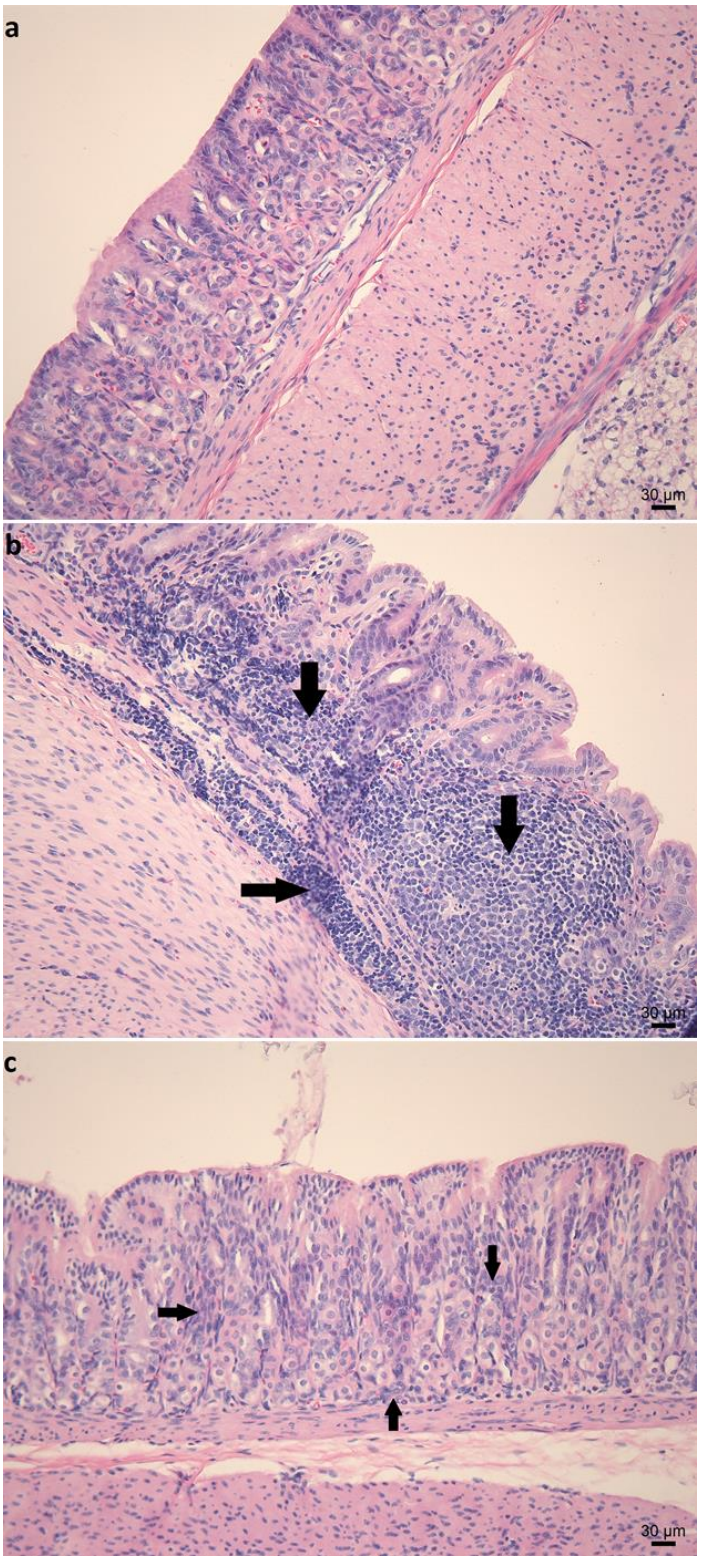

Figure 1. H&E staining of the antrum of a gerbil stomach. Normal histology of the antrum of a sham-inoculated negative control animal (a). Explicit lymphocytic infiltration of the lamina propria and the submucosa with the formation of lymphoid follicles (arrows) in the antrum of a gerbil inoculated with *H. heilmannii* s.s. ASB1 (b). Mild to absent lymphocytic infiltration (arrows) of the lamina propria in the antrum of a gerbil inoculated with *H. heilmannii* s.s. ASB7 (c). Bar = 30 µm.

Colonization capacity of *H. heilmannii* s.s. strains in the stomach and duodenum

Detection of *H. heilmannii* s.s. DNA with quantitative RT-PCR at 9 weeks post-infection revealed high-level colonization of ASB1, ASB2, ASB3 and ASB6 in the stomach (**Figure 2a-c**). In contrast, colonization of ASB7, ASB11, ASB13 and ASB14 was more restricted while ASB9 was not detected in the stomach (**Figure 2a-c**). In general, the colonization capacity in the fundus (**Figure 2a**) was lower than in the antrum (**Figure 2c**) for all strains tested and the lowest number of bacteria was detected in the duodenum (**Figure 2b**). In addition, a clear association was seen between the colonization capacity of the *H. heilmannii* s.s. strains and the gastric inflammation scores in the antrum of the stomach (**Figure 2c-d**).

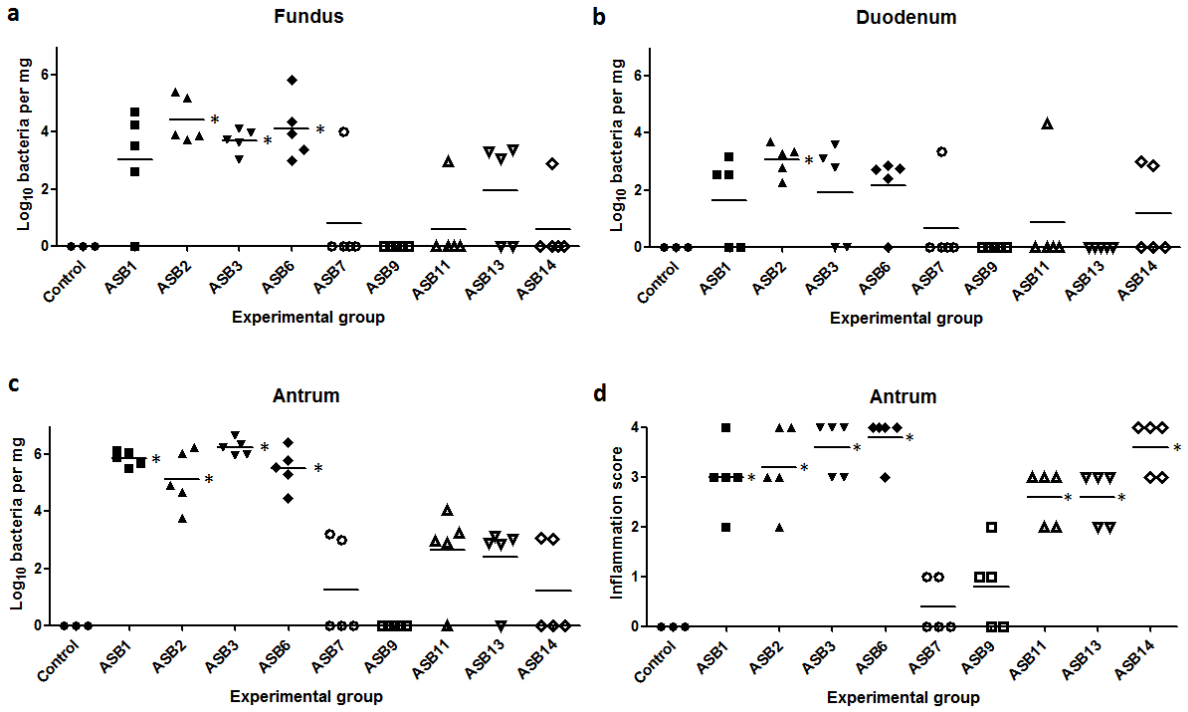

Figure 2. Colonization capacity of *H. heilmannii* s.s. strains and gastric inflammation score after experimental infection. Colonization capacity is shown as $\log_{10}$ values of *H. heilmannii* s.s. bacteria per mg tissue, detected with quantitative RT-PCR in the fundus (a) and the antrum (c) of the stomach and the duodenum (b). Results below detection limit (log 2.39 bacteria per mg tissue) were set as 0. Antral inflammation (d) was scored on a scale of 0 to 4 (0: no infiltration with mononuclear and/or polymorphonuclear cells; 1: mild diffuse infiltration with mononuclear and/or polymorphonuclear cells or the presence of one small (50–200 cells) aggregate of inflammatory cells; 2: moderate diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of 2–4 inflammatory aggregates; 3: marked diffuse infiltration with mononuclear and/or polymorphonuclear cells and/or the presence of at least five inflammatory aggregates; 4: diffuse infiltration of large regions with large aggregates of mononuclear and/or polymorphonuclear cells). Individual gerbils are depicted as figures around the mean (lines). Statistical significant differences compared to control animals are indicated by * (Mann-Whitney *U* test, $p < 0.05$).

Effect of *H. heilmannii* s.s. strains on gastric antral epithelial cell proliferation

Results of the gastric epithelial cell proliferation scoring in the antrum of the stomach are shown in **Figure 3c**. Significantly higher numbers of Ki67-positive proliferating epithelial cells were seen in the antrum of ASB1- and ASB6-infected gerbils, compared to the control group (ANOVA, $p < 0.05$, **Figure 3a-b**). Numbers of Ki67-positive cells were moderately increased in ASB2-, ASB3-, ASB11-, ASB13- and ASB14-infected gerbils, although not statistically significant. *H. heilmannii* s.s. strains ASB7 and ASB9 did not cause an increase of gastric epithelial cell proliferation. In addition, significantly higher numbers of proliferating epithelial cells were demonstrated in the antrum of ASB1-, ASB2-, and ASB6-infected gerbils, compared to gerbils infected with ASB7 and ASB9 (ANOVA, $p < 0.05$). In the fundus of all *H. heilmannii* s.s.-infected gerbils, the epithelial cell proliferation rate was not significantly higher compared to the control animals (Additional file 2).

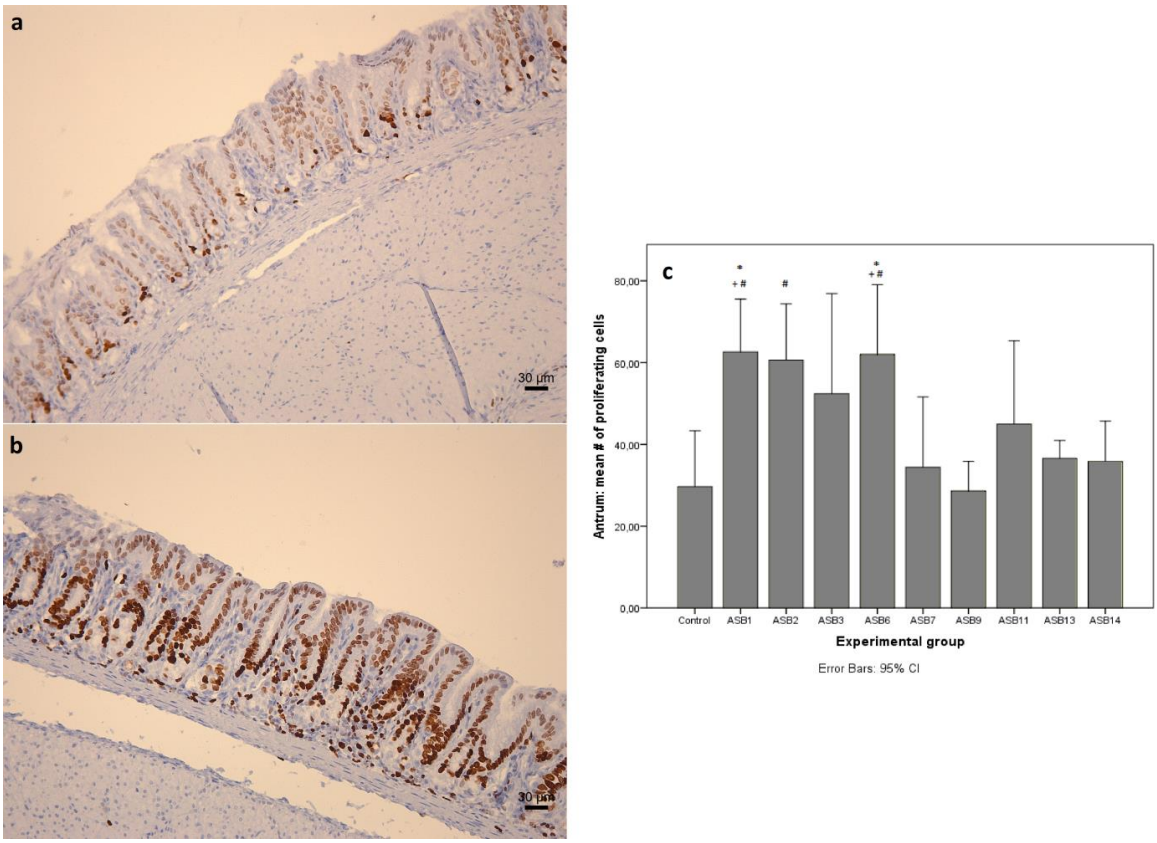

Figure 3. Gastric antral epithelial cell proliferation. Ki67 staining of the antrum of a gerbil inoculated with *H. heilmannii* s.s. ASB1 (b) showing a higher number of proliferating epithelial cells compared to a sham-inoculated negative control animal (a). The rate of epithelial cell proliferation was determined by counting Ki67-positive epithelial cells in 5 randomly chosen High Power Fields at the level of the gastric pits (magnification: 400×) in the antrum of the gerbil stomach (c). The mean numbers of Ki67-positive cells are shown in each experimental group. Significant differences between *H. heilmannii* s.s.-inoculated and control animals are indicated by * (ANOVA, $p < 0.05$). Significant differences in comparison with ASB7 and ASB9 inoculated groups are indicated by + and # respectively (ANOVA, $p < 0.05$).

Cytokine gene expression in the stomach in response to *H. heilmannii* s.s. infection

The local host immune response towards *H. heilmannii* s.s. infection was characterized by measuring the mRNA expression level of IFN-γ, IL-1β, IL-5, IL-6, IL-10, IL-12p40, IL-17 and TNF-α in the stomach of the gerbils. Results are shown in **Table 2** and in **Figure 4**. The pro-inflammatory cytokine IL-1β is a potent inhibitor of gastric acid secretion (El-Omar, 2001) and plays a role in the acute phase of inflammation (Yamaoka et al., 2005). Expression of IL-1β was up-regulated in the antrum of the stomach of gerbils infected with *H. heilmannii* s.s. ASB1, ASB2, ASB3, ASB6, ASB11, ASB13 and ASB14, compared to the negative control animals (**Figure 4a**). For gerbils inoculated with ASB7 and ASB9, no up-regulation of IL-1β was seen. The Th1 cytokine IFN-γ, a signature marker of the Th1-polarized response (Crabtree et al., 2004; Bamford et al., 1998), exhibited a decreased expression in the antrum of gerbils infected with ASB11 and ASB13 (**Figure 4b**), compared to the control animals. No significant differences in expression between infected and sham-inoculated gerbils could be observed for IL-5, IL-6, IL-10, IL-12p40, IL-17 and TNF-α.

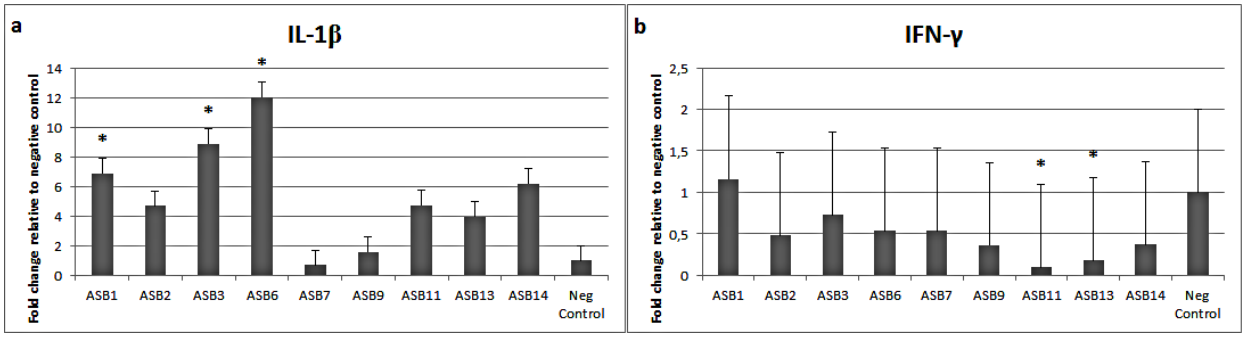

Figure 4. mRNA expression levels of cytokines IL-1β and IFN-γ in the antrum of the stomach. Cytokine mRNA expression levels in the fundus and antrum of the stomach were examined by quantitative RT-PCR. Expression levels of IL-1β (a) and IFN-γ (b) in the antrum are shown. Data are presented as the fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data are shown as means + standard deviation. Significant differences in expression level between inoculated groups and negative control group are indicated by * $p < 0.05$ (ANOVA).

Table 2. Statistical analysis of mRNA expression levels. Shown is the statistical analysis of IL-1β, IFN-γ and H^+/K^+ ATPase mRNA expression in the antrum and gastrin mRNA expression in the fundus of the gerbil stomach at 9 weeks after experimental infection. Cytokine expression was analyzed by analysis of variance with a Bonferroni post hoc test. H^+/K^+ ATPase and gastrin gene expression were compared between different infected groups and controls using Kruskall-Wallis analysis, followed by a Mann-Whitney *U* test. Differences were considered statistically significant at $p \leq 0.05$. SPSS Statistics 21 software (IBM) was used for all analyses.

	Antrum						Fundus	
	IL-1β		IFN-γ		H^+/K^+ ATPase		Gastrin	
	Mean Ct-Ctref[a]	*p*-value[b]	Mean Ct-Ctref	*p*-value	Mean Ct-Ctref	*p*-value	Mean Ct-Ctref	*p*-value
ASB1	6.92 ± 0.29 *	0.031	6.87 ± 0.60	1.000	3.70 ± 2.11 *	0.050	7.20 ± 1.68	0.101
ASB2	7.48 ± 1.42	0.231	8.15 ± 1.26	1.000	2.42 ± 5.01	0.513	5.94 ± 2.49 *	0.050
ASB3	6.55 ± 0.80 *	0.008	7.54 ± 0.37	1.000	3.49 ± 2.28 *	0.050	7.71 ± 1.17	0.101
ASB6	6.12 ± 0.67 *	0.001	7.99 ± 0.90	1.000	2.23 ± 1.99 *	0.050	5.33 ± 2.39 *	0.025
ASB7	10.25 ± 0.37	1.000	7.98 ± 0.52	1.000	0.45 ± 2.51	0.724	7.12 ± 1.54	0.180
ASB9	9.03 ± 2.54	1.000	8.55 ± 0.71	1.000	0.79 ± 0.03	0.564	8.11 ± 1.58	1.000
ASB11	7.47 ± 0.15	0.499	10.45 ± 0.95 *	0.004	1.32 ± 1.45	0.248	7.67 ± 1.25	0.289
ASB13	7.72 ± 1.17	0.523	9.54 ± 1.55 *	0.044	3.80 ± 0.34	0.083	8.10 ± 0.93	0.456
ASB14	7.07 ± 0.52	0.054	8.51 ± 0.97	1.000	3.81 ± 3.11	0.127	7.77 ± 2.96	0.881
Negative Control	9.71 ± 1.13	-	7.08 ± 0.65	-	0.15 ± 1.82	-	8.70 ± 0.62	-

[a] **Mean Ct-Ctref:** For each experimental group, the mean of the normalized Ct-values ± standard deviation are shown.

[b] **p-value:** the exact p-values are given.

* Statistically significant differences compared to the uninfected control group ($p \leq 0.05$).

Parietal cell H^+/K^+ ATPase mRNA expression is down-regulated in response to colonization with *H. heilmannii* s.s.

No clear loss of parietal cells could be visualized by immunohistochemical staining in the fundus and the antrum of the *H. heilmannii* s.s.-infected gerbils compared to the uninfected controls (data not shown). However, quantitative RT-PCR showed a clear decrease in the expression of gastric H^+/K^+ ATPase in the antrum of the gerbils infected with ASB1, ASB3 and ASB6 (**Figure 5** and **Table 2**). Compared to the control animals with mRNA expression levels set to 1.0, the mean relative expression was 0.09 ± 2.11 for ASB1-, 0.10 ± 2.28 for ASB3- and 0.24 ± 1.99 for ASB6-infected gerbils, respectively. No significant change in expression was seen in the fundus of the *H. heilmannii* s.s.-infected gerbils.

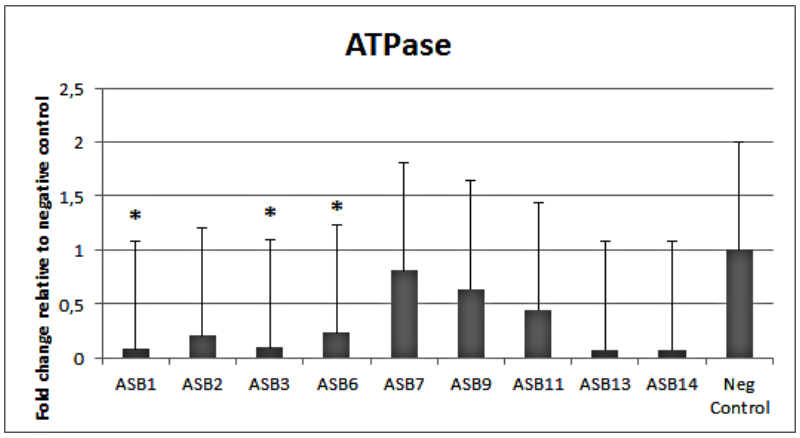

Figure 5. mRNA expression level of H^+/K^+ ATPase in the antrum of the stomach. Hydrogen potassium ATPase mRNA expression level in the stomach was examined by quantitative RT-PCR. H^+/K^+ ATPase mRNA expression level in the antrum is shown. Data are presented as the fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data are shown as means + standard deviation. Significant differences in expression level between inoculated groups and negative control group are indicated by * $p \leq 0.05$ (Mann-Whitney *U* test).

Virulent *H. heilmannii* s.s. strains induce increased gastrin expression in the fundus

The peptide hormone gastrin stimulates the secretion of gastric acid by parietal cells. A disturbance in its expression may lead to hypergastrinemia. The expression of gastrin was highly up-regulated in the fundus of gerbils infected with ASB2 and ASB6 at 9 weeks post-infection (**Figure 6** and **Table 2**). Compared to control animals, the mean relative expression was 6.79 ± 2.49 for ASB2- and 10.35 ± 2.39 for ASB6-infected gerbils. In the antrum of the stomach, no up-regulation of gastrin expression was detected.

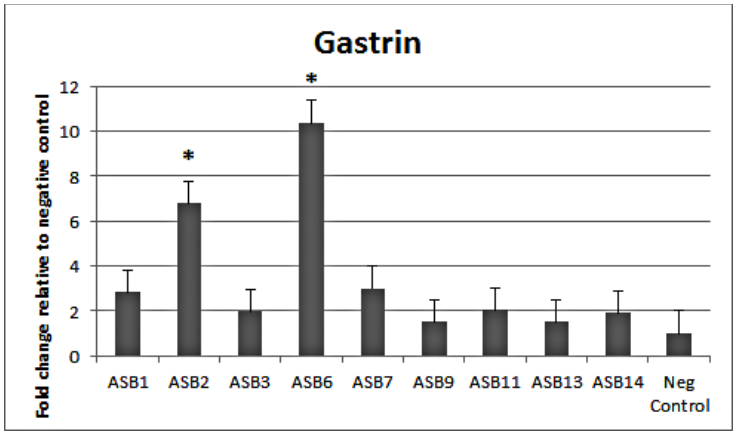

Figure 6. mRNA expression level of gastrin in the fundus of the stomach. Gastrin mRNA expression level in the stomach was examined by quantitative RT-PCR. Shown is the expression level in the fundus. Data are presented as the fold change in gene expression normalized to 3 reference genes and relative to the negative control group which is considered as 1. Data are shown as means + standard deviation. Significant differences in expression level between inoculated groups and negative control group are indicated by * $p \leq 0.05$ (Mann-Whitney *U* test).

Discussion

At 9 weeks post inoculation, a chronic active gastritis in the antrum of the stomach was observed in gerbils experimentally infected with 7 out of 9 *H. heilmannii* s.s. strains tested in this study (ASB1, ASB2, ASB3, ASB6, ASB11, ASB13 and ASB14). The lamina propria and submucosa were massively infiltrated with lymphocytes, resulting in the formation of lymphoid follicles. The *H. heilmannii* s.s. strains were mainly detected in the antrum and to a lesser extent in the fundus and the duodenum. In humans infected with NHPH, colonization and inflammation also mainly occur in the antrum of the stomach (Debongnie et al., 1998; Haesebrouck et al., 2009; Morgner et al., 1995; Morgner et al., 2000). This confirms that Mongolian gerbils are an appropriate model to study *H. heilmannii* s.s. infections in humans, as has also been shown for *H. suis* (Flahou et al., 2010) and *H. pylori* (O'Rourke and Lee, 2003; Rogers and Fox, 2004). No inflammation was seen in the fundus of the stomach of the *H. heilmannii* s.s.-infected gerbils. Wiedemann et al. (2009) demonstrated that in *H. pylori*-infected Mongolian gerbils a fundus-dominant gastritis is dependent on a functional Cag pathogenicity island. The *H. heilmannii* s.s. genome lacks a Cag pathogenicity island (Smet et al., 2013), which might explain the antrum-dominant gastritis and the absence of inflammation in the fundus.

The highest number of colonizing bacteria was seen in the antrum of gerbils inoculated with ASB1, ASB2, ASB3 and ASB6. The colonization capacity of ASB7, ASB11, ASB13 and ASB14 was more restricted. ASB9 could not be detected in the stomach nor the duodenum of the gerbils at 9 weeks after experimental infection, which might indicate that the infection was cleared within 9 weeks, or that ASB9 was not able to colonize the gastric mucosa. It would be interesting to examine these two options in a repeat study with ASB9, with samples taken at different time points. Also, *H. heilmannii* s.s. strains ASB7 and ASB9 did not cause explicit lymphocytic inflammation or gastric lesions. These results indicate that the capacity of *H. heilmannii* s.s. to colonize the stomach and to cause inflammation and lesions is strain-dependent.

The risk to develop MALT lymphoma is considered to be higher in humans infected with NHPH than in *H. pylori*-infected patients (Greiner et al., 1997; Haesebrouck et al., 2009; Knörr et al., 1999; Stolte et al., 1997) and MALT lymphoma-like lesions have been demonstrated in the stomach of Mongolian gerbils colonized with *H. suis* for 8 months (Flahou et al., 2010). MALT lymphoma is characterized by an extensive proliferation of B-lymphocytes which may be dependent on Th2-type cytokines. Indeed, experimental *H. suis* infections in mouse models have been shown to evoke a Th2-polarized response (Flahou et al., 2010; Flahou et al., 2012).

Surprisingly, in the present study, there was no up-regulation of the Th2-cytokine IL-5 in the stomach of the *H. heilmannii* s.s.-colonized gerbils at 9 weeks post inoculation. It remains to be determined if long-term colonization of Mongolian gerbils with *H. heilmannii* s.s. would induce a prolonged Th2-polarized response eventually resulting in MALT lymphoma-like lesions.

In the present study, mRNA levels of the pro-inflammatory cytokine IL-1β were up-regulated in the antrum of gerbils suffering from gastritis. Up-regulation of this cytokine has also been demonstrated in the stomach of *H. pylori*-infected gerbils (Wiedemann et al., 2009; Yamaoka et al., 2005). Another cytokine playing a role in gastric inflammation in *H. pylori*-infected Mongolian gerbils is the Th17 cytokine IL-17 (Sugimoto et al., 2009). *H. suis*-infection in mouse models has been shown to induce a predominant Th17 response as well (Flahou et al., 2012). In our study, there was no significant up-regulation of IL-17 at 9 weeks post-infection. Since IL-17 has been shown to be a key regulator of neutrophil infiltration (Algood et al., 2007; Flahou et al., 2010; Shiomi et al., 2008), the absence of a Th17 response might explain the low number of infiltrating neutrophils in the antral mucosa of the gerbils with gastritis. Examination of samples taken at other time points after infection will be needed to elucidate the importance of IL-17 in the maintenance and regulation of chronic gastric inflammation during an *H. heilmannii* s.s. infection.

A major difference with *H. pylori* infections is the absence of an up-regulation of IFN-γ in the stomach of gerbils infected with *H. heilmannii* s.s. An *H. pylori* infection in mice, gerbils and humans is indeed accompanied by a Th1-polarized response, characterized by a strong increase of IFN-γ (Shi et al., 2010; Sommer et al., 1998; Wiedemann et al., 2009; Yamaoka et al., 2005). In contrast, expression levels of IFN-γ were even lower in the stomach of gerbils infected with *H. heilmannii* s.s. strains ASB11 and ASB13 compared to sham-inoculated control gerbils. Absence of a Th1-polarized response has also been described for *H. suis* infection in mice (Flahou et al., 2012). This demonstrates that the pathogenesis of gastric NHPH infections is not identical to that of an *H. pylori* infection.

Gastric acid secretion is mediated by the gastric hydrogen potassium ATPase (H^+/K^+ ATPase), that functions as a proton pump in the gastric acid-secreting parietal cells (Forte et al., 1989). Although acid-secreting parietal cells are characteristic for the fundic epithelium, they are also observed in the gastric antrum of the Mongolian gerbil, albeit to a lesser extent (Flahou et al., 2010). In the present study, a reduction in the antral expression of H^+/K^+ ATPase was detected for 3 *H. heilmannii* s.s. strains (ASB1, ASB3 and ASB6), suggesting reduced gastric acid secretion which might lead to antral mucosal atrophy. Mucosal atrophy of the antrum has also

been described in Mongolian gerbils infected with *H. pylori* and with *H. suis* (Flahou et al., 2010; Wiedemann et al., 2009). However, the relevance of a reduced antral H^+/K^+ ATPase expression for the physiology of the stomach remains unclear, since the majority of parietal cells are located in the fundus of the stomach.

The peptide hormone gastrin is released by G-cells mainly in the antrum of the stomach in response to food intake and stimulates the secretion of gastric acid by parietal cells (Dockray, 1999). *H. pylori* infection in human patients and animal models is commonly associated with increased gastrin levels and is considered to be a reaction to the *H. pylori*-induced hypochlorhydria (Chittajallu et al., 1991; Tucker et al., 2010). In an attempt to repair acid homeostasis, gastrin stimulates histamine release from enterochromaffin-like (ECL) cells, inducing acid secretion (Furutani et al., 2003; Rieder et al., 2005; Takashima et al., 2001). Moreover, IL-1β, which is up-regulated after a *H. pylori* infection, stimulates gastrin release from antral G-cells and inhibits antral D-cells to express somatostatin, an inhibitor of gastrin-stimulated acid secretion (Wiedemann et al., 2009; Zavros and Merchant, 2005). This does, however, not result in increased production of hydrochloric acid due to a modulating effect of IL-1β on the *H. pylori*-mediated H^+/K^+ ATPase α-subunit promoter inhibition, contributing to reduced parietal cell H^+/K^+ ATPase gene and protein expression and thus to hypochlorhydria (Saha et al., 2007). As mentioned above, IL-1β was also up-regulated in the stomach of our gerbils with gastritis and in the present study, gastrin mRNA was up-regulated in the fundus of gerbils inoculated with strains ASB2 and ASB6. While G-cells were most abundant in the antrum of the stomach, some could also be seen at the edge of the fundus of the *H. heilmannii* s.s.-infected gerbils, in the transition zone between fundus and antrum (Additional file 3). It should be noted that an increased level of gastrin mRNA does not necessarily mean a higher level of active gastrin hormone, as the translated precursor protein progastrin has to be processed by posttranslational modifications into its active form gastrin (Dockray, 1999). In *H. pylori*-infected gerbils, gastrin levels started to increase only after 16 weeks of infection and mainly in antral tissue (Wiedemann et al., 2009). Further studies, measuring the levels of IL-1β, gastrin, histamine and somatostatin after long term experimental infection, are necessary to obtain additional insights into the influence of *H. heilmannii* s.s. on gastric homeostasis.

In conclusion and taking together the results of histopathology, antral epithelial cell proliferation, colonization capacity and cytokine, H^+/K^+ ATPase and gastrin expression, the present experimental infection studies in Mongolian gerbils indicate variation in bacterium-host interactions and virulence between different *H. heilmannii* s.s. isolates. Since the Mongolian gerbil model is considered to be a good model for human *Helicobacter*-induced pathology

(Flahou et al., 2010; O'Rourke and Lee, 2003; Rogers and Fox, 2004), this strain variation is most probably also relevant for human infections with this microorganism and might be important for infections in the natural hosts of *H. heilmannii* s.s., cats and dogs, as well. Future research is necessary to determine if the variation in virulence can be explained by specific virulence genes present in highly virulent strains, or by differences in expression of such genes between highly virulent and less virulent strains.

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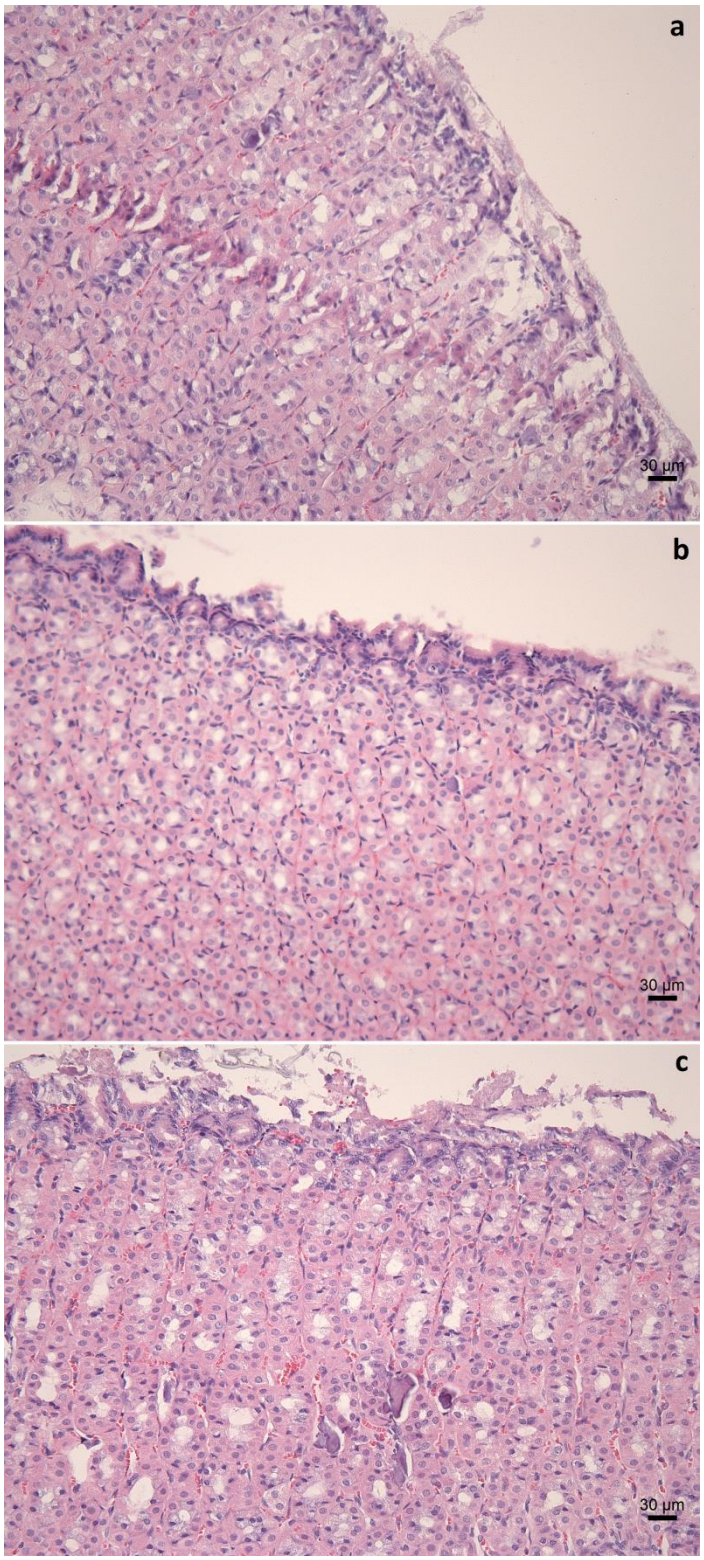
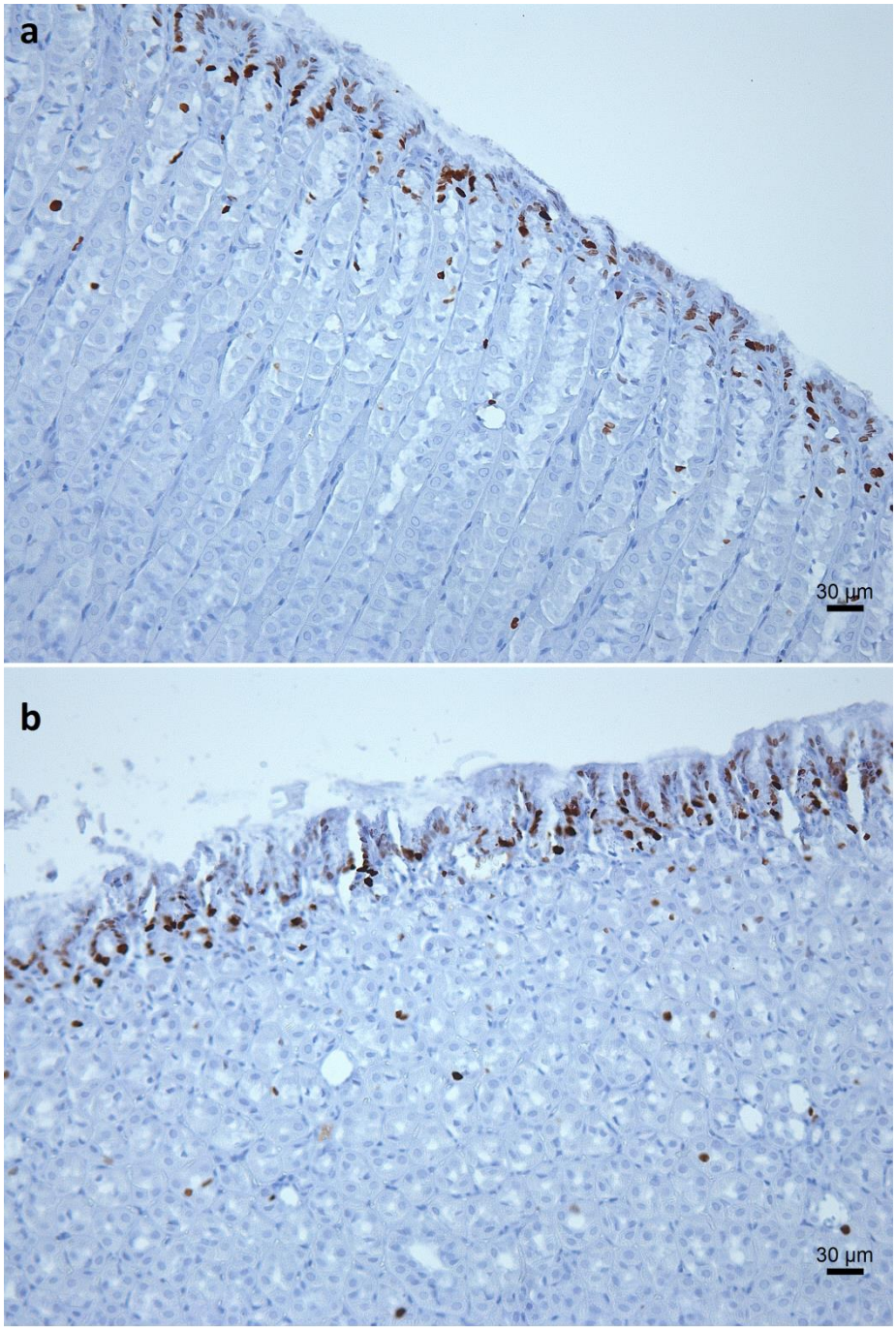
Additional files

Additional file 1: H&E staining of the fundus of a gerbil stomach.



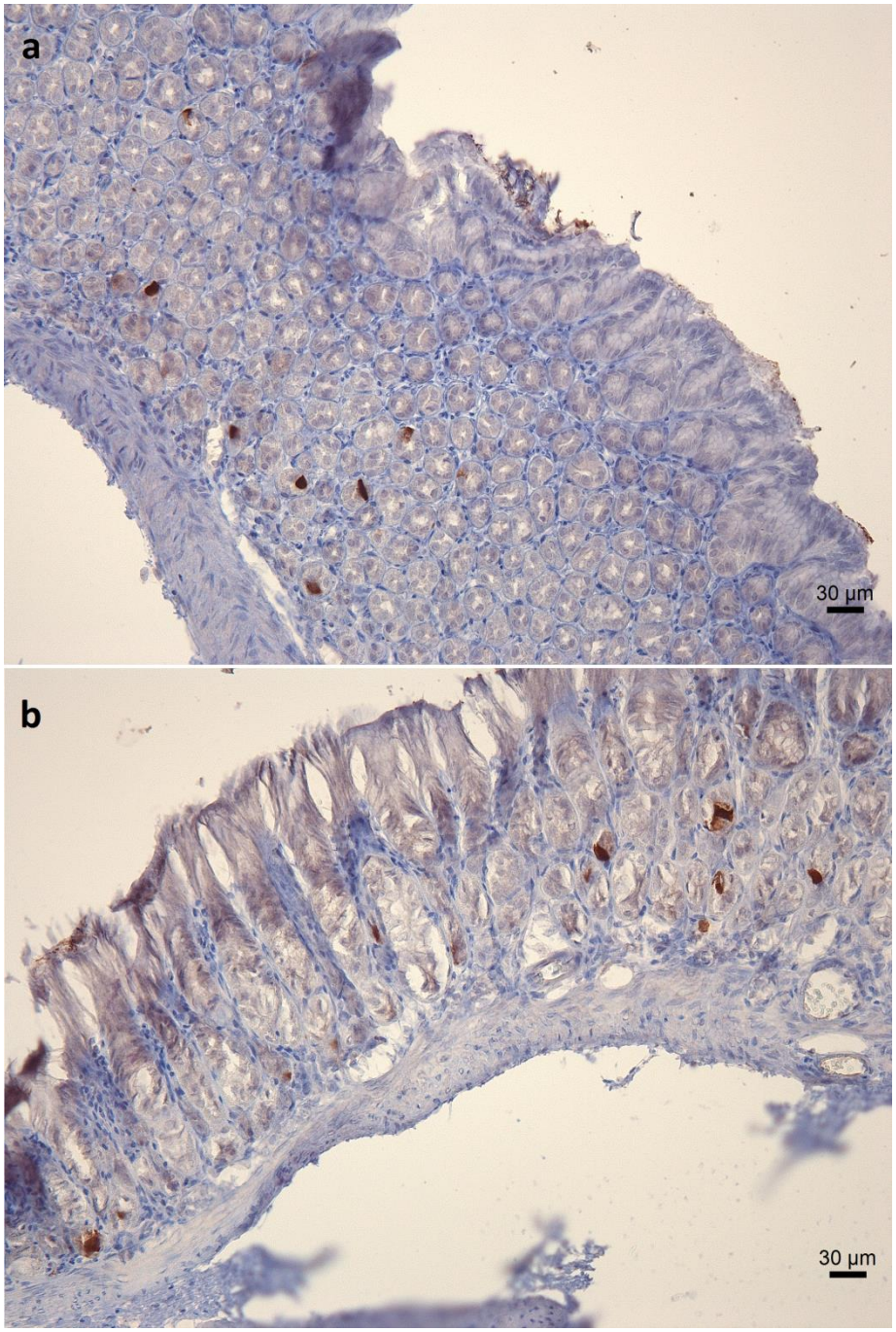
Normal histology of the fundus of a sham-inoculated negative control animal (a). Comparable normal histology of the fundus of a gerbil inoculated with *H. heilmannii* s.s. ASB1 (b) and *H. heilmannii* s.s. ASB7 (c). Bar = 30 µm.

Additional file 2: Ki67 staining of the fundus of a gerbil stomach.



Ki67 staining of the fundus of a sham-inoculated negative control animal (a) and of a gerbil inoculated with *H. heilmannii* s.s. ASB1 (b) showing an equal number of proliferating epithelial cells. Bar = 30 µm.

Additional file 3: Gastrin staining of the fundus of a gerbil stomach.



The presence of G-cells in the fundus of the stomach was analyzed by immunohistochemical staining using a polyclonal rabbit antigastrin-17 antibody (1/800 dilution, Code No A0568, DAKO A/S, Denmark). Some G-cells are located in the transition zone between the fundus and the antrum in gerbils inoculated with *H. heilmannii* s.s. ASB2 (a) or with *H. heilmannii* s.s. ASB6 (b). Bar = 30 µm.

Chapter 2

Divergence between the highly virulent zoonotic pathogen *Helicobacter heilmannii* and its closest relative, the low-virulence "*Helicobacter ailurogastricus*" sp. nov.

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Abstract

Helicobacter heilmannii naturally colonizes the stomachs of cats and dogs and has been associated with gastric disorders in humans. Nine feline *Helicobacter* strains, classified as *H. heilmannii* based on *ureAB* and 16S rRNA gene sequences, were divided into a highly virulent and a low-virulence group. The genomes of these strains were sequenced to investigate their phylogenetic relationships, to define their gene content and diversity, and to determine if the differences in pathogenicity were associated with the presence or absence of potential virulence genes. The capacities of these helicobacters to bind to the gastric mucosa were investigated as well. Our analyses revealed that the low-virulence strains do not belong to the species *H. heilmannii* but to a novel, closely related species for which we propose the name *Helicobacter ailurogastricus*. Several homologs of *H. pylori* virulence factors, such as IceA1, HrgA, and jhp0562-like glycosyltransferase, are present in *H. heilmannii* but absent in *H. ailurogastricus*. Both species contain a VacA-like autotransporter, for which the passenger domain is remarkably larger in *H. ailurogastricus* than in *H. heilmannii*. In addition, *H. ailurogastricus* shows clear differences in binding to the gastric mucosa compared to *H. heilmannii*. These findings highlight the low-virulence character of this novel *Helicobacter* species.

Introduction

Helicobacter pylori is considered one of the most successful human pathogens. Infection with this agent has been associated with a wide range of gastric disorders. However, *H. pylori* is not the only *Helicobacter* species causing gastric disease in humans. *Helicobacter heilmannii* (sensu stricto), a zoonotic bacterium naturally colonizing the stomachs of cats and dogs, has been associated with gastritis, peptic and duodenal ulcers, and mucosa-associated lymphoid tissue (MALT) lymphoma in humans (Baele et al., 2009; Haesebrouck et al., 2009; Hwang et al., 2002; O'Rourke et al., 2004a; O'Rourke et al., 2004b; Van den Bulck et al., 2005). This *Helicobacter* species is highly prevalent in the stomachs of clinically healthy cats and dogs as well as in those of animals showing chronic active gastritis (Haesebrouck et al., 2009; Hwang et al., 2002). Its pathogenic significance in these animals remains unclear and is probably strain dependent or related to host differences (Haesebrouck et al., 2009). Little information is available regarding the pathogenesis of *H. heilmannii* infections in humans (Bento-Miranda and Figueiredo, 2014; Haesebrouck et al., 2009). A recent experimental infection study, using Mongolian gerbils as an *in vivo* model to study *Helicobacter*-related gastric pathology in humans, investigated the colonization capacities and virulence of nine different *Helicobacter* strains (Joosten et al., 2013). These helicobacters had been isolated from the gastric mucosae of stray cats and had been classified as *H. heilmannii* on the basis of the *ureAB* and 16S rRNA gene sequences (Smet et al., 2012). At 9 weeks postinfection, the induction of an antrum-dominant chronic active gastritis associated with the formation of lymphocytic aggregates and upregulation of the proinflammatory cytokine interleukin 1β (IL-1β) was shown for seven strains. However, differences in the expression of IL-1β were noted, together with differences in the intensity of the observed gastritis. High-level antral colonization was seen for four strains, while the colonization levels of the other strains were lower in the antrum and the fundus of the stomach. Based on the differences seen in colonization capacity and virulence, these *Helicobacter* strains were divided into a highly virulent group and a low-virulence group (Joosten et al., 2013). In the present study, we sequenced the genomes of the highly virulent and low-virulence *H. heilmannii* strains in order to investigate their phylogenetic relationships, to define this species' gene content and diversity, and to determine whether the presence or absence of specific virulence-associated genes might help to explain the differences in pathogenicity between these helicobacters. Because of the differences seen in gastric colonization between the highly virulent and low-virulence *H. heilmannii* strains, several *in*

vitro binding assays were carried out to investigate possible differences in the capacity for adhesion to the gastric mucosa.

Material and Methods

Bacterial strains and whole-genome sequencing

Five highly virulent (ASB1^T, ASB2, ASB3, ASB6, and ASB14) and four low-virulence (ASB7^T, ASB9, ASB11, and ASB13) feline *Helicobacter* strains (Joosten et al., 2013) were cultivated in biphasic medium as described previously (Smet et al., 2012), and their genomic DNA was extracted by using the Qiagen, (Venlo, Netherlands) Blood & Tissue kit according to the manufacturer's guidelines. The genomes of ASB1^T and ASB7^T were obtained as described previously (Smet et al., 2013). An improved new assembly of the ASB1^T genome resulted in a genome size of 1,638,988 bp (**Table 1**), which is approximately 200 kb smaller than the previously published ASB1^T genome (Smet et al., 2013). An erratum will be submitted to adjust the previous publication. For whole-genome sequencing of the other seven strains, genomic DNA was normalized to 0.2 ng/µl, and a total of 1 ng was used for library generation. Sequencing libraries were prepared by using Nextera XT chemistry (Illumina Inc., San Diego, CA, USA) in accordance with the manufacturer's recommendations. Libraries were sequenced for a 250- or 300-bp paired-end sequencing run using the MiSeq personal sequencer (Illumina) (Perkins et al., 2013). All genomes were assembled with the CLC Genomics Workbench, version 7. Gene finding and automatic annotation were performed using the RAST server (Aziz et al., 2008, Overbeek et al., 2014).

Phylogenetic and evolutionary analyses

The list of fully annotated complete or draft genomes of different gastric and enterohepatic *Helicobacter* species obtained from the NCBI ftp server is shown in **Table S1** in the supplemental material. The GET_HOMOLOGUES software package (Contreras-Moreira and Vinuesa, 2013) was used to cluster genes in groups of orthologs. The bidirectional best-hit algorithm was used to define the core genes of these genomes. The GET_HOMOLOGUES output was further filtered by removing genes containing ambiguous nucleotides and selecting unique nucleotide sequences present in all genomes. Subsequently, a phylogenetic tree was created based on 303 concatenated core genes, as described previously (Abascal et al., 2010; Katoh and Standley, 2013; Kersulyte et al., 2013). The phylogenetic tree was built using PhyML (Guindon et al., 2010) by applying the -b 2, -m GTR, -f e, -c 6, -a e, -s BEST, and -o tlr

parameters and was visualized by MEGA6 software (Tamura et al., 2013). A distance matrix of the concatenated aligned core genes was calculated with DISTMAT implemented in jEMBOSS using the Kimura 2-parameter model (Rice et al., 2000). The ASB genomes were submitted to the Genome-to-Genome Distance Calculator (GGDC; http://ggdc.dsmz.de) in order to calculate whole-genome distances and define the degree of DNA-DNA hybridization (DDH) between them. Additionally, the average nucleotide identity (ANI) values among the ASB genomes were calculated using the online "average nucleotide identity calculator" tool (enve-omics.ce.gatech.edu/ani/index) (Konstantinidis and Tiedje, 2005).

TEM

The morphologies of two low-virulence strains, ASB7^T and ASB11, were characterized by means of transmission electron microscopy (TEM) as described previously (De Bock et al., 2006). Semithin sections (2 µm) were cut and stained with toluidine blue. Thereafter, selected regions were chosen for ultrathin sectioning (90 nm) with an ultramicrotome (Ultracut E; Reichert-Jung, Nussloch, Germany). The sections were stained with uranyl acetate and lead citrate solutions before examination under a JEOL EX II transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV. The morphologies of these two low-virulence strains were also studied by negative staining of bacterial culture samples with 2% (wt/vol) uranyl acetate.

Biochemical and tolerance tests

The isolates were examined for catalase activity by adding a 3% H_2O_2 solution and observing the reaction within 5 s. Oxidase activity was tested with Bactident Oxidase strips (Merck, Overijse, Belgium). The API Campy identification system (bio-Mérieux, Marcy L'Etoile, France) was used to study urease activity, nitrate reduction, esterase activity, hippurate hydrolysis, γ-glutamyltransferase activity, triphenyltetrazolium chloride (TTC) reduction, alkaline phosphatase activity, and pyrrolidonyl, L-arginine, and L-aspartate arylamidase activities. Tests were read after 24 h of incubation at 37°C under an aerobic atmosphere.

Comparative proteomic analyses

The complete set of predicted proteins from the *Helicobacter* species used in this study (see **Table S1** in the supplemental material) and those from the ASB strains were clustered in groups of orthologs by using the GET_HOMOLOGUES software and applying the OrthoMCL algorithm. The compare_cluster.pl and parse_pangenome_matrix.pl Perl scripts were then used to find proteins that are absent in other *Helicobacter* species and thus unique to *H. heilmannii*.

To identify proteins present in the highly virulent strains but absent in the low-virulence strains, and vice versa, all proteins of the highly virulent strain ASB1 were compared with those of the low-virulence strain ASB7 by reciprocal BLASTP. Orthologous proteins were identified using the BLAST score ratio, a powerful tool for determining the probability of sharing a recent common ancestor. The general "acceptable" BLAST score ratio cutoff of 0.4 (equivalent to 40%) was used to define two proteins as homologs. Subsequently, the presence or absence of these specific proteins was checked and confirmed in the other highly virulent (ASB2, ASB3, ASB6, and ASB14) and low-virulence (ASB9, ASB11, and ASB13) strains as well. The proteins obtained were then used as queries for BLASTP homology searches against the total NCBI database in order to find related sequences present in other gastric *Helicobacter* species. The putative outer membrane protein (OMP) sequences were extracted from the ASB genomes by using the HHomp tool (Remmert et al., 2009) and BLASTP against the OMP database (Tsirigos et al., 2011) and a set of well-known *H. pylori* OMPs using a BLASTP score ratio of 0.45.

Evolutionary analysis of the putative *vacA*-like genes present in all gastric *Helicobacter* species was performed using MUSCLE software (Edgar, 2004). Neighbor-joining tree data were calculated on the basis of the VacA-like amino acid sequence alignment.

***In vitro* binding assays. (i) Binding to human gastric mucins**

All ASB isolates, cultured for 24 h, were harvested, centrifuged at 2,500 × *g* for 4 min, and resuspended in 1% Blocking Reagent for ELISA (Roche, Stockholm, Sweden), containing 0.05% Tween 20 (blocking buffer). Two human gastric mucin samples were used, one derived from a healthy stomach and one from a patient with a gastric tumor. Mucin samples were diluted in 4 M guanidinium chloride to 4 mg/ml and were used to coat 96-well plates (PolySorp; Nunc A/S, Roskilde, Denmark) overnight at 4°C. The plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, and the wells were blocked for 1 h with blocking buffer. After the blocking buffer was discarded, the bacteria with an optical density at 600 nm (OD_{600}) of 0.1 were diluted 1:10 in blocking buffer containing 10 mM citric acid (at pH 2 and pH 7), and the dilutions were added to the wells. The 96-well plates were incubated for 2 h at 37°C in a shaker at 120 rpm. The plates were washed three times with PBS (plus 0.05% Tween 20) and were incubated with rabbit anti-*H. pylori* serum (1:1,000 dilution in blocking buffer) for 1 h at room temperature. Subsequently, the plates were washed three times and were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000 dilution in blocking buffer) for 1 h at room temperature. After further washing steps, a 3,3',5,5'-

tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich, Diegem, Belgium) was added to the wells, and the plates were incubated for 20 min. The reaction was stopped with an equivalent amount of 0.5M H_2SO_4, and the absorbance was measured in a microplate reader at 450 nm.

(ii) Binding to gastric epithelial cells

The human gastric epithelial cell line MKN7 (Riken Cell Bank, Japan) was cultured in RPMI 1640 medium with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Invitrogen) at 37°C under 5% CO_2.

GSM06 cells (a murine gastric surface mucous cell line) were cultured in Ham's F-12 medium (Invitrogen) and Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% (vol/vol) heat-inactivated FBS, 1% (vol/vol) insulin-transferrin-selenium-A supplement (ITS; Gibco, Life Technologies, Erembodegem-Aalst, Belgium), penicillin (50 U/ml), streptomycin (50 µg/ml), and 0.2% (vol/vol) epidermal growth factor (EGF; Sigma-Aldrich) at 37°C under 5% CO_2.

For cocultures with the highly virulent and low-virulence *Helicobacter* strains, the cell medium was changed to antibiotic-free medium.

Bacteria adhering to gastric epithelial cells were visualized by scanning electron microscopy (SEM). For this purpose, MKN7 cells and GSM06 cells were seeded at a concentration of 10^4/ml on coverslips in 24-wellplates and were incubated overnight at 37°C. After incubation, cells were washed twice with Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} (HBSS+; Life Technologies). Five hundred microliters of a bacterial suspension (at a concentration of 10^8 viable bacteria/ml of cell medium) at pH 2 or pH 7 was added to the cells, and they were further incubated for 1 h at 37°C under microaerobic conditions. Thereafter, coverslips were again washed twice with HBSS+. Finally, coverslips were fixed in 500 µl HEPES fixative (2% paraformaldehyde) and were prepared for SEM as described previously (De Spiegelaere et al., 2008). Briefly, the coverslip samples were fixed overnight in a HEPES-buffered glutaraldehyde solution. Samples were post-fixed in 1% buffered osmium tetroxide for 2 h and were dehydrated in an increasing alcohol series, followed by an increasing ethanol-acetone series up to 100% acetone. The samples were then dried to the critical point with a Balzers CPD 030 critical point dryer (Sercolab BVBA, Merksem, Belgium) and were further mounted on metal bases and sputtered with platinum using the JEOL JFC-1300 Auto Fine Coater (JEOL Ltd., Zaventem, Belgium). The samples were examined with a JEOL JSM 5600LV scanning electron microscope (JEOL Ltd.). The mean number of binding bacteria per cell was calculated by counting spiral and coccoid *Helicobacter* bacteria attached to 5 cells selected at random.

A quantitative fluorescence-based adherence assay was performed in order to confirm the SEM results. For this purpose, MKN7 and GSM06 cells were seeded at a concentration of 10^4/ml in 200 µl antibiotic-free cell medium in 96-well plates (Greiner Bio-One, Vilvoorde, Belgium) and were incubated overnight at 37°C. *Helicobacter* ASB strains were fluorescently labeled with fluorescein isothiocyanate isomer I (FITC; excitation wavelength, 492 nm; emission wavelength, 518 nm; Sigma-Aldrich). Briefly, bacteria (concentration, 10^8 viable bacteria/ml of *Brucella* broth) were harvested, washed three times by centrifugation at 2,000 × *g* for 5 min, and resuspended in PBS-0.05% Tween. Subsequently, pellets were resuspended in 0.1 M carbonate and 0.15 M NaCl buffer (pH 9.0). Ten microliters of FITC (10 mg/ml dimethyl sulfoxide [DMSO]) was added to 1 ml of the bacterial suspension, followed by incubation for 30 min in the dark. FITC-labeled bacteria were washed three times in blocking buffer (1% bovine serum albumin [BSA] in PBS–0.05% Tween). The viability of all FITC-labeled strains was examined by checking their motility using light microscopy. The antibiotic-free cell medium was removed from the 96-well plates, and 150 µl of the FITC-labeled bacterial suspension was added to the cells (5 replicates per strain), followed by incubation for 1 h at 37°C under microaerobic conditions (Smet et al., 2012). Thereafter, the cells were washed twice with HBSS+, and the emission of fluorescent light at a λ of 527 nm was measured with a fluorometer (Fluoroskan Ascent FL microplate fluorometer and luminometer; Thermo Scientific, Erembodegem-Aalst, Belgium). Wells without cells (bacterial suspension only) and wells without the bacterial suspension (cells only) were included as controls to correct for any possible background signal. The adherence assay was performed immediately after fluorescent labeling of the *Helicobacter* strains in order to minimize the possible loss in viability of the labeled helicobacters over time. Finally, the relative levels of FITC labeling of all ASB strains were analyzed by flow cytometry (FCM) on a BD FACSCanto II flow cytometer (Becton Dickinson, Erembodegem, Belgium). The mean fluorescence intensity of each labeled strain, measured by FCM, was used as a correction factor for differential FITC labeling of the nine different strains. For each strain, the ratio of the mean fluorescence intensity measured by the fluorometer (indicating bacterial adhesion) to the mean fluorescence intensity measured by FCM (indicating the relative FITC labeling per strain) was calculated.

(iii) Binding to the gastric mucosae of Mongolian gerbils and stray cats

Paraffin-embedded stomach tissues of 20 euthanized Mongolian gerbils (Joosten et al., 2013) and 5 euthanized stray cats (Smet et al., 2012) were used. The ASB1^T, ASB7^T, and ASB11 isolates were cultured for 24 h, harvested, washed twice by centrifugation at 2,500 × *g* for 4 min, and resuspended in PBS. The bacterial concentration was adjusted to an OD_{600} of 0.1 in

PBS, and bacteria were labeled by incubation with 100 µg/ml FITC for 5 min at room temperature. Labeled bacteria were recovered by centrifugation at 800 × *g* for 7 min, washed three times with PBS, and resuspended in blocking buffer.

Paraffin-embedded tissue sections were deparaffinized and were washed twice in water and once in PBS with 0.05% Tween 20. The slides were incubated with Blocking Reagent for ELISA (Roche) for 30 min at room temperature. The labeled bacteria were diluted 1:20 in blocking reagent containing 10 mM citric acid (pH 2 and pH 7), and 200 µl was added to each slide, followed by incubation in a humidified chamber for 1 h at room temperature. The slides were washed twice in PBS with 0.05% Tween 20 and once in water and were then mounted with ProLong Antifade Reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies).

Statistical analysis

The normality of data was assessed using the Shapiro-Wilk normality test, which is appropriate for smaller sample sizes up to 2,000. The variance homogeneity of data was analyzed by using Levene's test for homogeneity of variances (SPSS Statistics, version 22, Command Syntax Reference; IBM).

The quantities of bacteria binding MKN7 cells and GSM06 cells were compared between *H. heilmannii*- and *H. ailurogastricus*-infected cells by using Kruskal-Wallis analysis, followed by a Mann-Whitney *U* test. An unpaired *t* test was applied to compare the quantities of *H. heilmannii* and *H. ailurogastricus* bacteria bound to gastric mucins and DNA. The numbers of bacteria binding gastric glands and surface epithelium on paraffin-embedded tissue sections were analyzed by Kruskal-Wallis analysis, followed by a Mann-Whitney *U* test. Differences were considered statistically significant at a *P* value of < 0.05. SPSS Statistics software, version 22 (IBM), and GraphPad Prism, version 6, were used for the analyses.

Ethics statement

All experimental procedures were approved and carried out in accordance with the regulation and guidelines of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium (approval number EC2011/090), the IMIM-Hospital del Mar, Barcelona, Spain, and the Lund University Hospital, Lund, Sweden.

Nucleotide sequence accession numbers

The genome sequences determined in this study have been deposited in the EMBL database, and the accession numbers can be found via BioProject record numbers PRJEB7933 and PRJEB7975. (The accession numbers of the individual strains are shown in **Table S1** in the supplemental material.)

Results

Phylogenetic relationships and phenotypical characterization of the highly virulent and low-virulence strains

The draft genomes of the five highly virulent strains (ASB1^T, ASB2, ASB3, ASB6, and ASB14) and the four low-virulence strains (ASB7, ASB9, ASB11, and ASB13) (Joosten et al., 2013) were 1.57 to 1.67 Mb. General features of the genomes are listed in **Table 1**, and the phylogenetic positions of the ASB strains are shown in **Fig. 1**. The nodes in this phylogenetic tree were supported with Chi2-based parameter branch values of 99%. All ASB strains belonged to the clade of the gastric non-*H. pylori Helicobacter* species and clustered in the sister clade of *Helicobacter suis*, but the highly virulent group was separated from the low-virulence group by a long branch. The branch length was comparable to that separating *H. pylori* from *Helicobacter cetorum*.

The Kimura-2 corrected distance value between the two ASB groups, calculated on the basis of the 303 core genes, was approximately 19 substitutions per 100 bp (19%). The average distances among the highly virulent group and the low-virulence group were approximately 3% and 1%, respectively. Thus, at 19% substitution, these two ASB groups differed far more from each other than was expected on the basis of the 16S rRNA and *ureAB* genes. Compared to the *H. pylori* clade (**Fig. 1**), the average distance values between both the highly virulent ASB group and the low-virulence ASB group and *H. pylori*, *Helicobacter acinonychis*, or *H. cetorum* were approximately 50%. The average distance between the ASB group and *H. suis* was approximately 35%. Subsequently, additional tests were used to further investigate the relationship of the ASB strains to each other and, in particular, to determine whether they belong to the same species.

TABLE 1. General features of the genomes of *H. heilmannii* and *H. ailurogastricus*

Strain	Genome size (bp)	No. of contigs	% GC	No. of coding sequences	No. of hypothetical proteins	No. of RNAs
H. heilmannii						
ASB1	1,638,988	6	37.40	1,740	567	40
ASB2	1,570,832	93	37.46	1,783	627	38
ASB3	1,671,206	136	37.11	1,892	682	39
ASB6	1,606,820	84	37.50	1,781	642	40
ASB14	1,574,711	73	37.54	1,762	622	37
H. ailurogastricus						
ASB7	1,675,643	9	37.34	1,706	564	42
ASB9	1,584,457	71	37.35	1,688	531	38
ASB11	1,578,737	56	37.38	1,677	532	38
ASB13	1,580,529	68	37.33	1,697	539	38

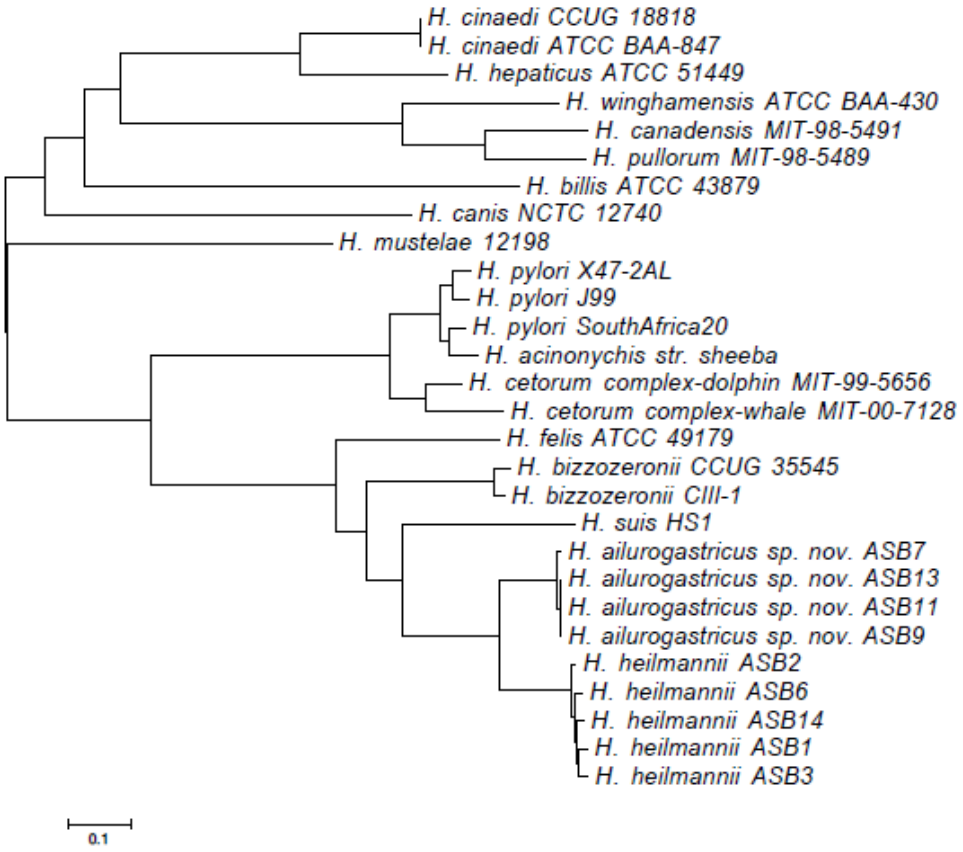

FIG 1. Phylogenetic tree. Shown is a phylogram representing a maximum-likelihood tree of gastric and enterohepatic *Helicobacter* spp. based on 303 aligned and concatenated core genes. All nodes are supported with approximate likelihood ratio test (aLRT) values of 99%, and the topology, branch length, and parameters of the starting tree were optimized. The enterohepatic *Helicobacter* spp. were used as an outgroup.

By use of the GGDC tool, it was shown that all the isolates previously called "low-virulence strains" did not belong to the *H. heilmannii* species. DNA-DNA hybridization (DDH)

parameters, estimated *in silico* by calculating whole-genome distances, yielded a probability via logistic regression of 96.30% that the five highly virulent isolates ASB1^T, ASB2, ASB3, ASB6, and ASB14 belong to the *H. heilmannii* species. The DDH estimates for the four low-virulence isolates ASB7, ASB9, ASB11, and ASB13 resulted in a probability of only 0.01% that they belong to the *H. heilmannii* species. DDH parameters among these low-virulence strains yielded a probability of ca. 98%, indicating that these strains belong to the same species. The average nucleotide identity (ANI) value among ASB1^T, ASB2, ASB3, ASB6, and ASB14 was 97.7%, whereas ASB7, ASB9, ASB11, and ASB13 shared an even higher ANI value of 99%. The ANI value between the two groups was only 84%, which is lower than the generally accepted threshold of 95% for belonging to the same species (Konstantinidis and Tiedje, 2005). The morphology of the low-virulence strains was characterized by transmission electron microscopy. In our previous experimental infection study with Mongolian gerbils (Joosten et al., 2013), strains ASB7 and ASB9, with low colonization capacity, did not cause antral inflammation, whereas chronic active gastritis was seen in the antra of the stomachs of gerbils infected with the low-colonization strains ASB11 and ASB13. Based on these differences among the four low-virulence strains, we selected ASB7 and ASB11 for the TEM study. As shown in **Fig. 2**, ASB7 and ASB11 presented as spiral bacteria with 4 to 5 turns that are 3.0 to 5.5 µm long and 0.5 to 0.7 µm wide and have 6 to 8 sheathed blunt-end flagella at both ends. No periplasmic fibrils were observed. These morphological characteristics are similar to those of *H. heilmannii* as described by Smet et al. (2012), with the exception that *H. heilmannii* has more spiral turns (as many as 9) and presents more bipolar flagella (as many as 10).

The biochemical characteristics of ASB7, ASB9, ASB11, and ASB13 were similar to those of *H. heilmannii* as well. Biochemical analysis revealed that these low-virulence strains were oxidase, catalase, and urease positive. They reduced nitrate and triphenyltetrazolium chloride (TTC) and tested positive for esterase, hippurate, and γ-glutamyltransferase. No pyrrolidonyl arylamidase activity, L-aspartate arylamidase activity, or indoxyl acetate hydrolysis was detected. In contrast to the result for *H. heilmannii*, alkaline phosphatase activity was present. This enzyme is produced by most gastric *Helicobacter* species, but not by *H. heilmannii* (Smet et al., 2012). An overview of the morphological and biochemical characteristics of the highly virulent and low-virulence ASB isolates is shown in **Table 2**.

All these data support the reclassification of these low-virulence strains as a novel species, for which we propose the name *Helicobacter ailurogastricus* sp. nov., with strain ASB7 as the type strain.

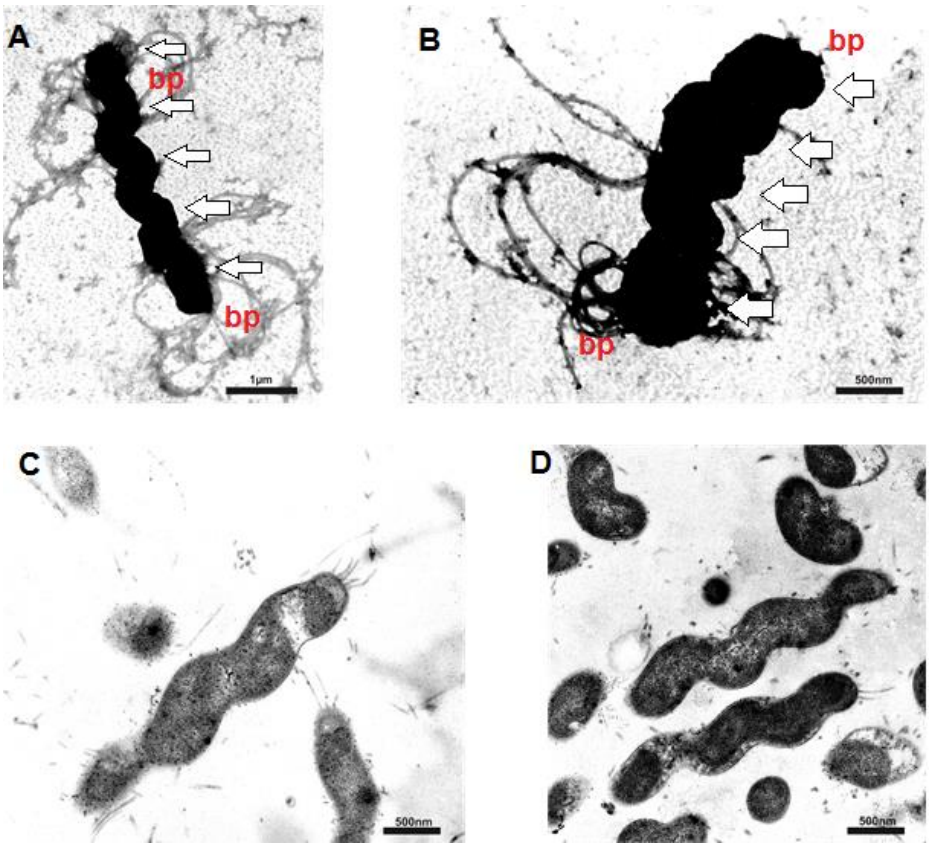

FIG 2. Transmission electron microscopic images of *H. ailurogastricus* strains ASB7^T (A and C) and ASB11 (B and D). (A and B) Negatively stained cells of *H. ailurogastricus* ASB7^T (A) and ASB11 (B) showing cells with as many as 5 turns (arrows) and bipolar blunt-end flagella (bp). (C and D) Uranyl acetate and lead citrate staining of *H. ailurogastricus* ASB7^T (C) and ASB11 (D).

TABLE 2. Morphological and biochemical characteristics of *H. heilmannii* and *H. ailurogastricus* compared to those of other gastric *Helicobacter* species

	Cell size (µm)			Flagella						
Helicobacter species[a]	Length	Width	Periplasmic fibril	No. per cell	Distribution[b]	Urease activity	Nitrate production	Alkaline phosphatase activity	Hydrolysis of indoxyl acetate	Growth at 42°C
H. heilmannii	3–6.5	0.6–0.7	−	4–10	BP	+	+	−	−	−
H. ailurogastricus	3–5.5	0.5–0.7	−	6–8	BP	+	+	+	−	−
H. felis	5–7.5	0.4	+	14–20	BP	+	+	+	−	−
H. bizzozeronii	5–10	0.3	−	10–20	BP	+	+	+	+	+
H. salomonis	5–7	0.8–1.2	−	10–23	BP	+	+	+	+	−
H. cynogastricus	10–18	0.8–1.0	+	6–12	BP	+	+	+	−	−
H. baculiformis	10	1	+	11	BP	+	+	+	−	−
H. suis	2.3–6.7	0.9–1.2	−	4–10	BP	+	−	+	−	−
H. pylori	2.5–5.0	0.5–1.0	−	4–8	MP	+	−	+	−	−

[a] Data for *H. ailurogastricus* are from this study. Data for the other species are from Smet et al., 2012 (*H. heilmannii*), Hänninen et al., 1996; Lee et al., 1988; Jalava et al., 1997 (*H. felis*), Hänninen et al., 1996 (*H. bizzozeronii*), Jalava et al., 1997 (*H. salomonis*), Van den Bulck et al., 2006 (*H. cynogastricus*), Baele et al., 2008b (*H. baculiformis*), Baele et al., 2008a (*H. suis*), Hänninen et al., 1996 and Jalava et al., 1997 (*H. pylori*). [b] BP, bipolar; MP, monopolar.

In silico proteome analysis

Examination of the annotated genomes of different gastric and enterohepatic *Helicobacter* species (see **Table S1** in the supplemental material) yielded a total of 50,899 predicted protein sequences. Based on OrthoMCL clustering, these proteins were divided into 12,216 groups of orthologs. A total of 132 (7.25%) *H. heilmannii* proteins and 82 (4.91%) *H. ailurogastricus* proteins had no orthologs in the other available genome-sequenced *Helicobacter* species (see **Table S1**) and thus might be unique to *H. heilmannii* and *H. ailurogastricus*, respectively. Reciprocal BLASTP analysis between the ASB1^T and ASB7^T proteomes identified 394 ASB1^T proteins (59 with putative function and 335 hypothetical proteins) with no significant homology to any *H. ailurogastricus* ASB7^T protein. Conversely, 303 ASB7^T proteins (59 with putative function and 244 hypothetical proteins) with no significant homology to any *H. heilmannii* ASB1^T protein were identified. An overview of the proteins with predicted functions is shown in **Table S2** in the supplemental material. The majority of genes present in *H. heilmannii* ASB1^T but absent in *H. ailurogastricus* ASB7^T and vice versa play roles in DNA replication, recombination, and repair, protection against the uptake of foreign DNA, chemotaxis (bacterial signaling), outer membrane and lipopolysaccharide (LPS) synthesis, lipid metabolism, and transport and metabolism of nucleotides, amino acids, and carbohydrates (see **Table S2**). Additionally, genes encoding proteins involved in DNA binding and transfer and associated with ulcer development were identified in ASB1^T but not in ASB7^T, whereas proteins that play roles in fermentation, iron uptake, cell division, and various metabolic processes were found in ASB7^T but were absent in ASB1^T. Similar results were obtained when other *H. heilmannii* strains were compared with the *H. ailurogastricus* strains (data not shown).

Genes possibly associated with differences in virulence and colonization capacity between *H. heilmannii* and *H. ailurogastricus*

Genes implicated in bacterium-host interactions that differ between *H. heilmannii* and *H. ailurogastricus* or that are present in *H. heilmannii* but absent in *H. ailurogastricus* merit special attention. The absence or presence of these genes in other gastric *Helicobacter* species was verified as well.

(i) *iceA1*. In *H. pylori*, the ulcer-associated protein restriction endonuclease (IceA) has been identified as a virulence factor associated with peptic ulcer disease and is induced by contact of the bacterium with epithelial cells. The *iceA* gene exists as two distinct genotypes, *iceA1* and *iceA2*, and only *iceA1* RNA is induced following adherence (Peek et al., 1998; Peek et al., 2000). The *iceA* allele is part of a restriction-modification (R-M) system and is located upstream

of the ulcer-associated adenine-specific DNA methyltransferase (*hpyIM*) (Peek et al., 2000). R-M systems function in self/nonself recognition and protect against genomic adulteration by foreign DNA. These systems also promote homologous recombination of species-specific or closely related DNA and thereby provide a rapid mechanism of genetic adaptation (Donahue and Peek, 2001; Lin et al., 2001). The *H. heilmannii* strains each contain an intact *iceA1* homolog (HHE01_10510 [see **Table S2** in the supplemental material], HHE02_06610, HHE03_16620, HHE06_03450, HHE014_17360), showing approximately 90% amino acid identity to one another and only 54% identity to *H. pylori* IceA1 homologs. As in *H. pylori*, this gene is located next to a homolog encoding an ulcer-associated adenine-specific DNA methyltransferase. Interestingly, an *iceA* homolog is absent in *H. ailurogastricus*, and only a gene encoding the DNA methyltransferase is present. The *iceA1*-DNA methyltransferase locus has also been found in the available genomes of *H. acinonychis* and *Helicobacter bizzozeronii* (see **Table S1** in the supplemental material) but is absent in *H. cetorum*, *H. suis*, and *Helicobacter felis*.

(ii) Putative *hrgA*. Another DNA R-M system described for *H. pylori* is the *hrgA*/*hpy*IIIR system, of which the endonuclease-replacing gene (*hrgA*) has been described as a clinical marker for virulence (Ando et al., 2002). A *hrgA*-like gene, though in the absence of its methyltransferase enzyme, is also present in the genomes of *H. heilmannii* and *H. bizzozeronii* but not in *H. ailurogastricus* and other non-*Helicobacter pylori* helicobacters. *H. heilmannii* HrgA shows 50% protein-level identity with *H. pylori* HrgA. Interestingly, this protein exhibited limited sequence identity among the HrgA proteins of the different *H. heilmannii* strains (HHE01_08490 showed sequence identities of 98.26% with HHE02_00920, 42.32% with HHE03_02210, 40.44% with HHE06_03280, and 42.01% with HHE014_12870; HHE02_00920 showed sequence identities of 44.79% with HHE03_02210, 42.71% with HHE06_03280, and 44.44% with HHE014_12870; HHE03_02210 showed sequence identities of 64.06% with HHE06_03280 and 99.38% with HHE014_12870; HHE06_03280 showed a sequence identity of 63.75% with HHE014_12870). Also for *H. pylori*, virulence factors are highly diverse between strains, and this diversity has been associated with different disease outcomes (Lu et al., 2014).

(iii) jhp0562-like glycosyltransferase. Besides proteins with functions in a R-M system, we identified a putative homolog of the *H. pylori* jhp0562 glycosyltransferase in the *H. heilmannii* strains (HHE01_14290, HHE02_07300, HHE03_07750, HHE06_15850, HHE014_01210) that is absent in *H. ailurogastricus*. The *H. pylori* LPS biosynthesis enzyme jhp0562 glycosyltransferase functions in the synthesis of both type I and type II Lewis (Le) antigens,

which are present on the LPS of the bacterial outer membrane (Pohl et al., 2012). Via intragenomic recombination of jhp0562, diverse Le antigens are generated, and this glycosyltransferase contributes to the process of phase variation in *H. pylori* (de Vries et al., 2002; Pohl et al., 2012; Weiser and Pan, 1998). Phase variation is one of the mechanisms used by *H. pylori* to escape the host immune response and to persist in the stomach. It creates phenotypical variation in a bacterial population by the reversible process of switching a gene on and off. Phase-variable bacterial genes, such as LPS biosynthesis genes, play roles in bacterial pathogenesis and virulence (de Vries et al., 2002; Weiser and Pan, 1998). Moreover, the presence of jhp0562 has been associated with peptic ulcer disease in children (Oleastro et al., 2010; Pohl et al., 2012). The jhp0562-like glycosyltransferases of the five *H. heilmannii* strains showed approximately 99% protein-level identity to one another but only 36% identity with *H. pylori* jhp0562 homologs. Homologs are also present in *H. bizzozeronii*, *H. felis*, *H. suis*, and *H. cetorum*, with protein-level identity between 35 and 40% with the jhp0562-like glycosyltransferase of *H. heilmannii*.

(iv) OMPs. Several outer membrane proteins (OMPs) of *H. pylori* play important roles in adhesion to and colonization of the human stomach (Oleastro and Ménard, 2013). *H. heilmannii* strains ASB1^{T}, ASB2, ASB3, ASB6, and ASB14 contain 53, 55, 56, 56, and 55 OMP-encoding genes, respectively, whereas the OMP repertoire of *H. ailurogastricus* consists of approximately 60 OMP-encoding genes. This gene number is in agreement with the ~64 well-annotated OMP-encoding genes described for *H. pylori* (Alm et al., 2000). The *H. pylori* (J99), *H. heilmannii* (ASB1^{T}, ASB2, ASB3, ASB6, and ASB14), and *H. ailurogastricus* (ASB7^{T}, ASB9, ASB11, and ASB13) OMPs were clustered in groups of orthologs. The results are shown in **Table S3** in the supplemental material. The analysis showed that *H. heilmannii* and *H. ailurogastricus* share only a few homologs of the *H. pylori* Hop, Hor, and Hom proteins. Remarkably, the well-studied *H. pylori* adhesins BabA and BabB (HopS and HopT), SabA (HopP), AlpA and AlpB (HopB and HopC), OipA (HopH), HopZ, HopQ, and HomB are absent in *H. heilmannii* and *H. ailurogastricus*. Only genes encoding homologs of the *H. pylori* Hof proteins, except for HofB, are present in *H. heilmannii* and *H. ailurogastricus*. The different Hof proteins each exhibit 98% identity among the different *H. heilmannii* strains. Similar findings were made for the *H. ailurogastricus* Hof proteins. The average levels of amino acid identity of the *H. heilmannii* Hof proteins to the *H. ailurogastricus* and *H. pylori* Hof proteins are about 88 to 90% and 55%, respectively. In contrast to those of *H. pylori*, the *H. heilmannii* and *H. ailurogastricus hof* genes are located in a ~10-kb locus. This locus is also present in other canine, feline, and porcine gastric helicobacters. Additionally, *H. heilmannii* and *H.*

ailurogastricus harbor several unique putative OMPs that are absent in *H. pylori* and whose biological function (e.g., interaction with the gastric mucosa) is unknown. Moreover, *H. heilmannii* harbors 6 putative OMPs that are absent in *H. ailurogastricus*. Two of these hypothetical OMPs are located in close proximity to each other on the same locus of approximately 13 kb (HHE01_09750 and HHE01_09730, HHE02_11280 and HHE02_11300, HHE03_06010 and HHE03_06030, HHE06_13950 and HHE06_13930, and HHE014_02600 and HHE014_02620 for ASB1, ASB2, ASB3, and ASB14, respectively [see **Table S3** in the supplemental material]).

(v) VacA-like autotransporter. One of the major protein toxins secreted by *H. pylori* is the vacuolating cytotoxin A (VacA), which belongs to an additional family of OMPs called autotransporters (Sause et al., 2012). The VacA toxin binds to host cells and is internalized, causing severe "vacuolation" characterized by the accumulation of large vesicles that possess hallmarks of both late endosomes and early lysosomes (Palframan et al., 2012). No homologs of the *H. pylori vacA* gene are present among the *H. heilmannii* and *H. ailurogastricus* genomes. This *vacA* gene is also absent in the other canine, feline, and porcine non-*H. pylori Helicobacter* species. Intact homologs of this gene have been reported only for *H. cetorum* (Kersulyte et al., 2013). Additionally, *H. pylori* contains three genes annotated as putative *vacA* paralogs, because the C-terminal autotransporter domains of the proteins they encode show approximately 30% identity to that of VacA. These three VacA-like autotransporters each enhance the capacity of *H. pylori* to colonize the stomach (Radin et al., 2013). The *H. heilmannii* and *H. ailurogastricus* strains also contain a gene encoding a VacA-like autotransporter (HHE01_12480, HHE02_13180, HHE03_15470, HHE06_06350, HHE014_11640, HAL07_13640, HAL09_00010, HAL011_16100, HAL013_02860). The VacA-like autotransporters of the different *H. heilmannii* strains exhibit approximately 95% identity to one another and 82% identity to those of *H. ailurogastricus*. An average identity of 98% was seen among the VacA-like autotransporters of the *H. ailurogastricus* strains. The *H. pylori* VacA-like autotransporters possess a conserved domain structure consisting of an N-terminal signal peptide, a nonconserved central passenger domain, and a C-terminal β-barrel domain. The presence of similar conserved domains in the VacA-like autotransporter proteins of *H. heilmannii* and *H. ailurogastricus* was predicted *in silico* and is shown in **Fig. 3**. These proteins show typical hallmarks of an autotransporter. The *H. heilmannii* autotransporter protein contains a short N-terminal cytoplasmic tail (ca. 1 to 53 amino acids [aa]) but without a predicted signal sequence, a transmembrane helix (ca. 23 aa), and a large noncytoplasmic part. The latter part contains the passenger domain with three VacA2 regions and a well-conserved

C-terminal autotransporter (β-barrel) domain (**Fig. 3**). A similar structure was predicted for the *H. ailurogastricus* VacA-like autotransporter protein but with a larger passenger domain. Interestingly, all *H. ailurogastricus* strains showed four VacA2 regions in their passenger domain (**Fig. 3**). The passenger domain represents the surface-exposed component of the protein and adopts an extended right-handed β-helix structure (Sause et al., 2012). Also other canine, feline, and porcine gastric *Helicobacter* species harbor a copy of a *vacA*-like autotransporter gene. Phylogenetic analysis of the VacA-like autotransporter proteins present among the different gastric *Helicobacter* species highlighted high divergence among these autotransporters between species; only their C-terminal parts were well conserved (see **Fig. S1** in the supplemental material). The *H. heilmannii* VacA-like protein exhibits approximately 40 to 50% protein-level identity to the *H. felis*, *H. bizzozeronii*, and *H. suis* homologs, while only 30% identity to the *H. pylori*, *H. acinonychis*, and *H. cetorum* homologs was noted.

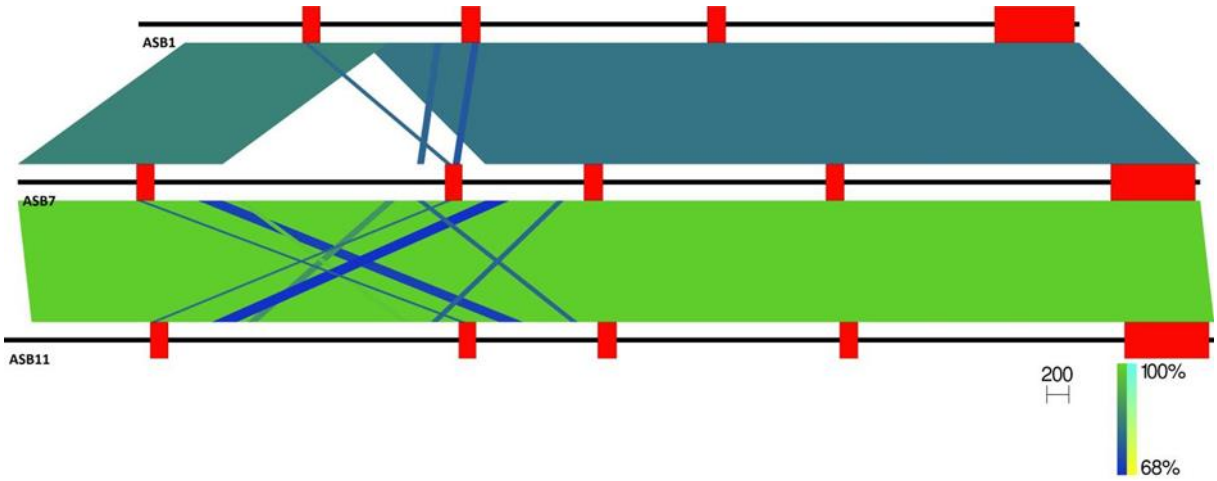

FIG 3. Schematic representation of the conserved domains present in the VacA-like autotransporter of *H. heilmannii* (ASB1^T) and *H. ailurogastricus* (ASB7^T and ASB11). No N-terminal signal sequence could be predicted by SignalP, version 3.0. The passenger domain of the ASB1^T VacA-like autotransporter contains three VacA2 regions, whereas those of ASB7^T and ASB11 harbor four VacA2 regions (small red rectangles). The main block of homology present in the autotransporter proteins of the two species is the C-terminal β-barrel domain (large red rectangle). (Top) The autotransporter of *H. heilmannii* ASB1^T is 8,583 bp long, with three VacA2 regions at bp 1,494 to 1,659, bp 2,946 to 3,120, and bp 5,190 to 5,358 and a β-barrel at bp 7,809 to 8,541. (Center) The autotransporter of *H. ailurogastricus* ASB7^T is 10,788 bp long, with four VacA2 regions at bp 1,083 to 1,248, bp 3,897 to 4,056, bp 5,166 to 5,340, and bp 7,374 to 7,542 and a β-barrel at bp 9,972 to 10,746. (Bottom) The autotransporter of *H. ailurogastricus* ASB11 is 11,040 bp long, with four VacA2 regions at bp 1,335 to 1,500, bp 4,149 to 4,308, bp 5,418 to 5,592, and bp 7,626 to 7,794 and a β-barrel at bp 10,224 to 10,998. The white triangle represents the region in the passenger domain that is absent in *H. heilmannii* ASB1^T but present in *H. ailurogastricus* ASB7^T and ASB11.

In vitro binding to the gastric mucosa

Because of the differences seen in gastric colonization between *H. heilmannii* and *H. ailurogastricus* (Joosten et al., 2013), we also investigated if there were differences in their capacities for binding to gastric mucins and epithelial cells.

(i) *In vitro* binding to human gastric mucins

The capacities of *H. heilmannii* and *H. ailurogastricus* for binding to human gastric mucin samples, derived from a healthy stomach and from a patient with a gastric tumor, were tested at pH 2 and pH 7. The binding of *H. pylori* to mucins at acidic pHs has been shown to be dependent on charge (Lindén et al., 2008). Mucins carry on the order of 100 different carbohydrate structures, including negatively charged carbohydrates. To distinguish between the charge-dependent binding mechanism and binding to other structures present on mucins, binding to DNA (as a marker for a negative charge) was also investigated. The results for the five *H. heilmannii* and four *H. ailurogastricus* isolates are displayed in **Fig. 4**. In general, the binding of *H. heilmannii* and *H. ailurogastricus* to the mucins was very weak at both pH values, whereas the level of binding to DNA at pH 2 was 10-fold higher (**Fig. 4E**). *H. heilmannii* had a higher capacity for binding to DNA than *H. ailurogastricus* (**Fig. 4E** and **F**) (*P*, <0.05 by an unpaired *t* test), and there also was a trend toward higher binding of *H. heilmannii* to the gastric mucins (**Fig. 4B** through **D**) (*P*, 0.07 to 0.11). There was no clear difference between binding to mucins at pH 2 and binding to mucins at pH 7.

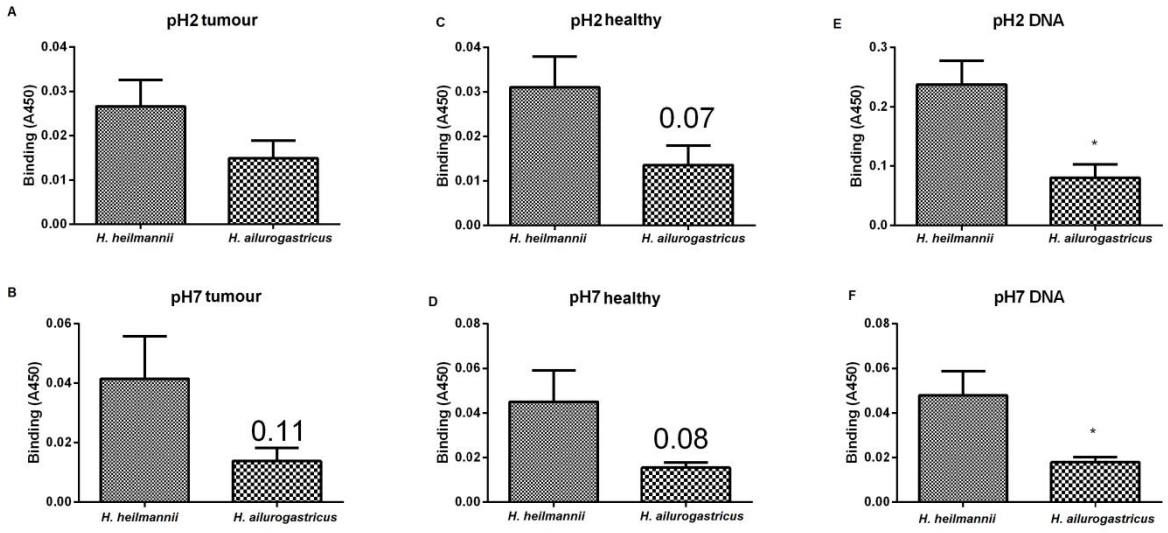

FIG 4. Capacity for *in vitro* binding to human gastric mucins and DNA. Shown is the *in vitro* binding of *H. heilmannii* (ASB1^T, ASB2, ASB3, ASB6, and ASB14) and *H. ailurogastricus* (ASB7^T, ASB9, ASB11, and ASB13) isolates to two human gastric mucins and to DNA at pH 2 (A, C, and E) and pH 7 (B, D, and F). Binding was quantified by measuring the OD at 450 nm. (A and B) Binding to a mucin sample derived from a gastric tumor; (C and D) binding to a mucin sample derived from a healthy stomach; (E and F) binding to DNA

as a marker for a negative charge. Significant differences in DNA binding between the two species are indicated by asterisks (*P*, <0.05 by an unpaired *t* test). *P* values given above bars indicate non-statistically significant differences in mucin binding between the two species (B through D).

(ii) *In vitro* binding to gastric epithelial cells

The *in vitro* capacities of *H. heilmannii* and *H. ailurogastricus* for binding to gastric epithelial cells were studied by scanning electron microscopy (SEM). An explicit difference between the capacities of *H. heilmannii* ASB1^T and *H. ailurogastricus* ASB7^T for binding to human-derived MKN7 cells and mouse-derived GSM06 cells was observed and is illustrated in **Fig. 5A** to **D**. Quantification of bacteria bound to cells showed higher numbers of *H. heilmannii* than of *H. ailurogastricus* bacteria binding to gastric epithelial MKN7 and GSM06 cells at both pH 2 and pH 7 (**Fig. 5E** to **H**) (*P*, <0.05 by the Mann-Whitney *U* test). At pH 7, more *H. heilmannii* bacteria were able to bind to MKN7 and GSM06 cells than at pH 2 (*P*, <0.05 by the Mann-Whitney *U* test). For each *H. heilmannii* and *H. ailurogastricus* isolate, the mean numbers of bacteria binding to MKN7 cells and GSM06 cells at pH 7 and pH 2 are shown in **Table S4** in the supplemental material. The *H. heilmannii* type strain, ASB1, had the highest capacity for binding to both cell lines.

A quantitative fluorescence-based adherence assay was performed to validate the SEM results. As shown in **Fig. 6**, a higher mean intensity of fluorescent light was emitted after the binding of FITC-labeled *H. heilmannii* bacteria to gastric epithelial MKN7 and GSM06 cells than after the binding of FITC-labeled *H. ailurogastricus* bacteria. Given that the intensity of emitted light is proportional to the quantity of cell-bound bacteria, this experiment confirms that *H. heilmannii* has a higher capacity for binding to gastric epithelial cells than *H. ailurogastricus*.

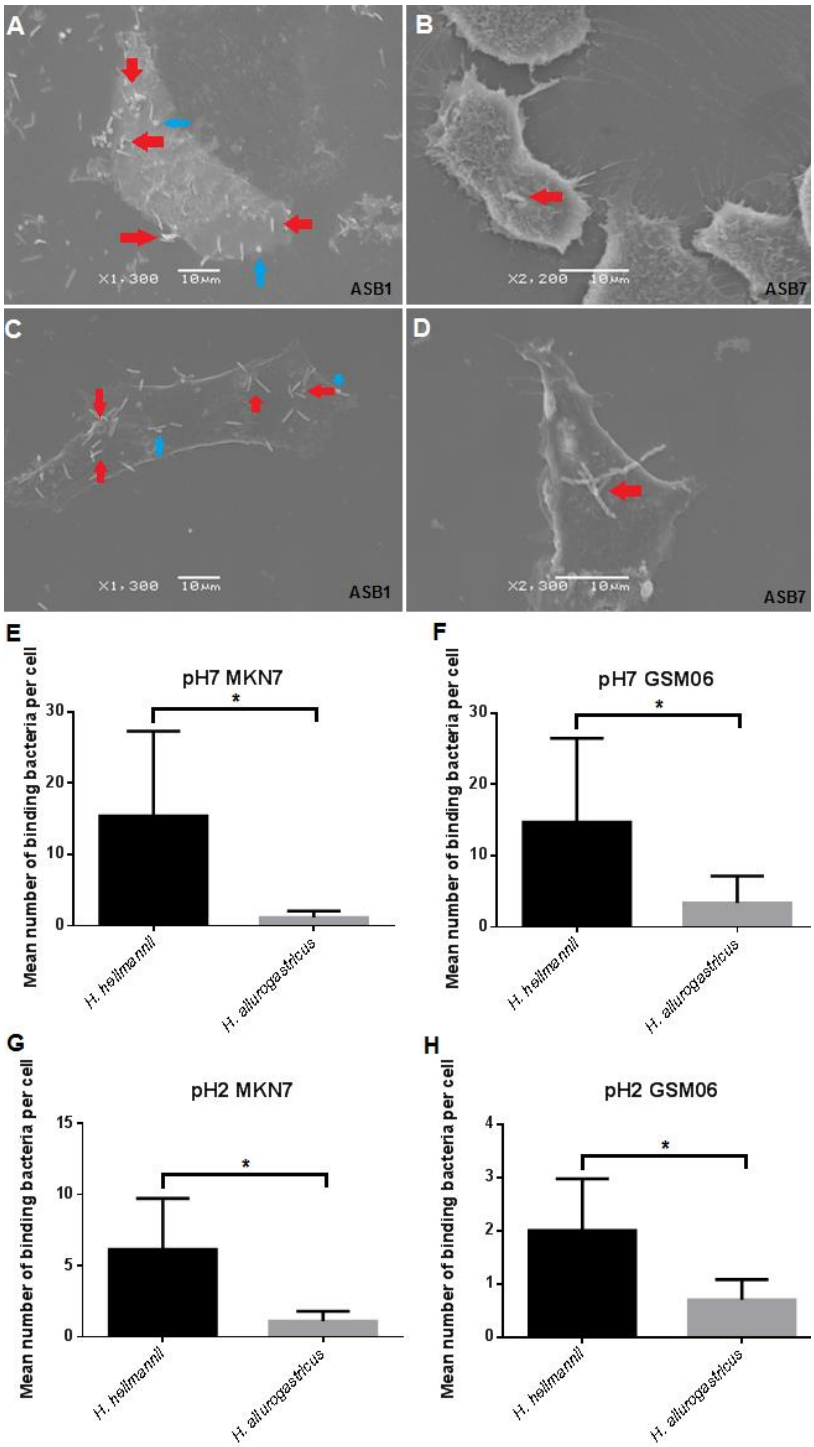

FIG 5. *In vitro* binding of *H. heilmannii* and *H. ailurogastricus* isolates to gastric epithelial cells. (A to D) Scanning electron microscopy was used for visualization of the binding of *H. heilmannii* strain ASB1^T (A and C) and *H. ailurogastricus* strain ASB7^T (B and D) to MKN7 (A and B) and GSM06 (C and D) cells. Binding bacteria are indicated by red arrows for spiral *Helicobacter* cells and blue arrows for coccoid *Helicobacter* cells. Bars, 10 µm. (E to H) The mean numbers of *H. heilmannii* (ASB1^T, ASB2, ASB3, ASB6, and ASB14) and *H. ailurogastricus* (ASB7^T, ASB9, ASB11, and ASB13) bacteria binding MKN7 cells and GSM06 cells at pH 7 (E and F) and at pH 2 (G and H) were calculated (binding bacteria per cell). The results showed higher numbers of *H. heilmannii* than of *H. ailurogastricus* bacteria binding gastric epithelial cells. Significant differences in binding between the two species are indicated by asterisks (P, <0.05 by the Mann-Whitney U test).

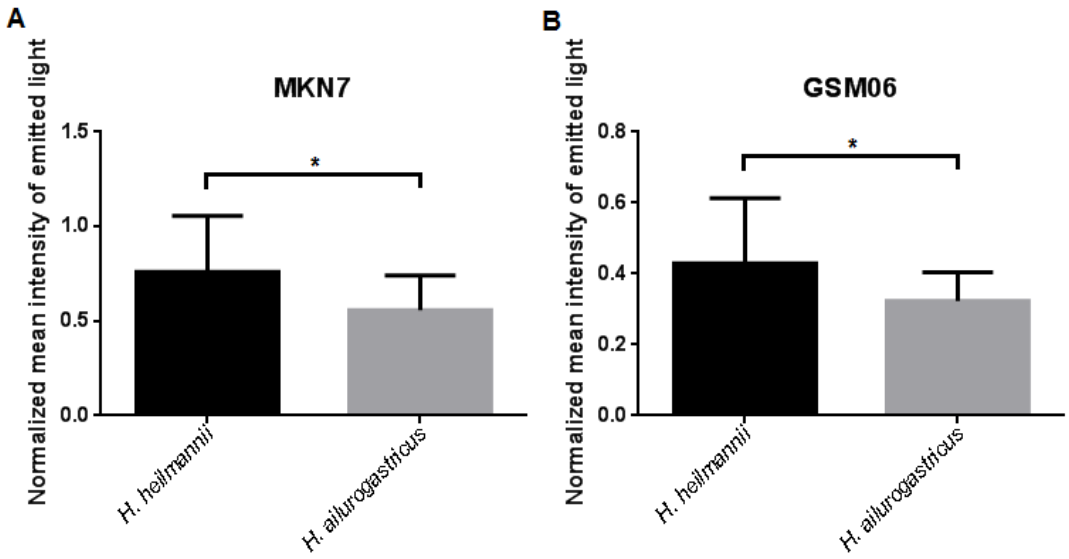

FIG 6. Fluorescence-based assay of the adherence of *H. heilmannii* and *H. ailurogastricus* isolates to gastric epithelial cells. The binding of FITC-labeled *H. heilmannii* (ASB1^T, ASB2, ASB3, ASB6, and ASB14) and *H. ailurogastricus* (ASB7^T, ASB9, ASB11, and ASB13) isolates to gastric epithelial MKN7 (A) and GSM06 (B) cells was quantified by measuring the emission of fluorescent light at a λ of 527 nm. To correct for differential labeling of *H. heilmannii* and *H. ailurogastricus* with FITC, the relative levels of FITC labeling of all ASB strains were analyzed by FCM. Therefore, data are presented as the mean intensity of the emitted light normalized to the relative level of FITC labeling of *H. heilmannii* and *H. ailurogastricus* strains. Significant differences in binding between *H. heilmannii* and *H. ailurogastricus* are indicated by asterisks (*P*, <0.05 by the Mann-Whitney *U* test).

(iii) *In vitro* binding to the gastric mucosae of Mongolian gerbils and stray cats

In vitro binding experiments (at pH 7 and pH 2) were also performed on paraffin-embedded gastric tissue samples from the antrum and the corpus of the stomach. Samples from Mongolian gerbils were used because this is a model used for studying *Helicobacter*-related gastric pathology in humans. Stomach samples from cats, the natural host of *H. heilmannii* and *H. ailurogastricus*, were included as well. Overall, similar binding patterns were obtained in the gastric mucosae of Mongolian gerbils and cats, and *H. heilmannii* and *H. ailurogastricus* showed equally strong capacities for binding to the samples. Results from Mongolian gerbils and cats were pooled in order to study the capacities for binding to the corpus versus the antrum of the stomach and to the surface epithelium versus the glands of the gastric mucosa. A clear difference in binding specificity between *H. heilmannii* and *H. ailurogastricus* (**Fig. 7**), which was more pronounced at pH 2, was observed. *H. heilmannii* ASB1^T bound mainly to the glandular cells of both the antrum and the corpus of the stomach (**Fig. 7A** and **D**), whereas *H. ailurogastricus* ASB7^T (**Fig. 7B** and **E**) and ASB11 (**Fig. 7C** and **F**) had a higher capacity for binding to the surface epithelium lining the gastric mucosa.

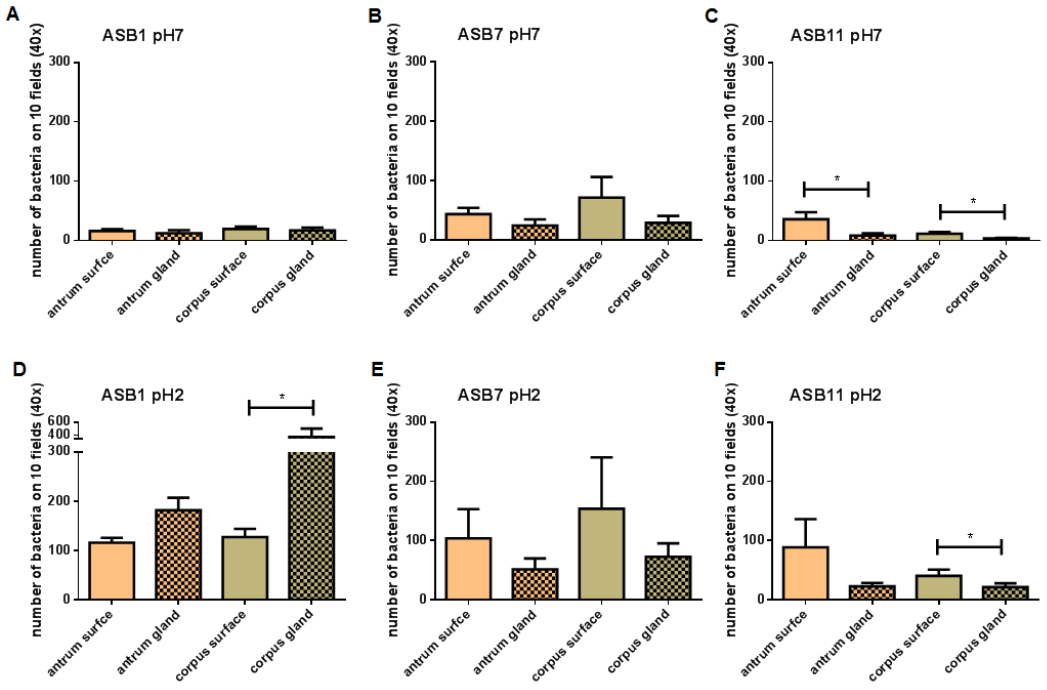

FIG 7. *In vitro* binding to the gastric mucosae of Mongolian gerbils and stray cats. The number of binding bacteria was counted in 10 randomly chosen high-power fields at the level of the surface epithelium and the gastric glands (magnification, ×40) in the antra and corpora of gerbil and cat stomachs, both at pH 7 (A to C) and at pH 2 (D to F). Results from gerbils and cats were pooled for each stomach region, and data are shown as means + standard deviations. Significant differences in binding are indicated by asterisks (*P*, <0.05 by the Mann-Whitney *U* test). (A and D) Binding of *H. heilmannii* ASB1^T; (B and E) binding of *H. ailurogastricus* ASB7^T; (C and F) binding of *H. ailurogastricus* ASB11.

Discussion

In the present study, comparative genomics and phylogenetic and phenotypical analyses of nine feline *Helicobacter* strains identified as *H. heilmannii* on the basis of their 16S rRNA and *ureAB* genes revealed that the previously defined low-virulence strains (Joosten et al., 2013) belong to a novel species, closely related to *H. heilmannii*, that has not been described before and for which we propose the name *Helicobacter ailurogastricus* sp. nov. *H. ailurogastricus* cannot be distinguished from *H. heilmannii* by means of its 16S rRNA and *ureAB* gene sequences, which have frequently been used for differentiation between gastric *Helicobacter* species (O'Rourke et al., 2004b, Smet et al., 2012). This implies that the discriminatory capacity of these gene sequences is not high enough for distinguishing between closely related gastric *Helicobacter* species. In this respect, our study underlines that genome-sequencing-based approaches are superior to traditional 16S rRNA sequence analysis for studying phylogeny, because they are based on the complete genome content and because they have better resolution for

distinguishing between both distantly and closely related bacteria (Vandamme and Peeters, 2014).

Phenotypically, *H. ailurogastricus* and *H. heilmannii* are also similar. Both species presented a spiral morphology with bipolar flagella but without periplasmic fibrils. The biochemical properties of *H. ailurogastricus* are very similar to those of *H. heilmannii*. Only the alkaline phosphatase activity differed between the two species: it was absent in *H. heilmannii* but present in *H. ailurogastricus*. The similar phenotypical characteristics contribute to the difficulty of distinguishing between these species. This reinforces the suggestion that a full genome sequence, combined with a minimal description of phenotypic characteristics, should become sufficient for the description of a novel species (Vandamme and Peeters, 2014).

Our proteomic analyses revealed that *H. ailurogastricus* lacks homologs of the *H. pylori* IceA1, HrgA, and jhp0562 glycosyltransferase proteins, which have been reported to be involved in the disease outcome of *H. pylori* infection (Ando et al., 2002; Pohl et al., 2012; Shiota et al., 2012; Xu et al., 2002). The absence of these virulence factors in *H. ailurogastricus* might thus contribute to the low-virulence character of this species. In contrast, genes encoding homologs of IceA1 and HrgA and a putative jhp0562-like glycosyltransferase are present in the *H. heilmannii* genomes. *H. heilmannii* has been associated with a number of different gastric disorders in humans, and the risk of developing mucosa-associated lymphoid tissue (MALT) lymphoma is higher after infection with *H. heilmannii* than after infection with *H. pylori* (Haesebrouck et al., 2009; Liu et al., 2014; Morgner et al., 2000). The biological function of these putative virulence-associated proteins and their exact role in the disease outcome of *H. heilmannii* infection remain to be investigated.

Bacterial outer membrane proteins (OMPs) are directly involved in the interactions of pathogenic bacteria with their hosts. *H. heilmannii* and *H. ailurogastricus* harbor several OMPs, but only a few are members of the *H. pylori* Hop, Hor, or Hom family. This suggests the presence of other OMP classes in the genus *Helicobacter*. Interestingly, both species lack all *H. pylori* adhesins described so far. Only genes encoding homologs of the *H. pylori* Hof proteins were well conserved in both species. In contrast to those of *H. pylori*, their *hof* genes are located in a large locus. This locus seems to be unique for the canine, feline, and porcine gastric helicobacters (Schott et al., 2011). Preliminary results obtained with *H. heilmannii* deletion mutants demonstrated that the *H. heilmannii* Hof locus plays a role in gastric colonization (unpublished results). Moreover, six putative unique OMPs were predicted in the *H. heilmannii* genome, whereas they were absent in *H. ailurogastricus*. These OMPs might be involved in the difference between the colonization capacities of the two species. Further research is necessary

to investigate the similarities and differences in these OMP genes and their expression, as well as the evolutionary events that are involved in their acquisition (e.g., gene conversion and phase variation).

Another virulence factor that merits particular attention is the VacA-like autotransporter. *H. pylori* harbors three such autotransporters, which belong to an additional OMP family and have been shown to enhance the ability of *H. pylori* to colonize the stomach (Radin et al., 2013; Sause et al., 2012). Only one copy is present among the canine, feline, and porcine gastric helicobacters. Although no signal sequence was predicted using available tools, whether these autotransporters really lack a signal sequence remains to be elucidated. Interestingly, the VacA-like autotransporters were highly divergent between the different gastric *Helicobacter* species at the amino acid level. Possibly, horizontal transfer events are involved in their acquisition. Further studies are necessary to reveal (i) how their divergent sequences affect the transport, actions, and interactions of the proteins they encode and (ii) the selective forces that drive their evolution. In *H. pylori*, the passenger domain of the VacA-like autotransporter, which likely confers the effector function of this protein, contains three VacA2 regions. Although these VacA2 regions show low similarity to the VacA toxin, they do not correspond to a functional portion of VacA (Sause et al., 2012). In our study, the passenger domains of the VacA-like autotransporters of all *H. heilmannii* strains also contained three VacA2 regions, whereas in *H. ailurogastricus* strains, which have low colonization capacity in Mongolian gerbils (Joosten et al., 2013), this passenger domain contained four VacA2 regions. In general, the major difference between the *H. heilmannii* and *H. ailurogastricus* VacA-like autotransporters was the size of the complete passenger domain, which was remarkably larger for the *H. ailurogastricus* VacA-like autotransporter. This highlights the potential role of the size of the passenger domain in gastric colonization, which merits further investigation.

In vitro binding assays revealed that *H. ailurogastricus* had a lower ability to bind to human- and mouse-derived gastric epithelial cells than *H. heilmannii*. The level of binding of *H. heilmannii* bacteria to gastric epithelial cells was higher at pH 7 than at pH 2. This is consistent with the physiological pH gradient in the stomach, ranging from pH 1 to 2 in the gastric lumen to pH 6 to 7 at the epithelial cell surface (Allen and Flemstrom, 2005; Bhaskar et al., 1992; O'Toole et al., 2005). Following this theory, we would expect better binding to human gastric mucins at pH 2, since they are located in the acidic lumen of the stomach. BabA- and SabA-independent adhesion of *H. pylori* to gastric mucins has indeed been shown to be more pronounced at low pHs, a phenomenon that is dependent on a charge/low-pH-dependent mechanism (Lindén et al., 2004; Lindén et al., 2008; Skoog et al., 2011). In the present study,

however, the *in vitro* capacities of *H. heilmannii* and *H. ailurogastricus* for binding to human gastric mucins were very low, and there was no difference in binding at low or neutral pHs. The binding capacities of *H. heilmannii* and *H. ailurogastricus* were 20- to 100-fold lower than the capacity of *H. pylori* for binding to the same human gastric mucins (Lindén et al., 2008; Skoog et al., 2011; Skoog et al., 2012). Mucins can carry on the order of 100 different carbohydrate structures, which provide the mucins with a bottle brush appearance and make them act as receptors for microorganisms (Klein et al., 1993). The absence of carbohydrates acting as receptors for *H. heilmannii* and *H. ailurogastricus* in the mucin samples used might explain the low binding capacities of both species. On the other hand, binding to DNA as a marker for a negative charge was markedly higher at pH 2 than at pH 7. Thus, the role of a charge/low-pH-dependent mechanism in the binding of *H. heilmannii* and *H. ailurogastricus* to gastric mucins needs to be further investigated.

Both *in vivo* (Haesebrouck et al., 2009; Joosten et al., 2013) and *in vitro* in our study, *H. heilmannii* is found mostly in the gastric glands. A recent study also highlighted the potential role of the gland mucin MUC6 in the early colonization process of *H. heilmannii* (Liu et al., 2014). *In vitro* binding studies might be interesting to further unravel the adhesion capacity of *H. heilmannii* to MUC6. In contrast, the binding of *H. ailurogastricus* to the surface epithelium is more pronounced, like that of *H. pylori* (Lindén et al., 2002).

In conclusion, we have described a new feline gastric *H. ailurogastricus* species, which is closely related to *H. heilmannii*. *H. ailurogastricus* lacks several homologs encoding *H. pylori* virulence and colonization factors and has a lower capacity for binding to gastric epithelial cells *in vitro*. This may explain why its virulence is lower than that of *H. heilmannii*.

Description of *Helicobacter ailurogastricus* sp. nov.

Helicobacter ailurogastricus (ai.lu.ro.gas'tri.cus. N.L. n. *ailurus*, cat, from Gr. n. *ailouros*, cat; N.L. masc. adj. *gastricus*, of the stomach, from Gr. n. *gaster*, stomach; *ailurogastricus*, N.L. masc. adj., of a cat's stomach).

Cells are tightly coiled spirals with as many as 5 turns that are approximately 3.0 to 5.5 µm long and approximately 0.5 to 0.7 µm wide. They have no periplasmic fibrils, and coccoid cells predominate in older cultures. They are motile by means of tufts of as many as 8 sheathed blunt-end flagella at both ends of the cells. Cells are Gram negative and nonsporulating.

Growth is detected under microaerobic conditions at 37°C, but not at 25 or 42°C. The organism is oxidase, catalase, and urease positive, reduces TTC and nitrate, and tests positive for esterase,

γ-glutamyltransferase, hippurate, L-arginine arylamidase, and alkaline phosphatase. Activity of pyrrolidonyl arylamidase, L-aspartate arylamidase, and indoxyl acetate hydrolysis is not detected. Its clinical significance in cats and humans is so far unknown. The type strain, ASB7^T (also referred to as DSM 100489^T or LMG 28648^T), was isolated from the gastric mucosa of a stray cat.

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Additional files

TABLE S1. Strains and species used in this study

	NCBI accession number	
Species and strain designation	**Chromosomes**	**Plasmids**
Helicobacter heilmannii		
ASB1	CDMK00000000	
ASB2	CDMP00000000	
ASB3	CDMJ00000000	
ASB6	CDMM00000000	
ASB14	CDMI00000000	
***Helicobacter ailurogastricus* sp. nov.**		
ASB7	CDMG00000000	
ASB9	CDMN00000000	
ASB11	CDML00000000	
ASB13	CDMH00000000	
Helicobacter pylori		
Feline strain (X47-2AL)	AWNG00000000	
J99	NC 000921	
SouthAfrica20	NC 022130	
Helicobacter acinonychis		
Sheeba str.	NC 008229	NC 008230
Helicobacter cetorum		
MIT-00-7128	NC 017737	NC 017738
MIT-99-5656	NC 017735	NC 017736
Helicobacter felis		
ATCC 49179	NC014810	
Helicobacter bizzozeronii		
CIII-1	NC 015674	NC015670
CCUG 35545	CAGP00000000	
Helicobacter suis		
HS1	ADGY00000000	

Helicobacter mustelae		
12198	NC 013949	
Helicobacter Canadensis		
MIT-98-5491	ACSF00000000	
Helicobacter canis		
NCTC 12740	AZJJ00000000	
Helicobacter bilis		
ATCC 43879	ACDN00000000	
Helicobacter cinaedi		
ATCC_BAA847	NC 020555	
CCUG A8818	ABQT00000000	
Helicobacter hepaticus		
ATCC 51449	NC 004917	
Helicobacter pullorum		
MIT-98-5498	ABQU00000000	
Helicobacter winghamensis		
ATCC_BAA-430	ACDO00000000	

TABLE S2. Genome comparison of the virulent *H. heilmannii* ASB1^T and the low virulent *H. ailurogastricus* ASB7^T. Shown are the locus tags of protein encoding genes that are 1, present in *H. heilmannii* ASB1^T and absent in *H. ailurogastricus* ASB7^T (left side of table, column 1) and 2, present in *H. ailurogastricus* ASB7^T and absent in *H. heilmannii* ASB1^T (right side of table, column 4). For these species-specific genes, also the predicted function (column 2 and column 5) and the presence of orthologous proteins in other *Helicobacter* species (column 3 and column 6) are indicated.

***H. heilmannii* ASB1**			***H. ailurogastricus* ASB7**		
Gene (locus tag) encoding:	**Function in:**	**Orthologue in:**	**Gene (locus tag) encoding:**	**Function in:**	**Orthologue in:**
HHE01_13610 Helix-turn-helix domain of resolvase protein	DNA binding and transfer	*H. felis*	**HAL07_12730** Liposaccharide core biosynthesis protein	Outer membrane synthesis	*H. mustelae*, *H. felis*
HHE01_13650 Methyl-directed repair DNA adenine methylase	DNA replication: DNA repair	-	**HAL07_12160** Alpha (1,3)-fucosyltransferase	LPS synthesis outer membrane	*H. bizzozeronii*
HHE01_13670 Phage (Mu-like) virion morphogenesis protein	Viral head morphogenesis bacteriophages	*H. felis*, *H. suis*	**HAL07_11980** Alcohol dehydrogenase zinc domain	Fermentation	*H. suis*
HHE01_13730 Plasmid partitioning protein ParA	-	-	**HAL07_11970** Transcriptional regulator, HxlR family	Transcription	-
HHE01_00570 Type I restriction-modification system, restriction subunit R	DNA replication and transcription	*H. suis*	**HAL07_11710** Apolipoprotein N-acyltransferase	Cell envelope biogenesis, outer membrane synthesis	*H. suis*, *H. bizzozeronii*, *H. felis*, *H. pylori*

HHE01_00580 Type I restriction-modification system, restriction subunit R	DNA replication and transcription	*H. suis*, *H. bilis*	**HAL07_11700** Putative ABC transporter ATP binding protein	Export of lipoprotein and macrolide, cell division	*H. bizzozeronii*, *H. suis*, *H. felis*, *H. cetorum*, *H. pylori*
HHE01_01100 Von Willebrand factor type A domain protein	Toxicity protection (plasmid R478)	*H. bilis*	**HAL07_11280** Alcohol dehydrogenase	Fermentation	*H. fennelliae*
HHE01_01190 Serine transporter	Amino acid transport and metabolism	*H. felis*, *H. mustelae*	**HAL07_11260** Protein trl	Unknown	*H. bizzozeronii*, *H. suis*
HHE01_04630 Putative transposase OrfB	Movement of transposons	*H. pylori*, *H. suis*, *H. bizzozeronii*	**HAL07_11180** Putative ATP binding protein	DNA replication	*H. bizzozeronii*
HHE01_04610 Serine transporter	Amino acid transport	*H. felis*, *H. mustelae*	**HAL07_11130** Anaerobic ribonucleoside-triphosphate reductase	DNA replication	*H. felis*
HHE01_04100 Putative UDP-N-acetylglucosamine 2-epimerase	Biosynthesis cell surface polysaccharides	*H. bizzozeronii*, *H. felis*	**HAL07_11120** ATPase AAA-2 domain-containing protein	DNA replication	*H. felis*
HHE01_04130 N-acetylneuraminate synthase	Cell envelope biogenesis, outer membrane	*H. bizzozeronii*	**HAL07_10970** Type IIS restriction enzyme R protein	DNA replication, recombination, and repair	*H. bizzozeronii*, *H. suis*
HHE01_03790 Acetate permease ActP (cation/acetate symporter)	Acetate transport system	*H. bizzozeronii*	**HAL07_10960** Type IIS restriction enzyme M1 protein	DNA replication, recombination, and repair	*H. bizzozeornii*
HHE01_03460 Possible restriction /modification enzyme	Protection from uptake foreign DNA	*H. bizzozeronii*, *H. felis*, *H. suis*, *H. mustelae*	**HAL07_10950** Type II DNA modification enzyme (methyltransferase)	DNA replication, recombination, and repair	*H. bizzozeronii*, *H. suis*
HHE01_08010 Methyl-accepting chemotaxis protein	Bacterial chemotaxis (signaling)	*H. bizzozeronii*, *H. felis*	**HAL07_10110** ATPase AAA	DNA replication	*H. bizzozeronii*
HHE01_07980 Phosphatidyl-glycerophosphatase A	Lipid metabolism	*H. felis*, *H. bizzozeronii*, *H. suis*	**HAL07_10000** Haloacid dehalogenase	Dehalogenation and hydrolysis of phosphate esters and phosphonates	*H. bizzozeronii*
HHE01_07880 ThiJ/PfpI family protein	Putative intracellular protease	-	**HAL07_09800** Branched-chain amino acid transport protein	Amino acid transport, translation	*H. felis*
HHE01_07740 ATP-dependent DNA helicase UvrD/PcrA	DNA replication, recombination and repair	*H. suis*, *H. bizzozeronii*, *H. felis*, *H. cetorum*	**HAL07_06070** Transmembrane glucose/galactose transporter	Glucose uptake and catabolism	*H. felis*
HHE01_07510 Adenine-specific methyltransferase	DNA replication, recombination and repair	*H. bizzozeronii*	**HAL07_05790** Sel1 domain-containing protein	Protein binding	*H. felis*
HHE01_07490 DNA-cytosine methyltransferase	Transcription and DNA repair	*H. bizzozeronii*, *H. rodentium*	**HAL07_05740** DNA helicase II	DNA replication, recombination, and repair	*H. suis*, *H. bizzozeronii*
HHE01_07400 Putative glycosyl transferase	Polysaccharide synthesis	-	**HAL07_05460** McrBC 5-methylcytosine restriction system component	DNA replication and transcription	*H. felis*
HHE01_16110 Type I restriction-modification system, DNA-methyltransferase subunit M	Protection against invasion of foreign DNA	*H. felis*	**HAL07_05450** McrBC restriction endonuclease system McrB subunit	DNA replication and transcription	*H. felis*
HHE01_06960 Magnesium citrate secondary transporter	Energy production and conversion	-	**HAL07_04790** Adenine specific DNA methyltransferase	DNA replication	*H. felis*
HHE01_06090 Modification methylase EcoRI	DNA replication and transcription	*H. bizzozeronii*	**HAL07_16050** Type I restriction enzyme R protein	DNA replication, recombination and repair	*H. felis*
HHE01_05550 Alpha-aspartyl dipeptidase Peptidase E	Amino acid transport and metabolism	*H. felis*, *H. bizzozeronii*	**HAL07_16000** ATP-dependent Clp protease ATP-binding subunit ClpX	DNA replication	*H. bizzozeronii*
HHE01_04970 Type II restriction enzyme EcoRI	DNA replication and transcription	*H. pylori*	**HAL07_15940** Fic family protein	Cell division	*H. felis*
HHE01_04960 Adenine-specific methyltransferase	DNA replication, recombination and repair	-	**HAL07_15570** Siderophore-mediated iron transporter	Iron uptake	*H. suis*
HHE01_02130	Protection against invasion of foreign DNA	-	**HAL07_04030** Sel1 domain-containing protein	Protein binding	*H. felis*

Type I restriction-modification system, DNA-methyltransferase subunit M					
HHE01_02160 Restriction and modification enzyme CjeI	DNA replication and transcription, protection against foreign DNA	-	**HAL07_03720** Translation elongation factor TS	DNA replication	*H. felis*
HHE01_02370 DNA-cytosine methyltransferase	DNA replication, recombination and repair	-	**HAL07_01520** Type I restriction-modification enzyme M subunit	Protection against invasion of foreign DNA	*H. sanguini*
HHE01_02730 Putative TYPE II DNA modification enzyme (methyltransferase)	DNA replication, recombination and repair	*H. felis*, *H. pylori*	**HAL07_01530** Type I restriction-modification enzyme M subunit	Protection against invasion of foreign DNA	*H. sanguini*
HHE01_02740 Type I restriction-modification system, DNA-methyltransferase subunit M	Protection against invasion of foreign DNA	-	**HAL07_01750** Membrane protein	Putative membrane protein	*H. suis*
HHE01_02750 Type I restriction-modification system, specificity subunit S	Protection against invasion of foreign DNA	-	**HAL07_01480** Restriction modification system DNA specificity domain	Protection against invasion of foreign DNA	-
HHE01_08240 Nucleoside 5-triphosphatase RdgB (dHAPTP,dITP,XTP-specific)	Nucleotide transport and metabolism	*H. felis*, *H. pametensis*, *H. bizzozeronii*, *H. pylori*	**HAL07_01490** DNA methylase-type I restriction-modification system	Protection against invasion of foreign DNA	-
HHE01_08490 HrgA	DNA replication and transcription	*H. pylori*	**HAL07_15350** D-beta-hydroxybutyrate dehydrogenase	Metabolism: Krebs Cycle	*H. mustelae*
HHE01_08590 LSU ribosomal protein L35p	Translation, ribosomal structure and biogenesis]	*H. suis*, *H.bizzozeronii*, *H. felis*, *H. pylori*	**HAL07_15230** Putative MCP-type signal transduction protein	Bacterial signaling : chemotaxis	*H. bizzozeronii*
HHE01_14290 Jhp0562-like glycosyltransferase	LPS biosynthesis (outer membrane)	*H. bizzozeronii*, *H. felis*, *H. suis*, *H. cetorum*, *H. pylori*	**HAL07_15220** (Alpha-(1,3)-fucosyltransferase	LPS synthesis outer membrane	*H. felis*
HHE01_14540 Transposase	Movement transposon in DNA replication, recombination and repair	*H. bizzozeronii*, *H. pylori*	**HAL07_00740** Putative CagY like protein	Protein transport	*H. felis*
HHE01_14550 Putative transposase OrfB	Movement transposon in DNA replication, recombination and repair	*H. pylori*	**HAL07_00710** Beta-1,4-N-acetylgalactosamyl-transferase	LPS synthesis outer membrane	*H. pylori*
HHE01_14900 Putative two-domain glycosyltransferase	LPS biosynthesis (outer membrane)	*H. felis*	**HAL07_00530** Ribonuclease HII	DNA replication and repair	*H. bizzozeronii*
HHE01_16860 Transposase	Movement transposon in DNA replication, recombination and repair	*H. bizzozeronii*, *H. pylori*	**HAL07_14160** NADH:flavin oxidoreductase/NADH oxidase	Oxygen reduction, production hydrogen peroxide	*H. bizzozeronii*
HHE01_16870 Putative transposase OrfB	Movement transposon in DNA replication, recombination and repair	*H. pylori*	**HAL07_13930** Membrane protein	-	-
HHE01_16900 Type II DNA modification methyltransferase M.TdeIII	DNA replication, recombination and repair	-	**HAL07_13870** 2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase	Fatty acid oxidation	*H. suis*
HHE01_16910 Type II restriction endonuclease TdeIII	DNA replication, recombination and repair	-	**HAL07_13730** Type I restriction-modification enzyme M	Protection against invasion of foreign DNA	-
HHE01_17150 Type I restriction-modification system, specificity subunit S	Protection against invasion of foreign DNA	-	**HAL07_13720** Type I restriction-modification enzyme M	Protection against invasion of foreign DNA	-
HHE01_09360 DNA-cytosine methyltransferase	DNA replication, recombination and repair	*H. pametensis*, *H. pylori*, *H. cetorum*	**HAL07_13690** Endonuclease	Unknown	-

HHE01_09600 Putative MCP-type signal transduction protein	Bacterial signaling (chemotaxis)	*H. felis*, *H. bizzozeronii*, *H. suis*	**HAL07_13400** L-2-hydroxyglutarate oxidase	Oxygen reduction	*H. felis, H bilis*
HHE01_09630 Mobile element protein, probable transposase	Movement transposon in DNA replication, recombination and repair	-	**HAL07_13390** Carbon starvation induced protein csiD	Iron ion binding	*H. felis*
HHE01_09640 Mobile element protein, probable transposase	Movement transposon in DNA replication, recombination and repair	*H. pylori*	**HAL07_13260** Outer membrane protein 15	Outer membrane protein	*H. bizzozeronii*
HHE01_10030 NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate transport and metabolism: glycolysis and glyconeogenesis	*H. suis*, *H. bizzozeronii*, *H. pylori*, *H. cetorum*	**HAL07_06620** Biotin-dependent carboxylase domain-containing protein	Biotin biosynthesis	*H. felis*
HHE01_10510 IceA1	type II restriction endonuclease, putatively associated with peptic ulcer disease	*H. pylori*, *H. hepaticus*, *H. rodentium*, *H. bizzozeronii*	**HAL07_06630** Allophanate hydrolase 2 subunit 1	Cyanuric acid metabolism	*H. bizzozeronii*
HHE01_11040 DNA adenine methylase	DNA replication, recombination and repair	-	**HAL07_06660** Lactam utilization protein LamB	Utilization of lactams	*H. bizzozeronii*
HHE01_11570 Modification methylase EcoRI	DNA replication and transcription: protection DNA from cleavage by the EcoRI endonuclease.	*H. felis*, *H. bizzozeronii*, *H. bilis*	**HAL07_09210** RloF	DNA replication	*H. bizzozeronii*
HHE01_11870 Type II restriction enzyme	DNA replication, recombination and repair	-	**HAL07_09470** Mobile element protein	Movement transposon in DNA replication, recombination and repair	*H. felis*, *H. bizzozeronii*, *H. pylori*
HHE01_11880 Type III restriction-modification system methylation subunit	DNA replication, recombination, and repair		**HAL07_09600** NADPH dependent preQ0 reductase	Oxygen reduction	*H. pylori*
HHE01_12430 Putative type II restriction endonuclease	DNA replication, recombination and repair (defense mechanism)	-	**HAL07_07460** Lipopolysaccharide 1,2-glucosyltransferase	LPS synthesis outer membrane	*H. pylori*
HHE01_12440 Modification methylase	DNA replication, recombination, and repair	-	**HAL07_08490** Putative lipopolysaccharide biosynthesis protein	LPS synthesis outer membrane	*H. bizzozeronii*
HHE01_13400 Short chain fatty acids transporter	Lipid metabolism	-	**HAL07_08500** Glycosyltransferase	Cell envelope biogenesis, outer membrane	*H. bizzozeronii*, *H. felis*
HHE01_13510 N-acetylmuramoyl-L-alanine amidase	Cell envelope biogenesis: Outer membrane synthesis	-	**HAL07_06240** Replication protein A	DNA replication	*H. felis*

TABLE S3. Identification of orthologous outer membrane proteins. By using the GET_HOMOLOGUES software, the *H. pylori* (J99), *H. heilmannii* (ASB1^T, ASB2, ASB3, ASB6 and ASB14) and *H. ailurogastricus* (ASB7^T, ASB9, ASB11 and ASB13) OMPs were clustered in group of homologs. Shown are the locus tags of the *H. pylori* OMP homologs in both species, the locus tags of the OMPs detected in *H. heilmannii* and *H. ailurogastricus* that are absent in *H. pylori*, and the locus tags of the OMPs that are only present in *H. heilmannii* or in *H. ailurogastricus*. Putative OMPs of *H. heilmannii* and/or *H. ailurogastricus* that clustered in the same homologous group are indicated by a number following the locus tag: 1-16 for the 16 putative OMPs that are present in both *H. heilmannii* and *H. ailurogastricus*, 1-6 for the 6 putative OMPs that are only present in *H. heilmannii*, and 1-8 for the 8 putative OMPs that are only present in *H. ailurogastricus.*

Well known *H. pylori* OMPs	**Homologs in *H. heilmannii* (locus tags)**		**Homologs in *H. ailurogastricus* (locus tags)**	
FecA	ASB1	**HHE01_07100, HHE01_11330, HHE01_11790**	ASB7	**HAL07_09720, HAL07_10750, HAL07_14920**
	ASB2	**HHE02_05770, HHE02_09250, HHE02_17010**	ASB9	**HAL09_13100, HAL09_05670, HAL09_16180**
	ASB3	**HHE03_17040, HHE03_03310, HHE03_17210**	ASB11	**HAL011_12030, HAL011_02340, HAL011_08880**
	ASB6	**HHE06_15320, HHE06_16950, HHE06_08210**	ASB13	**HAL013_06710, HAL013_16870, HAL013_01230**
	ASB14	**HHE014_08980, HHE014_08320, HHE014_06440**		
HofA	ASB1	**HHE01_01200**	ASB7	**HAL07_02840, HAL07_02830**
	ASB2	**HHE02_13400**	ASB9	**HAL09_06390, HAL09_06400**
	ASB3	**HHE03_18570, HHE03_12480**	ASB11	**HAL011_00030, HAL011_00040**
	ASB6	**HHE06_03750**	ASB13	**HAL013_13630, HAL013_13640**
	ASB14	**HHE014_06200, HHE014_06220**		
HofC	ASB1	**HHE01_16390**	ASB7	**HAL07_03820**
	ASB2	**HHE02_16270**	ASB9	**HAL09_08500**
	ASB3	**HHE03_18120**	ASB11	**HAL011_04140**
	ASB6	**HHE06_13140**	ASB13	**HAL013_12690**
	ASB14	**HHE014_14300**		
HofD	ASB1	**HHE01_16400**	ASB7	**HAL07_03830**
	ASB2	**HHE02_16260**	ASB9	**HAL09_08490**
	ASB3	**HHE03_18130**	ASB11	**HAL011_04130**
	ASB6	**HHE06_13150**	ASB13	**HAL013_12680**
	ASB14	**HHE014_14290**		
HofE	ASB1	**HHE01_16370**	ASB7	**HAL07_03790**
	ASB2	**HHE02_16290**	ASB9	**HAL09_08520**
	ASB3	**HHE03_18100**	ASB11	**HAL011_04160**
	ASB6	**HHE06_13120**	ASB13	**HAL013_12710**
	ASB14	**HHE014_14320**		
HofF	ASB1	**HHE01_16360**	ASB7	**HAL07_03780**
	ASB2	**HHE02_16300**	ASB9	**HAL09_08530**
	ASB3	**HHE03_18090**	ASB11	**HAL011_04170**
	ASB6	**HHE06_13110**	ASB13	**HAL013_12720**
	ASB14	**HHE014_14330**		
HofG	ASB1	**HHE01_16380**	ASB7	**HAL07_03800**
	ASB2	**HHE02_16280**	ASB9	**HAL09_08510**
	ASB3	**HHE03_18110**	ASB11	**HAL011_04150**
	ASB6	**HHE06_13130**	ASB13	**HAL013_12700**
	ASB14	**HHE014_14310**		
HofH	ASB1	**HHE01_05020, HHE01_16350**	ASB7	**HAL07_03770, HAL07_11310**
	ASB2	**HHE02_01410, HHE02_16310**	ASB9	**HAL09_08540, HAL09_10140**
	ASB3	**HHE03_18490, HHE03_18080**	ASB11	**HAL011_14990, HAL011_04180**
	ASB6	**HHE06_04940, HHE06_13100**	ASB13	**HAL013_08770, HAL013_12730**
	ASB14	**HHE014_05270, HHE014_14340**		
HomC-related	ASB1	**HHE01_00870**	ASB7	**HAL07_09950**
	ASB2	**HHE02_04750**	ASB9	**HAL09_05440**
	ASB3	**HHE03_10900**	ASB11	**HAL011_02110**
	ASB6	**HHE06_00330**	ASB13	**HAL013_16640**
	ASB14	**HHE014_12480**		
Hop-related (HopS/T, HopP, HopB/C, HopH, HopZ, HopQ)	Absent		Absent	
HopE (porin)	ASB1	**HHE01_15730**	ASB7	**HAL07_04240**
	ASB2	**HHE02_11160**	ASB9	**HAL09_16680**
	ASB3	**HHE03_04800**	ASB11	**HAL011_16430**
	ASB6	**HHE06_03190**	ASB13	**HAL013_15950**
	ASB14	**HHE014_13540**		
HopJ	Absent		ASB7	**HAL07_13260**
			ASB9	**HAL09_02660**
			ASB11	**HAL011_13190**
			ASB13	**HAL013_12050**
HopW	ASB1	**HHE01_02050**	ASB7	**HAL07_12930**
	ASB2	**HHE02_10190**	ASB9	**HAL09_02350**
	ASB3	**HHE03_08580**	ASB11	**HAL011_12880**
	ASB6	**HHE06_06690**	ASB13	**HAL013_11740**
	ASB14	**HHE014_11280**		
HorA	ASB2	**HHE02_11200**	Absent	
	ASB3	**HHE03_17980**		

	ASB6	**HHE06_14040**		
	ASB14	**HHE014_15920**		
HorB	ASB1	**HHE01_08570**	ASB7	**HAL07_02040**
	ASB2	**HHE02_16870**	ASB9	**HAL09_12580**
	ASB3	**HHE03_12680**	ASB11	**HAL011_00830**
	ASB6	**HHE06_16670**	ASB13	**HAL013_05720**
	ASB14	**HHE014_16960**		
HorD	ASB1	**HHE01_09950**	ASB7	**HAL07_05180**
	ASB2	**HHE02_12570**	ASB9	**HAL09_00940**
	ASB3	**HHE03_11510**	ASB11	**HAL011_10350**
	ASB6	**HHE06_00090**	ASB13	**HAL013_05460**
	ASB14	**HHE014_17590**		
HorE	ASB1	**HHE01_16810**	ASB7	**HAL07_04750**
	ASB2	**HHE02_04390**	ASB9	**HAL09_01960**
	ASB3	**HHE03_02450**	ASB11	**HAL011_05120**
	ASB6	**HHE06_06050**	ASB13	**HAL013_06360**
	ASB14	**HHE014_13750**		
HorF	ASB1	**HHE01_15440**	ASB7	**HAL07_00680**
	ASB2	**HHE02_03820**	ASB9	**HAL09_11980**
	ASB3	**HHE03_05570**	ASB11	**HAL011_11060**
	ASB6	**HHE06_04210**	ASB13	**HAL013_07810**
	ASB14	**HHE014_05890**		
HorH		Absent	Absent	
HorI		Absent	Absent	
HorL	ASB1	**HHE01_09500**	ASB7	**HAL07_03580**
	ASB2	**HHE02_09770**	ASB9	**HAL09_08750**
	ASB3	**HHE03_15160**	ASB11	**HAL011_04390**
	ASB6	**HHE06_07960**	ASB13	**HAL013_12940**
	ASB14	**HHE014_03040**		
TonB	ASB1	**HHE01_02040**	ASB7	**HAL07_12920**
	ASB2	**HHE02_10180**	ASB9	**HAL09_02340**
	ASB3	**HHE03_08590**	ASB11	**HAL011_12870**
	ASB6	**HHE06_06680**	ASB13	**HAL013_11730**
	ASB14	**HHE014_11290**		
OMP	ASB1	**HHE01_15550**	ASB7	**HAL07_10600**
	ASB2	**HHE02_03940**	ASB9	**HAL09_04260**
	ASB3	**HHE03_05440**	ASB11	**HAL011_08730**
	ASB6	**HHE06_04080**	ASB13	**HAL013_06000**
	ASB14	**HHE014_16990, HHE014_06020**		
OMP P1	ASB1	**HHE01_01590**	ASB7	**HAL07_03250**
	ASB2	**HHE02_03040**	ASB9	**HAL09_09070**
	ASB3	**HHE03_00880**	ASB11	**HAL011_04710**
	ASB6	**HHE06_17340**	ASB13	**HAL013_13260**
	ASB14	**HHE014_09340**		
OEP (efflux protein)	ASB1	**HHE01_04580**	ASB7	**HAL07_05820**
	ASB2	**HHE02_14970**	ASB9	**HAL09_15580**
	ASB3	**HHE03_05090**	ASB11	**HAL011_13480**
	ASB6	**HHE06_10940**	ASB13	**HAL013_14920**
	ASB14	**HHE014_00970**		
HefA	ASB1	**HHE01_15200**	ASB7	**HAL07_00450**
	ASB2	**HHE02_03570**	ASB9	**HAL09_11750**
	ASB3	**HHE03_01580**	ASB11	**HAL011_10830**
	ASB6	**HHE06_04460**	ASB13	**HAL013_07580**
	ASB14	**HHE014_14560**		
HefD	ASB1	**HHE01_02880**	ASB7	**HAL07_13160**
	ASB2	**HHE02_15920**	ASB9	**HAL09_02570**
	ASB3	**HHE03_18750**	ASB11	**HAL011_13100**
	ASB6	**HHE06_04050**	ASB13	**HAL013_11960**
	ASB14	**HHE014_01820**		
assembly factor YaeT	ASB1	**HHE01_10420**	ASB7	**HAL07_15460**
	ASB2	**HHE02_06700**	ASB9	**HAL09_14750**
	ASB3	**HHE03_12320**	ASB11	**HAL011_02720**
	ASB6	**HHE06_03360**	ASB13	**HAL013_10120**
	ASB14	**HHE014_17450**		
putative VacA (toxin like OMP)	ASB1	**HHE01_12480**	ASB7	**HAL07_13640**
	ASB2	**HHE02_13180**	ASB9	**HAL09_00010**
	ASB3	**HHE03_15470**	ASB11	**HAL011_16100**
	ASB6	**HHE06_06350**	ASB13	**HAL013_02860**
putative OMP	ASB1	**HHE01_11320**	ASB7	**HAL07_10760**
	ASB2	**HHE02_05780**	ASB9	**HAL09_13090**
	ASB3	**HHE03_03300**	ASB11	**HAL011_08890**
	ASB6	**HHE06_15310**	ASB13	**HAL013_06700**

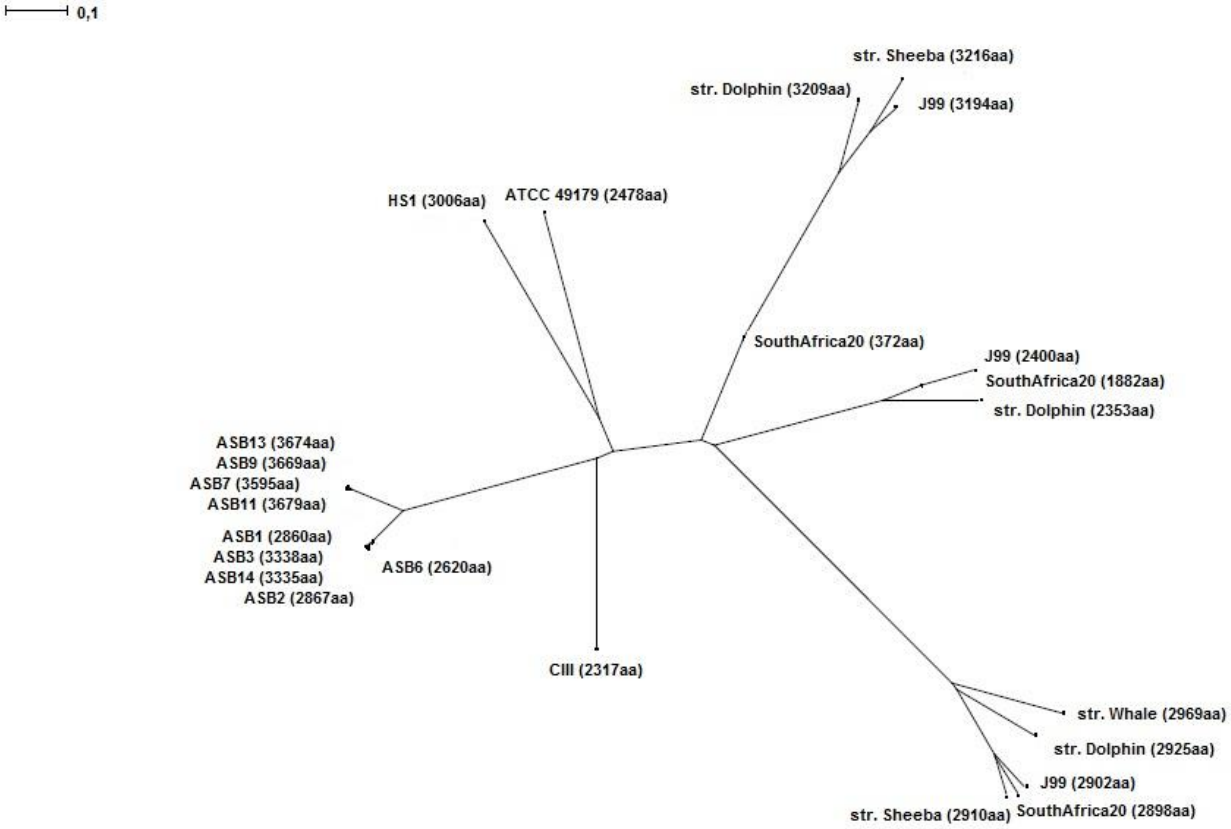
	ASB14	**HHE014_06450**		
OMPs absent in *H. pylori*, present in *H. heilmannii* and *H. ailurogastricus*				
	***H. heilmannii* (locus tags)**		***H. ailurogastricus* (locus tags)**	
HorA-related	ASB1	**HHE01_11780**	ASB7	**HAL07_09710**
	ASB2	**HHE02_17020**	ASB9	**HAL09_05680**
	ASB3	**HHE03_17030**	ASB11	**HAL011_02350**
	ASB6	**HHE06_16940**	ASB13	**HAL013_16880**
	ASB14	**HHE014_08310**		
HorC-related	ASB1	**HHE01_09230**	ASB7	**HAL07_01090**
	ASB2	**HHE02_07110**	ASB9	**HAL09_13840**
	ASB3	**HHE03_05780**	ASB11	**HAL011_08180**
	ASB6	**HHE06_10320**	ASB13	**HAL013_04990**
	ASB14	**HHE014_07870**		
OMP27/HorF-related	ASB1	**HHE01_11960**	ASB7	**HAL07_06900**
	ASB2	**HHE02_14340**	ASB9	**HAL09_05960**
	ASB3	**HHE03_11610**	ASB11	**HAL011_06580**
	ASB6	**HHE06_11540**	ASB13	**HAL013_02710**
	ASB14	**HHE014_00510**		
putative OEP/lipase	ASB1	**HHE01_01060**	ASB7	**HAL07_04520**
	ASB2	**HHE02_04930**	ASB9	**HAL09_16390**
	ASB3	**HHE03_11080**	ASB11	**HAL011_16720**
	ASB6	**HHE06_00150**	ASB13	**HAL013_11540**
	ASB14	**HHE014_12660**		
putative Imp (envelop biogenesis)	ASB1	**HHE01_06430**	ASB7	**HAL07_14330**
	ASB2	**HHE02_08600**	ASB9	**HAL09_01460**
	ASB3	**HHE03_06820**	ASB11	**HAL011_11430**
	ASB6	**HHE06_08860**	ASB13	**HAL013_01830**
	ASB14	**HHE014_03800**		
putative phopholipase A1 precursor	ASB1	**HHE01_03550**	ASB7	**HAL07_06350**
	ASB2	**HHE02_17670**	ASB9	**HAL09_06280**
	ASB3	**HHE03_09610**	ASB11	**HAL011_06050**
	ASB6	**HHE06_14220**	ASB13	**HAL013_10860**
	ASB14	**HHE014_16360**		
15 putative OMPs	ASB1	**HHE01_17290[1], HHE01_08720[2], HHE01_08740[3], HHE01_15890[4], HHE01_04310[5], HHE01_09380[6], HHE01_12010[7], HHE01_12000[8], HHE01_14510[9], HHE01_11210[10], HHE01_15170[11], HHE01_15510[12], HHE01_04660[13], HHE01_04680[13]**	ASB7	**HAL07_06160[1], HAL07_01900[2], HAL07_01890[3], HAL07_04400[4], HAL07_07820[5], HAL07_03680[6], HAL07_06850[7], HAL07_06860[8], HAL07_06990[9], HAL07_09250[10], HAL07_00420[11], HAL07_00770[12], HAL07_10570[13], HAL07_14900[14], HAL07_10540[15]**
	ASB2	**HHE02_09950[1], HHE02_16720[2], HHE02_16710[3], HHE02_02610[4], HHE02_05500[5], HHE02_09890[6], HHE02_06770[7], HHE02_14300[8], HHE02_01730[9], HHE02_05890[10], HHE02_03540[11], HHE02_03910[12], HHE02_01080[13]**	ASB9	**HAL09_11190[1], HAL09_12430[2], HAL09_12420[3], HAL09_16510[4], HAL09_03110[5], HAL09_08640[6], HAL09_05910[7], HAL09_05920[8], HAL09_12970[9], HAL09_04510[10], HAL09_11720[11], HAL09_09490[12], HAL09_04240[13], HAL09_16200[14]**
	ASB3	**HHE03_17430[1], HHE03_16160[2], HHE03_05280[3], HHE03_13980[4], HHE03_12520[5], HHE03_15280[6], HHE03_11550[7], HHE03_11570[8], HHE03_10530[9], HHE03_03190[10], HHE03_01550[11], HHE03_05480[12], HHE03_06200[14]**	ASB11	**HAL011_12690[1], HAL011_00980[2], HAL011_00990[3], HAL011_16600[4], HAL011_10000[5], HAL011_04280[6], HAL011_06530[7], HAL011_06540[8], HAL011_06680[9], HAL011_13900[10], HAL011_10800[11], HAL011_11150[12], HAL011_08710[13], HAL011_12010[14]**
	ASB6	**HHE06_11740[1], HHE06_11320[2], HHE06_11330[3], HHE06_16290[4], HHE06_02850[5], HHE06_07840[6], HHE06_11490[7], HHE06_11500[8], HHE06_01690[9], HHE06_15200[10], HHE06_04490[11], HHE06_04120[12], HHE06_08250[14]**	ASB13	**HAL013_01980[1], HAL013_05870[2], HAL013_05880[3], HAL013_11660[4], HAL013_00650[5], HAL013_12830[6], HAL013_02660[7], HAL013_02670[8], HAL013_02810[9], HAL013_09720[10], HAL013_07550[11], HAL013_07900[12], HAL013_02220[13], HAL013_01250[14]**
	ASB14	**HHE014_14760[1], HHE014_04970[2], HHE014_04980[3], HHE014_09700[4], HHE014_13190[5], HHE014_02920[6], HHE014_01690[7], HHE014_00470[8], HHE014_00460[9], HHE014_06560[10], HHE014_14530[11], HHE014_05980[12], HHE014_03170[13], HHE014_05630[15]**		
***H. heilmannii*-specific OMPs (locus tags)**				
6 putative OMPs	ASB1	**HHE01_10910[1], HHE01_09750[2], HHE01_09730[3], HHE01_07060[5]**		

	ASB2	**HHE02_06210[1], HHE02_11280[2], HHE02_11300[3], HHE02_02780[4], HHE02_09210[5], HHE02_05560[6]**
	ASB3	**HHE03_02780[1], HHE03_06010[2], HHE03_06030[3], HHE03_17990[4]**
	ASB6	**HHE06_14900[1], HHE06_13950[2], HHE06_13930[3], HHE06_05560[6]**
	ASB14	**HHE014_06860[1], HHE014_02600[2], HHE014_02620[3], HHE014_10440[4]**
***H. ailurogastricus*-specific OMPs (locus tags)**		
Hor-related	ASB7	**HAL07_09810**
	ASB9	**HAL09_05590**
	ASB11	**HAL011_02260**
	ASB13	**HAL013_16790**
8 putative OMPs	ASB7	**HAL07_04100[1], HAL07_15330[2], HAL07_05850[3], HAL07_06980[4], HAL07_04660[5]**
	ASB9	**HAL09_16820[1], HAL09_14620[2], HAL09_15550[3], HAL09_06020[4], HAL09_02060[5], HAL09_14610[6], HAL09_04220[7], HAL09_04950[8]**
	ASB11	**HAL011_16290[1], HAL011_02590[2], HAL011_13440[3], HAL011_06640[4], HAL011_08710[5], HAL011_02580[6], HAL011_08690[7], HAL011_08460[8]**
	ASB13	**HAL013_15810[1], HAL013_09990[2], HAL013_14950[3], HAL013_02770[4], HAL013_06460[5], HAL013_09980[6], HAL013_15700[7], HAL013_03820[8]**

TABLE S4. Quantification of *H. heilmannii* and *H. ailurogastricus* isolates binding to gastric epithelial cells. Shown is the mean number ± SD of *H. heilmannii* and *H. ailurogastricus* bacteria binding MKN7 cells and GSM06 cells (binding bacteria per cell) at pH7 and at pH2. Significant differences in binding between the ASB isolates are indicated by * $p < 0.05$ or ** $p < 0.01$ (Mann-Whitney *U* test), followed by the number of the isolate for which the significant difference was observed (1 (ASB1^T), 2 (ASB2), 3 (ASB3), 6 (ASB6), 14 (ASB14), 7 (ASB7^T), 9 (ASB9), 11 (ASB11) and 13 (ASB13).

	MKN7 cells		**GSM06 cells**	
	pH7	***pH2***	***pH7***	***pH2***
ASB1	35.80 ± 9.23 **2,3,6,7,9,11,13,14	11.40 ± 4.83 **7,9 *3,11,13,14	34.40 ± 9.61 **3,6,7,9,13 *2,11,14	1.20 ± 1.79
ASB2	10.00 ± 4.47 **1,7,9,13 *11	6.00 ± 1.00 **3,7,9,11,13 *14	16.40 ± 10.88 **1,9,13 *6,7	0.80 ± 0.84 *6
ASB3	10.60 ± 5.18 **1,7,9,11,13	2.60 ± 1.14 **2,6 *1,7,9	6.40 ± 2.88 **1,9 *13	2.40 ± 1.67 *7
ASB6	15.00 ± 4.06 **1,7,9,11,13 *14	7.60 ± 2.41 **3,7,9,11,13 *14	6.40 ± 4.72 **1 *2,9,13	3.20 ± 1.48 **7 *2,9,11,13
ASB14	15.60 ± 4.39 **1 *6,7,9	3.20 ± 1.30 **7 *1,2,6,9	10.00 ± 10.12 **9,13 *1	2.40 ± 1.82 *7
ASB7	0.40 ± 0.89 **1,2,3,6 *14	0.40 ± 0.55 **1,2,6,14 *3	3.00 ± 4.00 **1 *2	0.20 ± 0.45 **6 *3,14
ASB9	0.60 ± 0.89 **1,2,3,6 *14	0.60 ± 0.89 **1,2,6 *3,14	0.80 ± 0.84 **1,2,3,11,14 *6	0.60 ± 0.89 *6
ASB11	2.40 ± 1.89 **1,2,3,6	1.60 ± 1.14 **2,6 *1	8.80 ± 8.98 **9 *1,13	1.00 ± 1.41 *6
ASB13	1.20 ± 1.30 **1,2,3,6	1.80 ± 1.79 **2,6 *1	0.80 ± 1.30 **1,2 *3,6,11	1.00 ± 1.00 *6

Figure S1. Evolutionary analysis of the *vacA*-like genes present among all gastric *Helicobacter* species (*H. heilmannii* (ASB1^T, ASB2, ASB3, ASB6 and ASB14), *H. ailurogastricus* (ASB7^T, ASB9, ASB11 and ASB13), *H. suis* (HS1), *H. felis* (ATCC 49179), *H. bizzozeronii* (CIII), *H. pylori* (J99, SouthAfrica20), *H. acinonychis* (str. Sheeba) and *H. cetorum* (str. Dolphin and str. Whale)). Neighbour-joining tree data were calculated based on VacA-like amino-acid sequence alignment using the Muscle software. The size of each VacA-like protein is indicated in amino acids (aa) between brackets.



Chapter 3

Proteomic and phylogenetic analysis of the outer membrane protein repertoire of gastric *Helicobacter* species

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Abstract

Helicobacter pylori is considered as the most important risk factor for gastric malignancies worldwide. Its outer membrane is equipped with a large set of outer membrane proteins (OMPs) of which several are involved in colonization of the human gastric mucosa. Besides *H. pylori*, zoonotic non-*H. pylori* helicobacters (NHPH) have also been associated with gastric disease in humans. Currently, it is largely unknown which OMPs play a role in their colonization process. Therefore, the OMPs from all known gastric *Helicobacter* species with fully annotated genome sequences were characterized in the present study. The OMPs from 4 enterohepatic helicobacters, 2 closely-related *Campylobacter* species and *Escherichia coli* were included for comparison. All genomes were *in silico* screened for genes encoding OMPs and the identified OMPs were classified into distinct families based on their sequence homology. Phylogenetic analyses were applied and OMPs with a possible function in colonization or virulence were characterized in more detail. Several well-conserved OMP families were identified, including TonB-dependent receptors, an outer membrane factor, an outer membrane phospholipase and Imp/OstA. Moreover, several OMP families were only detected in *Helicobacter* and primarily in gastric species. These included SfpA/LpxR, two porin families and a VacA-like cytotoxin. In addition, gastric *Helicobacter* species harbored proportionally the most OMPs and various species-specific families were found in canine and feline helicobacters. In conclusion, we identified several OMP families that are of interest for future NHPH virulence and colonization studies.

Importance

NHPH originating from animals have been microscopically detected in 0.2-6% of gastric tissue biopsies from humans with severe gastric pathologies. However, it remains difficult to diagnose NHPH infections due to their fastidious nature and due to the lack of specific diagnostic tests in practice. Therefore, the actual prevalence of these zoonotic helicobacters is most probably even higher. In contrast to the well-studied *H. pylori*, the colonization mechanisms and virulence factors of NHPH species are largely unknown. All the known *H. pylori* OMPs that function as adhesins during colonization are commonly absent in NHPH. The present study is the first to characterize the OMP repertoire of zoonotic helicobacters and to identify OMP families that are possibly involved in colonization and virulence. Results from this study are valuable for future *in vitro* and *in vivo* research concerning the importance of these OMPs during NHPH colonization.

Introduction

Helicobacter pylori colonizes the stomach of 50% of the human population. Infection with this microbe is considered to be the most important risk factor for gastric malignancies worldwide (De Falco et al., 2015; Wang et al., 2014). In contrast to most other Gram-negative bacteria, the outer membrane of *H. pylori* is equipped with a remarkably large set of outer membrane proteins (OMPs) (Oleastro and Ménard, 2013). There are approximately 64 well-annotated OMPs present in *H. pylori*, encoded by 4% of its genome (Alm et al., 2000). This unusually large set of OMPs possibly originates as a response of this gastric bacterium to the hostile environment of the stomach. The *H. pylori* OMPs can be divided into five paralogous gene families. Family 1 is composed of the Hop (*H. pylori* OMP) and Hor (Hop-related) proteins, family 2 of the Hof OMPs (*Helicobacter* OMP) and family 3 of the Hom (*Helicobacter* outer membrane) proteins. Family 4 consists of iron-regulated OMPs and family 5 of efflux pump OMPs. Other OMPs, not classified in one of these families are also present (Alm et al., 2000; Oleastro and Ménard, 2013). Most of the *H. pylori* OMPs belonging to the Hop family, function as porins or as adhesins that promote binding of the bacterium to the gastric mucosa (Exner et al., 1995; Ilver et al., 1998; Mahdavi et al., 2002; Oleastro and Ménard, 2013). BabA (HopS), SabA (HopP), AlpA (HopC) and AlpB (HopB), OipA (HopH), HopQ and HopZ are the best characterized *H. pylori* Hop adhesins that play a role in its colonization process (Backert et al., 2011). Moreover, LabA (HopD), HomB and HorB may also be involved in *H. pylori* adherence (Loh et al., 2008; Oleastro et al., 2008; Oleastro and Ménard, 2013; Rossez et al., 2014; Snelling et al., 2007).

Besides *H. pylori*, also non-*H. pylori Helicobacter* species (NHPH), originating from domestic animals, have been associated with gastric disease in humans, including gastritis, gastric and duodenal ulcers and mucosa-associated lymphoid tissue lymphoma. These gastric NHPH include *H. suis* from pigs and *H. heilmannii*, *H. bizzozeronii*, *H. felis* and *H. salomonis* from cats and dogs (Haesebrouck et al., 2009). Recent comparative genomic studies highlighted that gastric NHPH lack all known *H. pylori* adhesins described so far (Arnold et al., 2011; Joosten et al., 2015; Schott et al., 2011; Vermoote et al., 2011). Furthermore, they share only few homologs of the Hor and Hom family. Only genes encoding homologs of the *H. pylori* Hof protein family are present in these animal-associated helicobacters, but in contrast to *H. pylori*, their *hof* genes are located in a ~10-kb locus (Joosten et al., 2015). For *H. heilmannii*, it has recently been shown that HofE and HofF play an important role in adhesion to the gastric mucosa, albeit with a higher affinity for gastric epithelial cells than for mucins (Liu et al., 2016).

Based on the above findings, it is clear that gastric NHPH harbor a different OMP repertoire compared to *H. pylori* and that the mechanisms by which these NHPH colonize the gastric mucosa remain largely unknown. Therefore, in the present study, we screened the genomes *in silico* from all gastric *Helicobacter* species known so far, for the presence of genes encoding outer membrane proteins. The identified OMPs were then classified into families based on their protein sequence homology, using 3 different protein databases. Finally, phylogenetic analyses of the OMP sequences were applied to study the evolutionary relationships among the OMPs and to unravel the OMPs that could play a role in the colonization and virulence properties of gastric NHPH. In addition, the genomes of 4 enterohepatic *Helicobacter* species and 2 closely-related *Campylobacter* species were included in the proteomic and phylogenetic analyses for comparison. The genome of *Escherichia coli*, for which the biological functions of the OMPs are well characterized, was included as well.

In this study, several OMP families, that are possibly important for colonization and virulence, were identified in gastric *Helicobacter* species. These families include TonB-dependent OMPs with a predicted function in iron-uptake, an outer membrane factor (OMF) and Imp/OstA involved in antimicrobial resistance, an outer membrane phospholipase (OMPLA) with a possible role in colonization capacity and virulence and a SfpA/LpxR family with a predicted function in immune evasion. Additionally, two *Helicobacter*-specific outer membrane porin families with putative functions in adhesion, and a *Helicobacter*-specific VacA-like cytotoxin family with a potential role in colonization capacity were identified.

Material and Methods

Escherichia coli, *Campylobacter* and *Helicobacter* species included

The genomes from 12 gastric *Helicobacter* species, namely *H. acinonychis*, *H. ailurogastricus*, *H. baculiformis*, *H. bizzozeronii*, *H. cetorum*, *H. cynogastricus*, *H. felis*, *H. heilmannii*, *H. mustelae*, *H. pylori*, *H. salomonis* and *H. suis* were analyzed in this study. For comparison, the genomes from 4 enterohepatic *Helicobacter* species, namely *H. cinaedi*, *H. equorum*, *H. hepaticus* and *H. trogontum* and from the *Campylobacter* species *C. coli* and *C. jejuni*, which are closely related to *Helicobacter*, were included as well. Also, the genomes from different *E. coli* strains were analyzed, since the biological functions of most *E. coli* OMPs are well known. An overview of the analyzed species and strains, their origin and their accession numbers is shown in **Table S1**.

Data management and integration

Genomic data was modeled using the Django object relational mapper (ORM) (http://www.djangoproject.com) and database tables were automatically created using the management command '*syncdb*'.

The FASTA protein sequences of putative outer membrane proteins (OMP) of the selected strains of *E. coli*, *Campylobacter* and *Helicobacter* were extracted from the genomes by using the HHomp tool (Remmert et al., 2009) as described before (Joosten et al., 2015). They were deposited in the EMBL databases and the accession numbers are shown in **Table S1**. Subsequently, all OMPs were combined to a single '*combined.fasta*' file using the Unix '*cat*' command. Next, a Phython script was written using IPhython Notebook (http://ipython.org/notebook.html), to load all sequences to the database.

Classification into OMP families

The seed alignments from the 90 OMP families that are defined in OMPdb.org were downloaded. For each of these families, the seed alignments were converted to HMM profiles using the HMMER3 suite of programs (http://hmmer.janelia.org). Specifically, the '*hmmbuild'* program was executed for each seed alignment as follows: '*hmmbuild <hmmfile_out> <seed_alignment>'*. The generated HMM profiles were then stored to the database. Next, the HMM profiles for all of the OMP families were collected and "pressed" for faster searches using the program '*hmmpress'* in HMMER3, resulting in an HMM database of OMP families. Then, each OMP protein sequence was searched against this HMM database in order to classify them into any of those OMP families.

The remaining unclassified proteins were searched against the Pfam database using '*hmmscan*' in the HMMER3 web server.

Finally, the last remaining 257 unclassified proteins were clustered into groups with CD-HIT using the settings 'word length = 2, identity cutoff = 40%' in order to produce the fewest possible number of clusters. The clusters with single members with an amino acid length of < 120 (31 sequences in total) were dropped.

The distribution of the OMP families from the *Escherichia*-, *Campylobacter*- and *Helicobacter* genera was determined with '*pandas*', a data processing package in Python (http://pandas.pydata.org). For plotting and visualization, the '*matplotlib'* plotting package was used (http://matplotlib.org).

Alignment and phylogenetic analysis

Families with at least 3 members were subjected to phylogenetic analysis. The OMP sequences of these families were written into multi-sequence FASTA files (one for each family). Multiple sequence alignment was performed using the Clustal Omega program (http://www.clustal.org/omega/). The alignments were trimmed with 'TrimAl' program (http://trimal.cgenomics.org) in order to remove sequence regions that potentially blur the phylogenetic signal such as highly variable loop regions.

The trimmed alignments were then fed into the 'FastTree' phylogenetic tree-building program (http://www.microbesonline.org/fasttree) The phylogenetic trees were visualized and edited by the online tool 'Interactive Tree Of Life' (iTol) (Letunic and Bork, 2016). The best-fitting root was selected with TempEst v1.5 (formerly known as 'Path-O-Gen') (Rambaut et al., 2016). OMP sequences per family were subjected to protein BLAST (ncbi) to obtain a protein name and/or function and in that way to identify possible subgroups of proteins in each OMP family.

Results

Identification of OMP families

A total of 3,359 putative OMPs was identified among the different strains of *E. coli* and the genera *Campylobacter* and *Helicobacter* (see **Table S1** and **Table S2**). An overview of the number of OMPs per species and per strain is presented in **Table S2**. For the *C. coli* and *C. jejuni* strains, the total number of OMPs ranges between 21 and 27 OMPs. Among the *E. coli* strains, the number of OMPs were 88, 89, 97, 110 and 116 for K12-W3110, IAI1, IAI39, 536 and TW14359, respectively. The mean number of total OMPs was lower for the 4 enterohepatic helicobacters (a mean of 33 OMPs with a minimum of 24 OMPs for *H. equorum* eqF1 and a maximum of 55 OMPs for *H. trogontum* R3554), compared to gastric *Helicobacter* species (a mean of 66 OMPs with a minimum of 49 OMPs for *H. mustelae* ATC 12198 and a maximum of 118 OMPs for *H. cetorum* MIT 00-7128). Subsequently, these OMPs were classified into families based on their sequence homology. From the 3,359 OMPs, 2,794 proteins could be classified into one of the 90 families from the OMPdb database. The results are listed in **Table 1**. For each of the 90 OMP families, the name and function is shown as well as the OMPs from the *E. coli*, *Campylobacter* and *Helicobacter* strains that clustered into that family.

The remaining 565 protein sequences were then searched against the Pfam protein database, resulting in the classification of 308 proteins into 31 OMP families with unknown biological function. These families were assigned a family ID from "X1" to "X31". The names of these

31 OMP families and the OMPs from the *E. coli*, *Campylobacter* and *Helicobacter* strains that clustered among these families, are shown in **Table 2**.

Finally, the remaining 257 unclassified protein sequences were clustered into groups using the CD-HIT program with algorithm settings to produce the fewest possible number of clusters. With this method, 106 clusters were produced. The clusters with single members that had a length of less than 120 amino acids (a total of 31 sequences) were excluded. This final clustering revealed 75 families without known name or function, which were assigned a family ID of "Y1" to "Y75" (**Table 3**).

Distribution plots were created for the 3 groups of OMP families in order to visualize the genus-specific OMP families as well as the OMP families that are present in both *E. coli* and the *Campylobacter* and *Helicobacter* species. Distribution plots specific for the *Helicobacter* OMPs in these 3 groups of families were included as well to distinguish between canine and feline helicobacters, *H. pylori*, *H. cetorum*, *H. acinonychis*, *H. suis*, *H. mustelae* and enterohepatic helicobacters (**Figure 1**). The OMP families from the OMPdb database contained the highest number of members and seemed to be well-conserved among *E. coli* and *Helicobacter* species, and to a lesser extent in *Campylobacter* species (**Figure 1A**). From the 90 OMPdb families, 20 families were unique for *E. coli*, 8 families for the *Helicobacter* genus (from which 4 specific for gastric - and 1 for enterohepatic helicobacters) and only 1 family for the *Campylobacter* genus. Two families were only shared among *E. coli*, *Campylobacter* and enterohepatic helicobacters, but not in gastric *Helicobacter* species (family 1 and family 45). Family 51 was found only in *C. jejuni* and *H. cinaedi* (see also **Table 1**). The majority of the X-families from the Pfam database were found to be genus-specific (**Figure 1B**). From the 31 X-families, 16 families were unique for *E. coli*, 2 families for the *Campylobacter* genus and 10 families for the *Helicobacter* genus (from which 8 specific for gastric - and 1 for the enterohepatic helicobacters tested). Most families were present only in 1 species and often not in all analyzed strains (see also **Table 2**). Also the CD-HIT Y-families were mostly found in only one species and often not in all analyzed strains. From the 75 Y-families, 56 families were unique for the *Helicobacter* genus (53 specific for gastric - and 3 for the enterohepatic helicobacters tested here), 4 families for the *Campylobacter* genus (3 specific for *C. coli* and 1 for *C. jejuni*) and 13 families were unique for *E. coli* (**Figure 1C** and **Table 3**).

According to the *Helicobacter*-specific distribution plots (**Figure 1D**), 9 families of the OMPdb database were found well-conserved among gastric and enterohepatic helicobacters, whereas the other 21 families were specific only to a few species and strains (**Figure 1D1**). From the X-families, only family X2 was well-conserved among the genus *Helicobacter* and family X3

among the gastric species. OMPs from family X1 were only found in *H. pylori*, *H. cetorum* and *H. acinonychis* and OMPs from family X5 in *H. felis* and *H. bizzozeronii*. OMPs from the other 7 X-families were only present in 1 *Helicobacter* species (**Figure 1D2**). Each Y-family was mainly composed of OMPs from only 1 *Helicobacter* species. Most of these Y-families were found in canine and feline gastric helicobacters and in *H. acinonychis* (**Figure 1D3**).

TABLE 1. Overview of the 90 OMP families from the OMPdb database. Shown are the OMP family names (left column), their function (middle column) and the strain names of *E. coli* and the *Campylobacter* and *Helicobacter* species with homolog OMP sequences (right column). For strains with multiple OMP sequences belonging to the same family, the number of sequences is indicated in superscript.

	OMP Family	**Function**	**Species (strain)**
1	Outer membrane-localized lipid A 3-O-deacylase (PagL)	Enzyme	*C. coli* (15-157360) *E. coli* (536, IAI1, IAI39, K12-W3110, TW14359) *H. hepaticus* (ATC 51449)
2	Outer Membrane Protein Insertion Porin (OmpIP/Omp85)	Biogenesis/Secretion	*C. coli* (15-157360, 76339, N29710) *C. jejuni* (00-2425, 4031, M1) *E. coli* (536^{2}, IAI1^{2}, IAI39^{2}, K12-W3110^{2}, TW14359^{2}) *H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. ailurogastricus* (ASB7, ASB9^{2}, ASB11, ASB13) *H. baculiformis* (M50) *H. bizzozeronii* (10, 14, CIII, M7) *H. cetorum* (MIT 00-7128, MIT 99-5656) *H. cinaedi* (BAA_847) *H. cynogastricus* (JKM4) *H. equorum* (eqF1) *H. felis* (CS1, CS6, CS7, DS1) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14) *H. hepaticus* (ATCC 51449) *H. mustelae* (ATC 12198^{2}) *H. pylori (*Puno120, G27, F30, J99, India7) *H. salomonis* (M45, R1053, KokIII) *H. suis* (HS2, HS4, HS7, HS9) *H. trogontum* (R3554)
3	Outer Membrane Receptor (OMR-TonB Dependent Receptor)	Receptor	*C. coli* (15-157360^{2}, 76339^{3}, N29710^{2}) *C. jejuni* (00-2425^{3}, 4031, M1) *E. coli* (536^{12}, IAI1^{5}, IAI39^{8}, K12-W3110^{5}, TW14359^{7}) *H. acinonychis* (Hacino1^{3}, Hacino2^{3}, Hacino3^{3}, Hacino4^{3}) *H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13) *H. baculiformis* (M50) *H. cetorum* (MIT 00-7128^{2}, MIT 99-5656^{3}) *H. cinaedi* (BAA_847^{3}) *H. cynogastricus* (JKM4) *H. equorum* (eqF1^{2}) *H. felis* (CS1, CS6, CS7, DS1) *H. heilmannii* (ASB1^{2}, ASB2, ASB3, ASB6, ASB14) *H. hepaticus* (ATCC 51449^{3}) *H. mustelae* (ATC 12198^{3}) *H. pylori* (Puno120^{2}, G27^{2}, F30^{4}, J99^{2}, India7^{3}, SouthAfrica7^{3}) *H. suis* (HS2^{2}, HS4, HS7^{2}, HS9^{2}) *H. trogontum* (R3554^{7})

4	Glucose-selective OprB Porin (OprB)	Specific channels	-
5	OmpG Porin (OmpG)	Non-specific channels	-
6	Two-Partner Secretion (TPS)	Biogenesis/Secretion	*C. coli* (76339[3], N29710) *E. coli* (536, TW14359)
7	Autotransporter (AT)	Biogenesis/Secretion	*C. coli* (15-157360[2], 76339[3], N29710[2]) *C. jejuni* (00-2425, 4031[2], M1[2]) *E. coli* (536[8], IAI1[6], IAI39[7], K12-W3110[6], TW14359[8]) *H. cetorum* (MIT 99-5656[3]) *H. equorum* (eqF1) *H. mustelae* (ATC 12198[3]) *H. pylori* (G27, F30) *H. trogontum* (R3554[2])
8	Outer Membrane Protein X (OmpX)	Adhesion	*E. coli* (TW14359)
9	General Bacterial Porin (GBP-1) Family 1	Non-specific channels	*E. coli* (536, IAI1[2], IAI39[4], K12-W3110, TW14359) *H. bizzozeronii* (10, CIII)
10	Intracellular Bacteria Surface Antigen	Adhesion	*E. coli* (IAI39[2])
11	Outer Membrane Fimbrial Usher Porin (FUP)	Biogenesis/Secretion	*E. coli* (536[11], IAI1[12], IAI39[8], K12-W3110[11], TW14359[9])
12	FadL Outer Membrane Protein (FadL)	Receptor	*C. coli* (15-157360, 76339, N29710) *H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. ailurogastricus* (ASB7[2], ASB9[2], ASB11[2], ASB13) *H. baculiformis* (M50[3]) *H. bizzozeronii* (10, 14, CIII[2], M7) *H. cetorum* (MIT 00-7128, MIT 99-5656[2]) *H. cinaedi* (BAA_847) *H. cynogastricus* (JKM4) *H. felis* (CS1[3], CS6[3], CS7[3], DS1[3]) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14) *H. hepaticus* (ATCC 51449) *H. mustelae* (ATC 12198) *H. pylori* (Puno120, G27, F30, J99, India7) *H. salomonis* (M45, R1053, KokIII) *H. suis* (HS2, HS4, HS7, HS9) *H. trogontum* (R3554[2])
13	*Helicobacter* Outer Membrane Porin (HOP-1) Family 1	Adhesion	*C. coli* (76339) *C. jejuni* (00-2425) *H. acinonychis* (Hacino1[37], Hacino2[38], Hacino3[40], Hacino4[37]) *H. ailurogastricus* (ASB7[27], ASB9[28], ASB11[28], ASB13[28]) *H. baculiformis* (M50[27]) *H. bizzozeronii* (10[27], 14[30], CIII[30], M7[31]) *H. cetorum* (MIT 00-7128[68], MIT 99-5656[55]) *H. cinaedi* (BAA_847[2]) *H. cynogastricus* (JKM4[29]) *H. equorum* (eqF1[2]) *H. felis* (CS1[28], CS6[25], CS7[27], DS1[25]) *H. heilmannii* (ASB1[20], ASB2[20], ASB3[19], ASB6[21], ASB14[21]) *H. hepaticus* (ATCC 51449[2]) *H. mustelae* (ATC 12198[11]) *H. pylori (*Puno120[36], G27[38], F30[37], J99[39], India7[39], SouthAfrica7[38]) *H. salomonis* (M45[17], R1053[17], KokIII[17])

			H. suis (HS2[21], HS4[22], HS7[21], HS9[21]) *H. trogontum* (R3554[7])
14	Imp/OstA	Biogenesis/Secretion	*C. coli* (15-157360, 76339, N29710) *C. jejuni* (00-2425, 4031, M1) *H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13) *H. baculiformis* (M50) *H. bizzozeronii* (10, 14, CIII, M7) *H. cetorum* (MIT 00-7128, MIT 99-5656) *H. cinaedi* (BAA_847) *H. cynogastricus* (JKM4) *H. equorum* (eqF1) *H. felis* (CS1, CS6, CS7, DS1) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14) *H. hepaticus* (ATCC 51449) *H. mustelae* (ATC 12198) *H. pylori* (Puno120, G27, F30, J99, India7, SouthAfrica7) *H. salomonis* (M45, R1053, KokIII) *H. suis* (HS2, HS4, HS7, HS9)
15	General Bacterial Porin (GBP-2) Family 2	Non-specific channels	-
16	Omptin (OmpT)	Enzyme	*E. coli* (536, IAI39, K12-W3110, TW14359)
17	OmpA	Structural	*C. coli* (76339, N29710) *C. jejuni* (00-2425, 4031, M1) *E. coli* (536, IAI1[3], IAI39[5], K12-W3110, TW14359[12])
18	Neisserial Surface Protein A (NspA)	Adhesion	*E. coli* (536[2], IAI1, K12-W3110) *H. baculiformis* (M50) *H. equorum* (eqF1) *H. pylori* (SouthAfrica7)
19	Outer Membrane Porin (OprD)	Specific channels	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
20	OprF Porin	Structural	*H. felis* (CS1, CS6, CS7, DS1)
21	OmpW	Non-specific channels	*E. coli* (TW14359) *H. hepaticus* (ATCC 51449[2]) *H. salomonis* (M45, R1053, KokIII) *H. suis* (HS2, HS4, HS7, HS9)
22	*Pseudomonas* OprP Porin (POP)	Specific channels	-
23	MipA/OmpV	Structural	*E. coli* (IAI1, IAI39, K12-W3110, TW14359)
24	Oligogalacturonate-specific Porin (KdgM)	Specific channels	-
25	YfaZ Outer Membrane Protein	Unknown	*E. coli* (536, IAI1[2], IAI39, K12-W3110, TW14359)
26	Nucleoside-specific Channel-forming Outer Membrane Porin (Tsx)	Receptor	*E. coli* (536[4], IAI1, IAI39[3], K12-W3110[2], TW14359)
27	Opacity (OpcA)	Adhesion	-
28	Copper resistance protein B (CopB)	Specific channels	-
29	Salt-stress induced outer membrane protein (SspA)	Unknown	*E. coli* (536[3], IAI1, IAI39, K12-W3110, TW14359) *H. hepaticus* (ATCC 51449)
30	General Bacterial Porin (GBP-4) Family 4	Non-specific channels	*E. coli* (536[4], IAI1[4], IAI39[2], K12-W3110[4], TW14359[3])
31	Sugar Porin (SP)	Specific channels	*E. coli* (536[3], IAI1[3], IAI39[2], K12-W3110[2], TW14359)
32	*Campylobacter jejuni* Major Outer Membrane Porin (MomP)	Non-specific channels	*C. coli* (76339)
33	*Helicobacter* Outer Membrane Porin (HOP-2) Family 2	Adhesion	*H. acinonychis* (Hacino1[8], Hacino2[7], Hacino3[8], Hacino4[8])

			H. ailurogastricus (ASB7[9], ASB9[9], ASB11[9], ASB13[9]) *H. baculiformis* (M50[9]) *H. bizzozeronii* (10[9], 14[9], CIII[10], M7[9]) *H. cetorum* (MIT 00-7128[9], MIT 99-5656[10]) *H. cinaedi* (BAA_847[3]) *H. cynogastricus* (JKM4[11]) *H. equorum* (eqF1[3]) *H. felis* (CS1[10], CS6[11], CS7[11], DS1[10]) *H. heilmannii* (ASB1[8], ASB2[10], ASB3[10], ASB6[10], ASB14[9]) *H. hepaticus* (ATCC 51449[2]) *H. mustelae* (ATC 12198[4]) *H. pylori (*Puno120[8], G27[8], F30[8], J99[8], India7[8], SouthAfrica7[8]) *H. salomonis* (M45[7], R1053[7], KokIII[7]) *H. suis* (HS2[8], HS4[8], HS7[8], HS9[8]) *H. trogontum* (R3554[2])
34	Type Specific Antigen (TSA)	Unknown	-
35	Outer Membrane Protein beta-barrel domain	Unknown	*C. coli* (76339) *C. jejuni* (00-2425, 4031, M1) *E. coli* (536[3], IAI1, IAI39[3], K12-W3110[2], TW14359[5]) *H. acinonychis* (Hacino1[3], Hacino2[2], Hacino3[3], Hacino4[3]) *H. ailurogastricus* (ASB7[2], ASB9[2], ASB11[2], ASB13[2]) *H. baculiformis* (M50[2]) *H. bizzozeronii* (10[2], 14[2], CIII[4], M7[2]) *H. cetorum* (MIT 00-7128[6], MIT 99-5656[2]) *H. cinaedi* (BAA_847[3]) *H. cynogastricus* (JKM4[3]) *H. equorum* (eqF1[2]) *H. felis* (CS1[5], CS6[5], CS7[4], DS1[5]) *H. heilmannii* (ASB1[3], ASB2[3], ASB3[3], ASB6[2], ASB14[3]) *H. hepaticus* (ATCC 51449[2]) *H. mustelae* (ATC 12198[3]) *H. pylori (*Puno120[3], G27[4], F30[2], J99[2], India7[3], SouthAfrica7[5]) *H. salomonis* (M45[5], R1053[5], KokIII[5]) *H. suis* (HS2[4], HS4[3], HS7[3], HS9[4]) *H. trogontum* (R3554[6])
36	Systemic factor protein A (SfpA/LpxR)	Enzyme	*E. coli* (TW14359) *H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13) *H. baculiformis* (M50[2]) *H. bizzozeronii* (10[2], 14[2], CIII[2], M7[2]) *H. cetorum* (MIT 00-7128, MIT 99-5656) *H. cynogastricus* (JKM4[2]) *H. felis* (CS1[2], CS6[2], CS7[2], DS1[2]) *H. heilmannii* (ASB1[2], ASB2[2], ASB3[2], ASB6, ASB14) *H. mustelae* (ATC 12198) *H. pylori* (Puno120, G27, F30, J99, India7, SouthAfrica7) *H. salomonis* (M45[2], R1053[2], KokIII[2]) *H. suis* (HS2, HS4, HS7, HS9) *H. trogontum* (R3554)
37	Antimicrobial peptide resistance and lipid A acylation protein (PagP)	Enzyme	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)

38	Outer Membrane Phospholipase (OMPLA)	Enzyme	*C. coli* (15-157360, 76339, N29710) *C. jejuni* (00-2425, 4031, M1) *E. coli* (536, IAI1, IAI39, K12-W3110, TW14359) *H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13 *H. baculiformis* (M50) *H. bizzozeronii* (10, 14, CIII, M7) *H. cinaedi* (BAA_847) *H. cetorum* (MIT 99-5656) *H. cynogastricus* (JKM4) *H. felis* (CS1, CS6, CS7, DS1) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14)) *H. hepaticus* (ATCC 51449) *H. pylori* (Puno120, F30, J99, India7, SouthAfrica7) *H. salomonis* (M45, R1053, KokIII) *H. suis* (HS2, HS4, HS7, HS9) *H. trogontum* (R3554[4])
39	*Borrelia* Porin p13 (BP-p13)	Non-specific channels	-
40	*Borrelia* Oms28 porin	Non-specific channels	*H. suis* (HS2, HS4, HS7, HS9)
41	Bacterial Cellulose Synthase Operon Protein C (BcsC)	Biogenesis/Secretion	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
42	Outer Membrane Factor (OMF)	Biogenesis/Secretion	*C. coli* (15-157360[3], 76339[3], N29710[3]) *C. jejuni* (00-2425[3], 4031[3], M1[3]) *E. coli* (536[5], IAI1[5], IAI39[6], K12-W3110[5], TW14359[7]) *H. acinonychis* (Hacino1[4], Hacino2[4], Hacino3[4], Hacino4[4]) *H. ailurogastricus* (ASB7[4], ASB9[4], ASB11[4], ASB13[4]) *H. baculiformis* (M50[4]) *H. bizzozeronii* (10[4], 14[4], CIII[5], M7[4]) *H. cetorum* (MIT 00-7128[4], MIT 99-5656[4]) *H. cinaedi* (BAA_847[4]) *H. cynogastricus* (JKM4[4]) *H. equorum* (eqF1[4]) *H. felis* (CS1[4], CS6[4], CS7[4], DS1[4]) *H. heilmannii* (ASB1[4], ASB2[4], ASB3[4], ASB6[4], ASB14[4]) *H. hepaticus* (ATCC 51449[4]) *H. mustelae* (ATC 12198[4]) *H. pylori (*Puno120[4], G27[4], F30[4], J99[4], India7[4], SouthAfrica7[4]) *H. salomonis* (M45[4], R1053[4], KokIII[4]) *H. suis* (HS2[4], HS4[4], HS7[4], HS9[4]) *H. trogontum* (R3554[4])
43	*Brucella*-Rhizobium Porin (BRP)	Non-specific channels	-
44	Autotransporter-2 (AT-2)	Biogenesis/Secretion	*E. coli* (536[2], IAI1[2], IAI39[2], K12-W3110[2], TW14359[3]) *H. trogontum* (R3554)
45	Secretin	Biogenesis/Secretion	*C. coli* (15-157360, 76339, N29710) *C. jejuni* (00-2425, 4031, M1) *E. coli* (536[3], IAI1[2], IAI39[2], K12-W3110[2], TW14359[3]) *H. cinaedi* (BAA_847) *H. equorum* (eqF1) *H. hepaticus* (ATCC 51449) *H. trogontum* (R3554)
46	*Serpulina hyodysenteriae* variable surface protein	Unknown	-

47	*Treponema* Porin Major Surface Protein (TP-MSP)	Adhesion	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359) *H. baculiformis* (M50) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14)
48	*Borrelia* Oms66/Omp66	Non-specific channels	-
49	Chlamydial Porin (CP)	Non-specific channels	-
50	*Leptospira* Porin OmpL1 (LP-OmpL1)	Non-specific channels	-
51	*Campylobacter* omp50	Non-specific channels	*C. jejuni* (4031) *H. cinaedi* (BAA_847)
52	*Francisella tularensis* fslE	Receptor	-
53	*Legionella pneumophila* major outer membrane protein (LP-MOMP)	Adhesion	-
54	Intimin/Invasin	Adhesion	*C. coli* (15-157360, 76339, N29710) *C. jejuni* (00-2425, 4031, M1) *E. coli* (536[4], IAI1[3], IAI39[5], K12-W3110[2], TW14359[5])
55	N4 bacteriophage Receptor (nfrA)	Unknown	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
56	wzi	Biogenesis/Secretion	-
57	Alginate Export Porin (algE)	Biogenesis/Secretion	-
58	*Thermus thermophilus* HB27 TtoA	Unknown	-
59	*Acinetobacter baumannii* 34-kDa outer membrane protein	Specific channels	-
60	Cyclodextrin Porin (CymA)	Specific channels	*E. coli* (IAI1)
61	Fusobacterial Outer Membrane Porin (fomA)	Non-specific channels	-
62	Carbapenem resistance-associated outer membrane protein (carO)	Specific channels	-
63	*Treponema* Major outer membrane protein (mspA)	Unknown	-
64	*Geobacter* ompJ	Structural	-
65	*Salmonella typhi* STY4528	Unknown	-
66	yaiO Outer Membrane Protein	Unknown	*E. coli* (536[2], IAI1[2], IAI39[3], K12-W3110[2], TW14359)
67	Oms38 Spirochaetes	Unknown	-
68	*Haemophilus influenza* (hmw1B) Outer Membrane Translocator	Biogenesis/Secretion	-
69	*Aggregatibacter actinomycetemcomitans* omp67/morC	Biogenesis/Secretion	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
70	Raffinose Porin (rafY)	Specific channels	*E. coli* (536[2], IAI39[2], TW14359)
71	Short Chain Amide and Urea Porin (SAP)	Specific channels	-
72	*Porphyromonas* Major Outer Membrane (MOMP/OmpA)	Structural	*H. cetorum* (MIT 00-7128, MIT 99-5656) *H. pylori* (F30)
73	DUF1597	Unknown	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
74	DUF3308	Unknown	*H. cynogastricus* (JKM4) *H. mustelae* (ATC 12198)
75	*Porphyromonas gingivalis* PorT	Biogenesis/Secretion	*E. coli* (536, IAI1, IAI39[2], K12-W3110, TW14359) *H. ailurogastricus* (ASB7[3], ASB9[3], ASB11[3], ASB13[3]) *H. bizzozeronii* (10[2], CIII, M7) *H. cetorum* (MIT 00-7128[7], MIT 99-5656[3])

			H. cynogastricus (JKM4[2]) *H. felis* (CS1[2], CS6[2], CS7[3], DS1) *H. heilmannii* (ASB1[2], ASB2, ASB3[3], ASB6[2], ASB14) *H. hepaticus* (ATCC 51449) *H. pylori* (Puno120, F30[2], J99[2], India7) *H. salomonis* (M45[3], R1053[3], KokIII[3]) *H. trogontum* (R3554[2])
76	DUF2320	Unknown	-
77	DUF3374	Unknown	*E. coli* (IAI1, K12-W3110)
78	Outer membrane protein beta-barrel	Unknown	*C. coli* (15-157360[2], N29710[2]) *C. jejuni* (4031[2], M1[2]) *E. coli* (536, IAI1[2], K12-W3110[2], TW14359[2]) *H. cetorum* (MIT 00-7128) *H. cinaedi* (BAA_847[3]) *H. cynogastricus* (JKM4) *H. felis* (CS6) *H. mustelae* (ATC 12198)
79	F plasmid transfer operon (TraF)	Unknown	*E. coli* (TW14359) *H. ailurogastricus* (ASB13) *H. baculiformis* (M50) *H. bizzozeronii* (14, M7) *H. cetorum* (MIT 00-7128) *H. cinaedi* (BAA_847[2]) *H. equorum* (eqF1[2]) *H. hepaticus* (ATCC 51449[2]) *H. salomonis* (M45, R1053, KokIII) *H. trogontum* (R3554)
80	DUF2490	Unknown	*C. coli* (15-157360, N29710) *C. jejuni* (00-2425, 4031, M1) *E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
81	DUF3575	Unknown	*H. baculiformis* (M50) *H. bizzozeronii* (14) *H. cetorum* (MIT 00-7128) *H. cynogastricus* (JKM4) *H. felis* (CS1, CS6, CS7, DS1) *H. hepaticus* (ATCC 51449) *H. salomonis* (M45, R1053, KokIII)
82	DUF2860	Unknown	*H. bizzozeronii* (10, 14) *H. hepaticus* (ATCC 51449)
83	DUF4289	Unknown	-
84	DUF3078	Unknown	-
85	DUF3138	Unknown	-
86	DUF560	Unknown	*C. coli* (15-157360, 76339, N29710) *C. jejuni* (00-2425, 4031, M1) *E. coli* (IAI39)
87	Putative MetA-pathway of phenol degradation	Unknown	*E. coli* (536)
88	DUF1302	Unknown	-
89	DUF3187	Unknown	*H. equorum* (eqF1)
90	Gcw_chp	Unknown	-

TABLE 2. Overview of the 31 OMP families (x1-x31) from the Pfam protein database. Shown are the OMP family names (left column) and the strain names of *E. coli* and the *Campylobacter* and *Helicobacter* species with homolog OMP sequences (right column). For strains with multiple OMP sequences belonging to the same family, the number of sequences is indicated in superscript.

	OMP Family	**Species (strain)**
x1	Vacuolating cyotoxin	*H. acinonychis* (Hacino1[8], Hacino2[9], Hacino3[18], Hacino4[8]) *H. cetorum* (MIT 00-7128, MIT 99-5656) *H. pylori (*Puno120, G27, F30, J99, India7, SouthAfrica7)
x2	TonB-dependent Receptor Plug Domain	*E. coli* (536, IAI1, IAI39, K12-W3110[2], TW14359) *H. acinonychis* (Hacino1[3], Hacino2[3], Hacino3[3], Hacino4[3]) *H. ailurogastricus* (ASB7[3], ASB9[3], ASB11[3], ASB13[3]) *H. baculiformis* (M50[2]) *H. bizzozeronii* (10[4], 14[5], CIII[4], M7[4]) *H. cetorum* (MIT 00-7128[5], MIT 99-5656) *H. cinaedi* (BAA_847[2]) *H. cynogastricus* (JKM4) *H. equorum* (eqF1) *H. felis* (CS1, CS6, CS7, DS1) *H. heilmannii* (ASB1[2], ASB2[3], ASB3[3], ASB6[3], ASB14[3]) *H. hepaticus* (ATCC 51449) *H. mustelae* (ATC 12198[4]) *H. pylori* (Puno120[3], G27[4], F30[3], J99[4], India7[3], SouthAfrica7[3]) *H. suis* (HS2[2], HS4[3], HS7[2], HS9[2]) *H. trogontum* (R3554[5])
x3	Putative vacuolating cytotoxin	*H. acinonychis* (Hacino1[2], Hacino2[2], Hacino3[2], Hacino4[2]) *H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13) *H. baculiformis* (M50) *H. bizzozeronii* (14, CIII, M7) *H. cetorum* (MIT 00-7128[2], MIT 99-5656[3]) *H. cynogastricus* (JKM4) *H. felis* (CS1, CS6, CS7, DS1) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14) *H. pylori (*Puno120[2], G27[3], F30[2], J99[3], India7[3], SouthAfrica7[3]) *H. salomonis* (M45, R1053, KokIII) *H. suis* (HS2, HS4, HS7, HS9) *H. trogontum* (R3554)
x4	Protein of unknown function (DUF2622)	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
x5	Sel1 repeat	*H. bizzozeronii* (10, 14, CIII, M7[2]) *H. felis* (CS1, CS6, CS7, DS1)
x6	AhpC/TSA family	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
x7	PapC C-terminal domain	*E. coli* (IAI1)
x8	Tetratricopeptide repeat	*E. coli* (K12-W3110, TW14359)
x9	Bacterial putative lipoprotein (DUF940)	*E. coli* (536, IAI1[2], IAI39, K12-W3110[2], TW14359[2])
x10	Polysaccharide biosynthesis/export protein	*C. jejuni* (00-2425, 4031, M1) *E. coli* (536, IAI1[2], IAI39[2], K12-W3110[2], TW14359[2])
x11	Fimbrial protein	*E. coli* (IAI1, K12-W3110, TW14359)
x12	Glutathionylspermidine synthase preATP-grasp	*H. felis* (CS6)
x13	FimH, mannose binding	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
x14	Stringent starvation protein B	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
x15	Aspartate/ornithine carbamoyltransferase, carbamoyl-P binding domain	*H. mustelae* (ATC 12198)
x16	Fibronectin type III protein	*E. coli* (TW14359[2])

x17	POTRA domain, ShlB-type	*C. jejuni* (00-2425, 4031[3], M1[3]) *E. coli* (TW14359)
x18	OstA-like protein	*H. trogontum* (R3554)
x19	Pregnancy-associated plasma protein-A	*H. cetorum* (MIT 99-5656)
x20	Transposase	*E. coli* (536)
x21	Extended Signal Peptide of Type V secretion system	*E. coli* (IAI39, K12-W3110, TW14359)
x22	Bacterial Ig-like domain (group 1)	*E. coli* (536)
x23	Tripartite tricarboxylate transporter family receptor	*C. coli* (15-157360, N29710)
x24	Capsule biosynthesis GfcC	*E. coli* (TW14359)
x25	Protein of unknown function (DUF997)	*E. coli* (536)
x26	SLBB domain	*C. coli* (76339, N29710)
x27	tRNA synthetase class II core domain (G, H, P, S and T)	*H. bizzozeronii* (M7)
x28	Peptidyl-tRNA hydrolase	*H. bizzozeronii* (M7)
x29	Pili and flagellar-assembly chaperone, PapD N-terminal domain	*E. coli* (K12-W3110)
x30	ATP:corrinoid adenosyltransferase BtuR/CobO/CobP	*E. coli* (IAI1, IAI39, K12-W3110, TW14359)
x31	Essential protein Yae1, N terminal	*H. mustelae* (ATC 12198)

TABLE 3. Overview of the 75 hypothetical OMP families (y1-y75) clustered with CD hit. Shown are the strain names of *E. coli* and the *Campylobacter* and *Helicobacter* species with homolog OMP sequences. For strains with multiple OMP sequences belonging to the same family, the number of sequences is indicated in superscript.

Hypothetical OMP families with unknown function (y)	**Species (strain)**
y1	*H. mustelae* (ATC 12198)
y2	*H. mustelae* (ATC 12198^{2})
y3	*E. coli* (536, TW14359)
y4	*C. coli* (76339) *H. trogontum* (R3554)
y5	*H. mustelae* (ATC 12198)
y6	*H. heilmannii* (ASB1, ASB2, ASB3, ASB14) *H. mustelae* (ATC 12198)
y7	*H. baculiformis* (M50) *H. heilmannii* (ASB6)
y8	*E. coli* (536, IAI1, IAI39)
y9	*H. mustelae* (ATC 12198)
y10	*E. coli* (536, IAI1, IAI39, TW14359)
y11	*H. cetorum* (MIT 00-7128, MIT 99-5656^{2})
y12	*H. felis* (CS1, DS1)
y13	*E. coli* (536, IAI39)
y14	*H. acinonychis* (Hacino1, Hacino2, Hacino4) *H. cetorum* (MIT 00-7128)
y15	*H. bizzozeronii* (10, 14, M7^{2}) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14) *H. felis* (DS1)
y16	*H. acinonychis* (Hacino1) *H. cetorum* (MIT 00-7128, MIT 99-5656)
y17	*H. ailurogastricus* (ASB7, ASB9, ASB11^{2}, ASB13) *H. heilmannii* (ASB6)
y18	*H. bizzozeronii* (10) *H. pylori (*Puno120) *H. suis* (HS9)
y19	*H. baculiformis* (M50^{2})
y20	*H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13) *H. heilmannii* (ASB2, ASB14)
y21	*H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14)
y22	*H. bizzozeronii* (10, 14, CIII, M7)
y23	*H. cetorum* (MIT 00-7128) *H. salomonis* (KokIII)
y24	*H. suis* (HS2, HS7, HS9)
y25	*E. coli* (K12-W3110)
y26	*H. cetorum* (MIT 99-5656)
y27	*H. cinaedi* (BAA_847)
y28	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. cetorum* (MIT 00-7128, MIT 99-5656) *H. pylori (*Puno120, G27, F30, J99, India7, SouthAfrica7)
y29	*H. suis* (HS2, HS4, HS7, HS9)
y30	*E. coli* (TW14359)
y31	*E. coli* (TW14359)
y32	*H. felis* (CS6, CS7)

y33	*H. bizzozeronii* (CIII)
y34	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4)
y35	*H. baculiformis* (M50) *H. bizzozeronii* (10)
y36	*C. coli* (N29710)
y37	*C. coli* (N29710)
y38	*E. coli* (TW14359)
y39	*H. acinonychis* (Hacino1, Hacino2^{2}, Hacino3, Hacino4)
y40	*H. acinonychis* (Hacino1^{2}, Hacino2, Hacino3^{2}, Hacino4^{2})
y41	*H. heilmannii* (ASB14)
y42	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4)
y43	*C. coli* (N29710)
y44	*H. suis* (HS7)
y45	*E. coli* (536)
y46	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4)
y47	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. bizzozeronii* (14, M7)
y48	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4)
y49	*H. bizzozeronii* (10, M7)
y50	*E. coli* (536)
y51	*H. trogontum* (R3554)
y52	*H. heilmannii* (ASB3)
y53	*E. coli* (536)
y54	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4)
y55	*H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14)
y56	*C. jejuni* (00-2425, 4031) *H. trogontum* (R3554)
y57	*H. acinonychis* (Hacino1, Hacino3, Hacino4)
y58	*H. bizzozeronii* (10, M7)
y59	*H. acinonychis* (Hacino1, Hacino2, Hacino3^{2}, Hacino4)
y60	*H. acinonychis* (Hacino1^{3}, Hacino2, Hacino3^{2}, Hacino4)
y61	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4)
y62	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4)
y63	*E. coli* (536, IAI1)
y64	*H. cetorum* (MIT 00-7128, MIT 99-5656)
y65	*H. acinonychis* (Hacino1, Hacino4)
y66	*C. jejuni* (00-2425, 4031, M1)
y67	*E. coli* (536, IAI39)
y68	*H. acinonychis* (Hacino1, Hacino2, Hacino4)
y69	*H. suis* (HS2, HS4, HS7, HS9)
y70	*H. bizzozeronii* (10^{2}, M7) *H. salomonis* (M45, R1053, KokIII)
y71	*H. acinonychis* (Hacino2, Hacino3, Hacino4)
y72	*H. acinonychis* (Hacino1, Hacino2, Hacino3^{2}, Hacino4)
y73	*H. cynogastricus* (JKM4) *H. felis* (CS7)
y74	*H. acinonychis* (Hacino2, Hacino4)
y75	*H. trogontum* (R3554^{2})

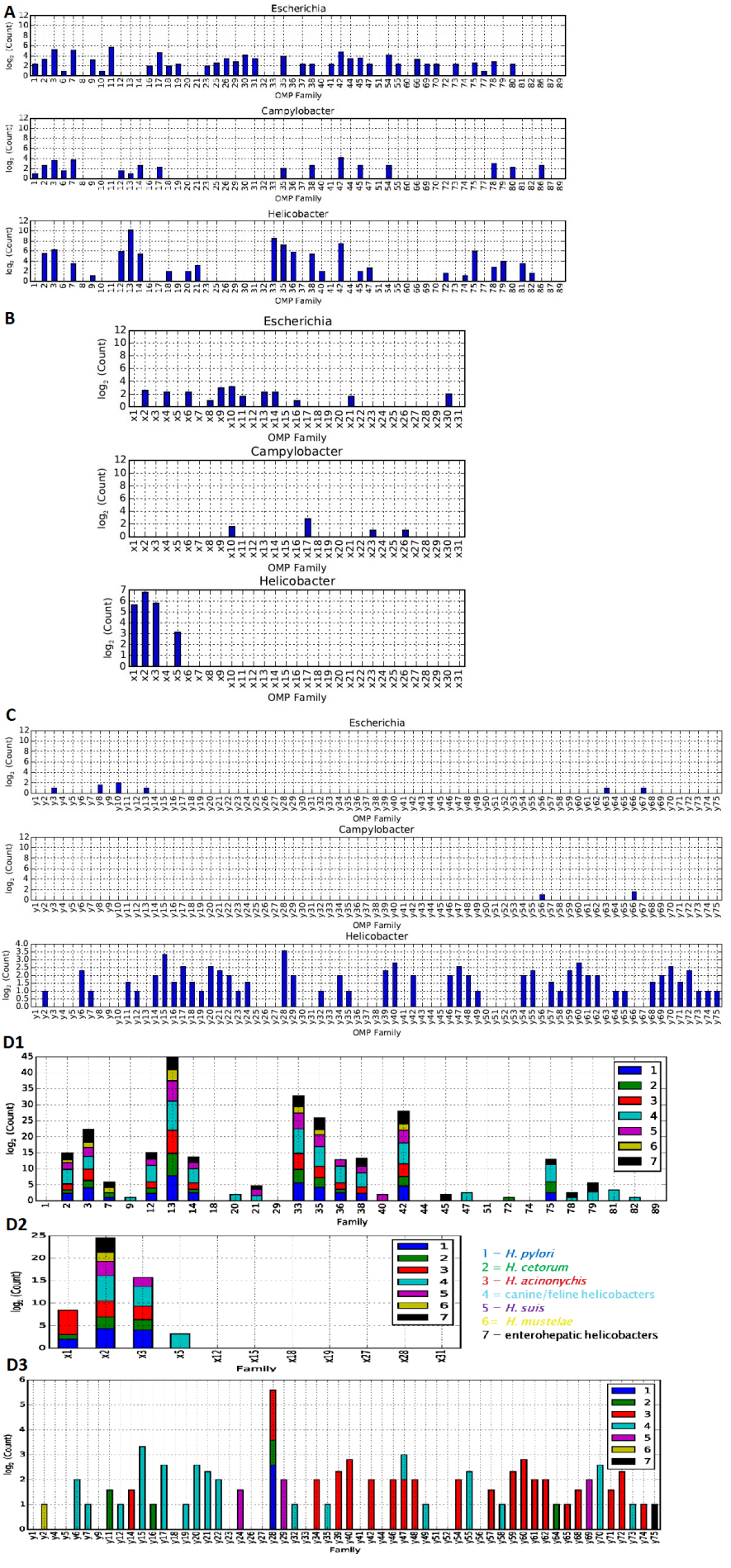
A
Escherichia
Campylobacter
Helicobacter
log2 (Count)
OMP Family
B
Escherichia
Campylobacter
Helicobacter
log2 (Count)
OMP Family
C
Escherichia
Campylobacter
Helicobacter
log2 (Count)
OMP Family
D1
log2 (Count)
Family
1
2
3
4
5
6
7
D2
log2 (Count)
Family
1 – H. pylori
2 = H. cetorum
3 – H. acinonychis
4 = canine/feline helicobacters
5 – H. suis
6= H. mustelae
7 – enterohepatic helicobacters
D3
log2 (Count)
Family

FIGURE 1. Distribution plots of (A) the 90 OMPdb families, (B) the 31 Pfam-derived "X" families and (C) the 75 "Y" families derived from CD-HIT-generated clusters. Shown are the numbers of members (log2 values) in each OMP family for the *Escherichia*-, *Campylobacter*- and *Helicobacter* genera. (D) Distribution plots of the *Helicobacter* OMPs from the OMPdb-, "X"- and "Y" families. Dark blue = *H. pylori*, green = *H. cetorum*, red = *H. acinonychis*, light blue = canine and feline gastric helicobacters, purple = *H. suis*, yellow = *H. mustelae*, black = enterohepatic helicobacters. For these 7 groups, the numbers of members (log2 values) in each OMP family are shown.

Phylogenetic analyses of the identified OMP families

The OMP families with at least 3 identified members were subjected to phylogenetic analysis. The protein sequences were aligned and a phylogenetic tree was built for each family. The well-conserved OMP families that are possibly involved in adhesion, colonization or virulence of *Helicobacter* species are presented below.

1) OMPs with a predicted function in iron-uptake

The cell membrane of Gram-negative bacteria is equipped with several iron-uptake systems for the acquisition of iron from the environment, an essential nutrient required for their growth (Andrews et al., 2008). Ferric iron (Fe^{3+}, the oxidized form of Fe^{2+}) is bound by siderophores, which are small iron-chelating compounds that are produced by Gram-negative bacteria (Miethke et al., 2007). The transport of the ferric-siderophore complex across the outer membrane requires energy from the proton motive force of the cytoplasmic membrane via the TonB/ExbB/ExbD proteins. The interaction between the energized form of TonB and TonB-dependent outer membrane receptors allows translocation of the ferric-siderophore complex into the periplasm (Higgs et al., 1998; Holden et al., 2012). The TonB-dependent outer membrane receptors have also been shown to be required for bacterial virulence (Hu et al., 2012). In the present study, two TonB-dependent outer membrane receptor families were identified.

Family 3 - Outer Membrane Receptor (OMR-TonB Dependent Receptor)

Family 3 of the OMPdb database (**Table 1**) comprised the TonB-dependent outer membrane receptors. A total of 123 orthologous OMP proteins clustered in this family. Members of this family are present in all examined strains of *E. coli*, *Campylobacter* and *Helicobacter* with the exception of *H. bizzozeronii* and *H. salomonis*. The number of orthologs in each strain is indicated in superscript after the strain names in **Table 1**.

The phylogenetic tree of OMP family 3 is shown in **Figure 2**. Remarkably, the TonB-dependent receptors of *H. mustelae*, which is described as a gastric *Helicobacter* species, clustered together with those of enterohepatic helicobacters. Moreover, the TonB-dependent receptors from enterohepatic helicobacters clustered together with those from *Campylobacter* species.

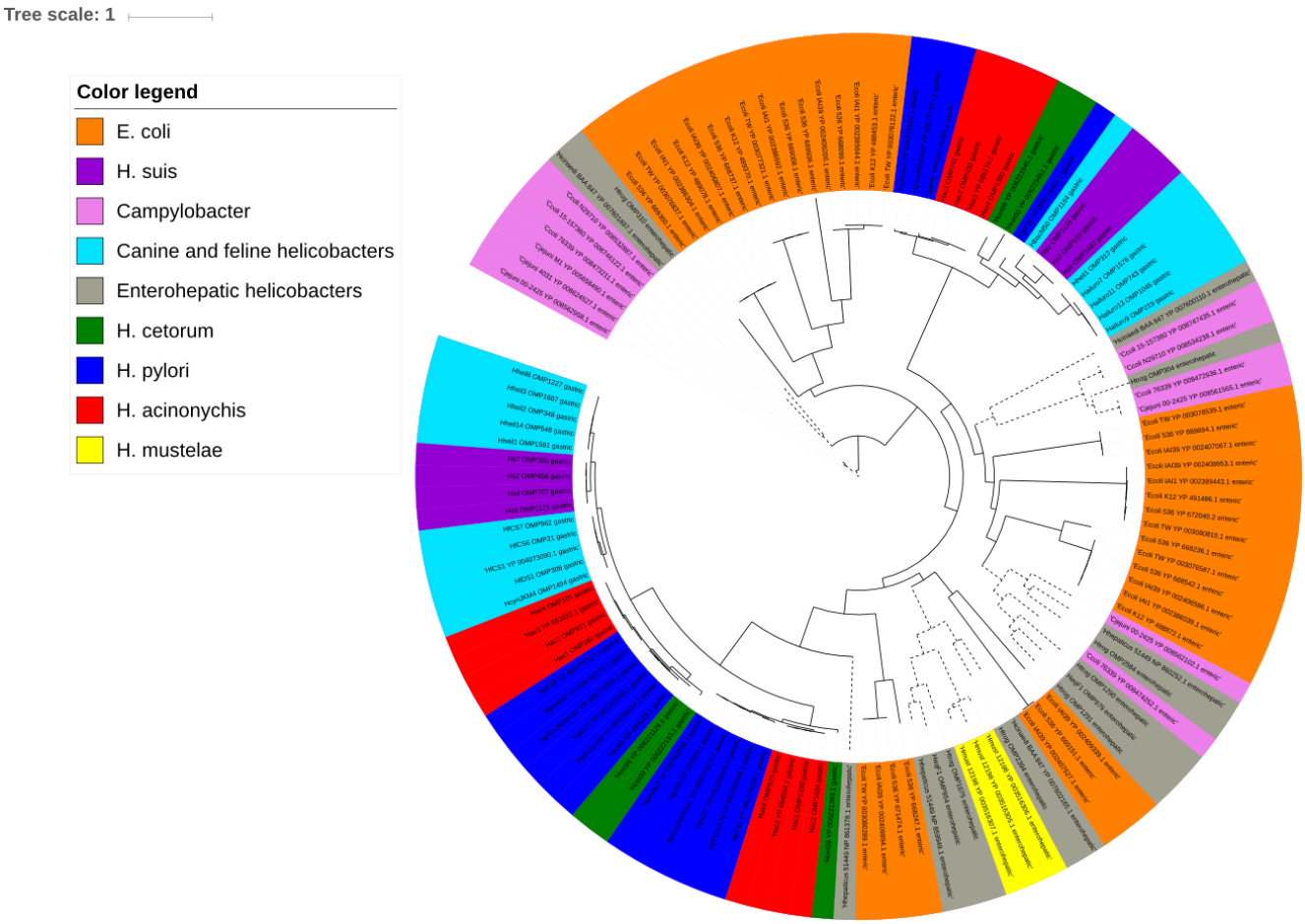

FIGURE 2. Phylogenetic tree of Family 3 – the outer membrane TonB-dependent receptors. Orthologous OMPs are present in all examined strains of *E. coli*, *Campylobacter* and *Helicobacter* with the exception of *H. bizzozeronii* and *H. salomonis*. OMPs of *H. mustelae* (yellow) cluster together with those of enterohepatic helicobacters (grey). OMPs of enterohepatic helicobacters (grey) cluster together with those of *Campylobacter* (pink). OMPs of *H. mustelae*, enterohepatic helicobacters and *Campylobacter* species are indicated by dashed clade lines.

Family X2 - TonB-dependent Receptor Plug Domain

Family X2 of TonB-dependent receptor plug domains comprised 116 orthologous proteins (Family X2 of the Pfam database, **Table 2**). Members of this family were present in all examined strains of *E. coli* and *Helicobacter* with the exception of *H. salomonis*. The number of orthologs in each strain is indicated in superscript after the strain names in **Table 2**. OMPs

belonging to the TonB-dependent receptor plug domain family were absent in *C. coli* and *C. jejuni*.

The phylogenetic tree of OMP family X2 is shown in **Figure 3**. Here, the OMPs from *H. mustelae* clustered in between those of gastric- and enterohepatic helicobacters.

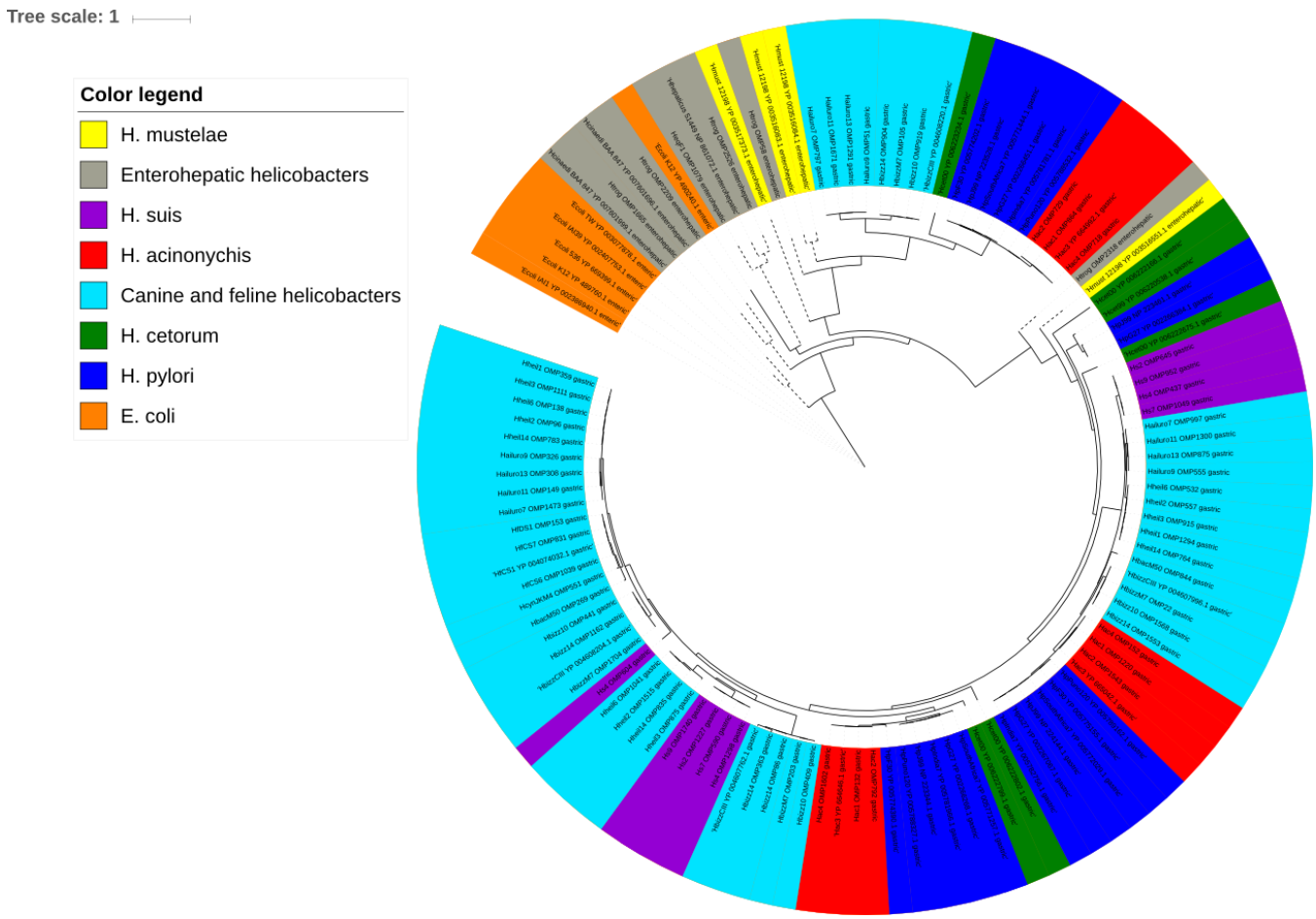

FIGURE 3. Phylogenetic tree of Family X2 – TonB-dependent receptor plug domains. Orthologous OMPs are present in all examined strains of *E. coli* and *Helicobacter* with the exception of *H. salomonis*, and are absent in *C. coli* and *C. jejuni*. OMPs from *H. mustelae* (yellow) clustered in between those of gastric- and enterohepatic (grey) helicobacters. OMPs of *H. mustelae* and enterohepatic helicobacters are indicated by dashed clade lines.

2) OMPs with a function in adhesion to the gastric mucosa

Some OMPs of Gram-negative bacteria and in particular, outer membrane porins, are able to interact with host tissues for adhesion to and invasion of cells and for evasion of host-defense mechanisms. Therefore, porins might play a fundamental role in the initial host-pathogen interaction (Galdiero et al., 2012). In our study, two different *Helicobacter* outer membrane porin families were identified.

Family 13 - *Helicobacter* Outer Membrane Porin Family 1

A total of 1,155 orthologous OMP proteins were clustered among the *Helicobacter* outer membrane porin family 1 (Family 13 of the OMPdb database, **Table 1**). Orthologs were present in all examined gastric and enterohepatic *Helicobacter* species. The number of orthologs in each strain is indicated in superscript after the strain names in **Table 1**. Only one orthologous protein was present in *C. coli* (76339) and *C. jejuni* (00-2425) and there were no members of this family detected in *E. coli*.

The phylogenetic tree of this OMP family is shown in **Figure 4** and roughly 6 subgroups could be distinguished. The *H. pylori* Hop, Hor and Hom adhesins, including BabA (HopS), SabA (HopP), AlpA (HopC) and AlpB (HopB), OipA (HopH), HopZ, HopQ, LabA (HopD), HorB and HomB clustered into this family. The phylogenetic tree clearly illustrates that the Hop adhesins (blue clades) which are specific for *H. pylori* (dark blue) were absent in canine, feline and porcine gastric NHPH. Only orthologs of AlpA, AlpB, BabB, HopA, HopD, HopF, HopI, HopG, HopL and OipA were present in *H. acinonychis* (red) whereas orthologs of AlpA, AlpB, HopA, HopF, HopI, HopG, HopL, OipA and SabA were found in *H. cetorum* (green) (see also **Figure 5**).

Subgroups corresponding to the *H. pylori* HorA/HopK/HopJ; HorC/HorH/HorI/HorB/HorJ; HorF/HorK; HopE/HorE/HorM/HorD/HorG/HomA/HomB/HomC/HomD OMPs were also detected (**Figure 4**). BLAST analyses revealed that several putative OMPs from the canine, feline and porcine gastric NHPH species clustered into these subgroups. For the enterohepatic *Helicobacter* species and *H. mustelae*, only orthologous putative OMPs of HorD and HorG were present in this family. The one orthologous OMP each from *C. coli* (76339) and from *C. jejuni* (00-2425) also clustered in this subgroup. A last subgroup that could be distinguished in the phylogenetic tree (**Figure 4**) contained putative OMPs that were only present in canine, feline and porcine *Helicobacter* species.

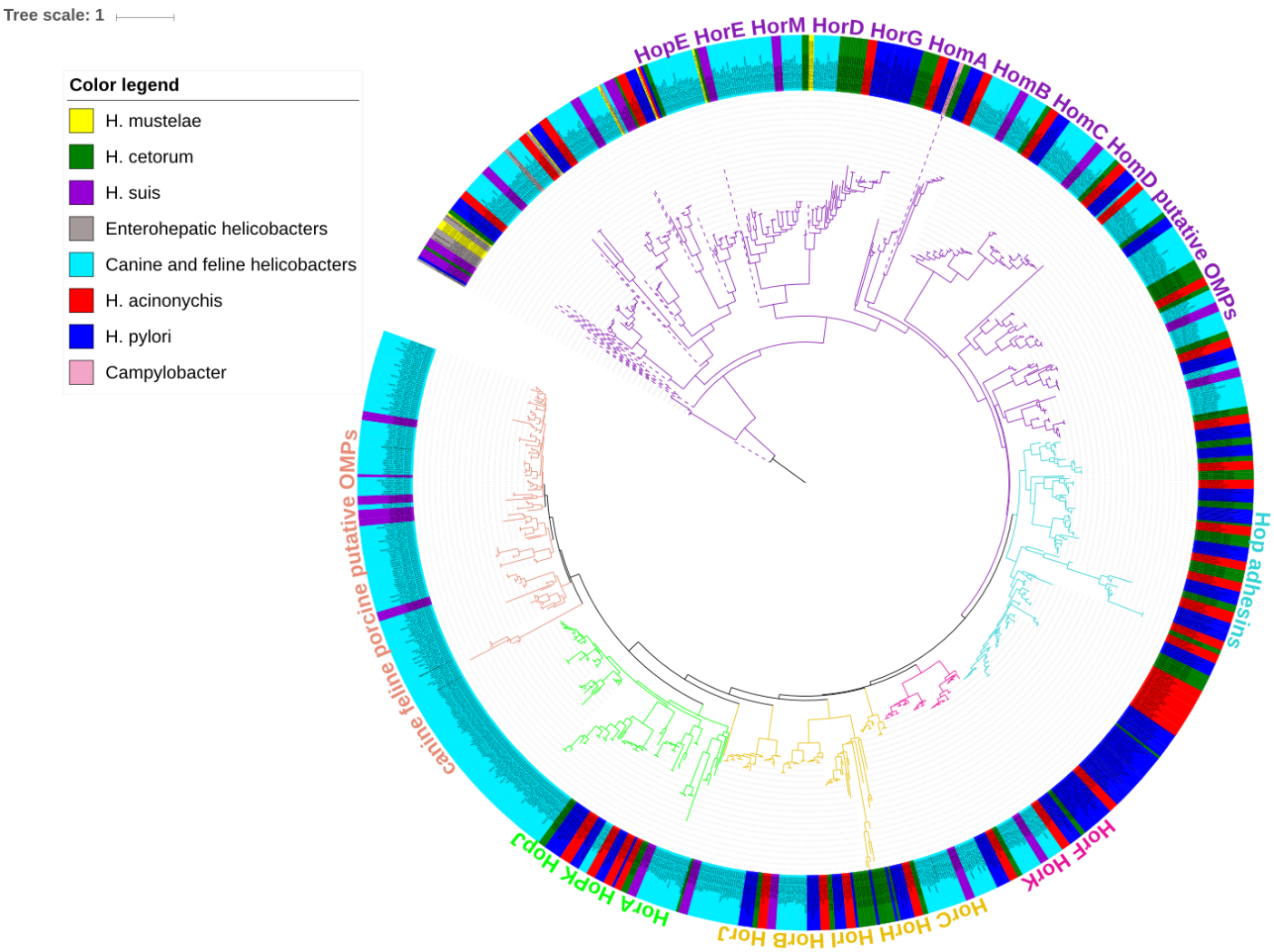

FIGURE 4. Phylogenetic tree of Family 13 – the *Helicobacter* outer membrane porin family 1. Orthologous OMPs are present in all examined gastric and enterohepatic *Helicobacter* species, only one orthologous protein is present in *C. coli* (76339) and *C. jejuni* (00-2425) and there are no orthologous OMPs detected in *E. coli*. Indicated are 6 different subgroups: putative OMPs specific for canine, feline and porcine helicobacters (light red clades); *H. pylori* HorA, HopK and HopJ (green clades) with orthologous putative OMPs in all other gastric NHPH species, except *H. mustelae*; *H. pylori* HorC, HorH, HorI, HorB and HorJ (yellow clades) with orthologous putative OMPs in all other gastric NHPH species, except *H. mustelae*; *H. pylori* HorF and HorK (pink clades) with orthologous putative OMPs in other gastric NHPH species, except *H. baculiformis* and *H. mustelae*; *H. pylori* Hop adhesins (blue clades) that are absent in other gastric NHPH species except for *H. acinonychis* and *H. cetorum*; *H. pylori* HopE, HorE, HorM, HorD, HorG, HomA, HomB, HomC and HomD (purple clades) with orthologous putative OMPs in all other gastric NHPH species. Also the 1 orthologous OMP from *C. coli* (76339) and *C. jejuni* (00-2425) (pink) cluster in the latter subgroup, as well as a few OMPs from *H. mustelae* (yellow) and enterohepatic helicobacters (grey). The position of the different *H. pylori* OMPs in each subgroup are clockwise indicated. OMPs of *H. mustelae*, enterohepatic helicobacters and *Campylobacter* species are indicated by dashed clade lines.

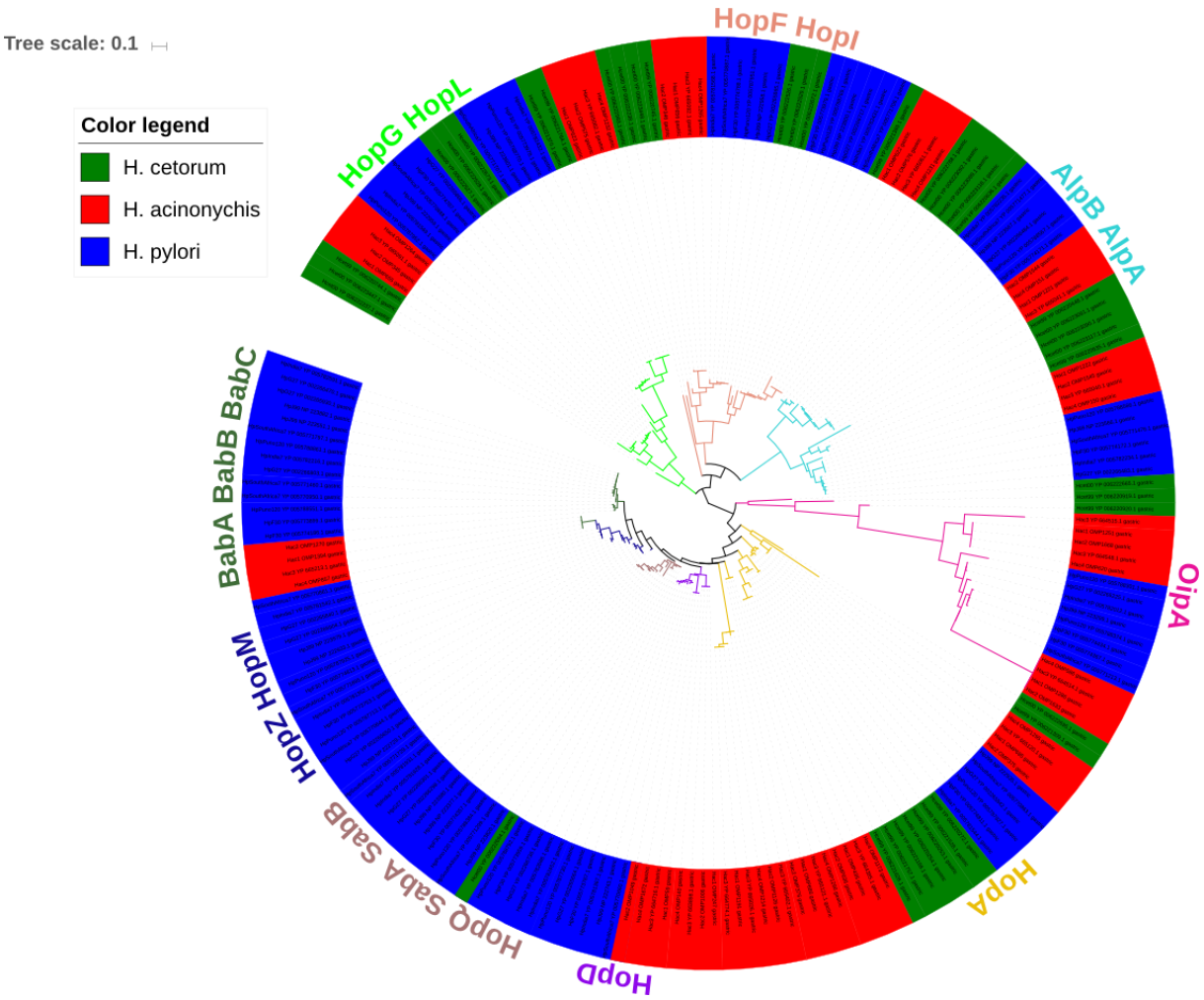

FIGURE 5. Phylogenetic subtree of the *H. pylori* Hop adhesins. Shown are 9 different subgroups of the *H. pylori*-specific Hop adhesins: HopG and HopL (light green clades), HopF and HopI (light red clades), AlpB and AlpA (light blue clades); OipA (pink clades) and HopA (yellow clades) with orthologs in *H. acinonychis* and *H. cetorum*; HopD (purple clades) with orthologs in *H. acinonychis*; HopQ, SabA and SabB (brown clades) with orthologs of SabA in *H. cetorum*; HopZ and HopM (dark blue clades); BabA, BabB and BabC (dark green clades) with orthologs of BabB in *H. acinonychis*.

Family 33 - *Helicobacter* Outer Membrane Porin Family 2

A total of 347 orthologous OMP proteins clustered in the *Helicobacter* outer membrane porin family 2 (Family 33 of the OMPdb database, **Table 1**). This family comprises orthologs of the *H. pylori* Hof proteins and members of these Hof OMPs were present in all examined *Helicobacter* species. The number of orthologs in each strain is indicated in superscript after the strain names in **Table 1**. There were no members of this family detected in *C. coli*, *C. jejuni* or *E. coli*. The phylogenetic tree of this porin OMP family is shown in **Figure 6**. A total of 8 subgroups could be distinguished, corresponding with the *H. pylori* HofA, HofB, HofC, HofD, HofE, HofF, HofG and HofH proteins. These 8 subgroups were highly conserved among

canine, feline and porcine NHPH species, *H. acinonychis* and *H. cetorum*. *H. mustelae* and the enterohepatic helicobacters only contained a few putative OMPs that did not cluster with one of the 8 Hof proteins.

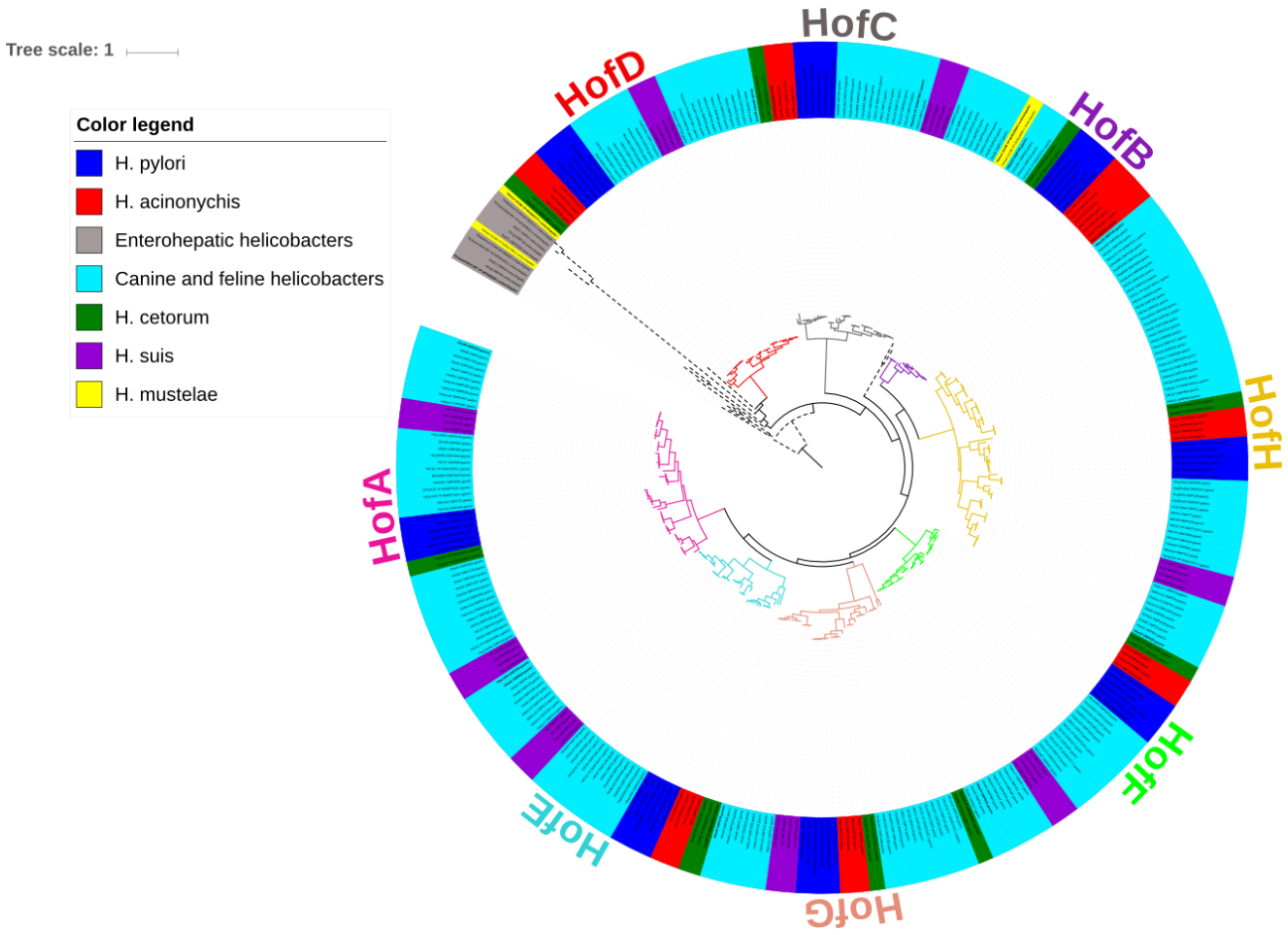

FIGURE 6. Phylogenetic tree of Family 33 – the *Helicobacter* outer membrane porin family 2. Orthologous OMPs are present in all examined *Helicobacter* species, but are absent in *Campylobacter* and *E. coli*. Indicated are 8 different subgroups, corresponding to the different *H. pylori* Hof proteins: HofA (pink clades) with orthologous putative OMPs in gastric NHPH except *H. acinonychis*, *H. salomonis* and *H. mustelae*; HofB (purple clades) with orthologous putative OMPs in *H. acinonychis*, *H. bizzozeronii* and *H. cetorum*; HofC (grey clades), HofD (dark red clades), HofE (blue clades), HofF (green clades), HofG (light red clades) and HofH (yellow clades) with orthologous putative OMPs in all gastric *Helicobacter* species except *H. mustelae*. *H. mustelae* (yellow) and enterohepatic helicobacters (grey) contain a few uncharacterized OMPs that cluster separately. OMPs of *H. mustelae* and enterohepatic helicobacters are indicated by dashed clade lines.

3) OMPs with a function in antimicrobial resistance

Family 14 - Imp/OstA

Imp (increased membrane permeability) or OstA (organic solvent tolerance) is an organic solvent tolerance protein in Gram-negative bacteria that participates in cell envelope biogenesis

(Aono et al., 1994; Sampson et al., 1989). The Imp/OstA protein has been associated with membrane permeability, organic solvent tolerance and resistance to antibiotics in *H. pylori* (Chiu et al., 2007).

We identified 48 orthologous outer membrane proteins in the Imp/OstA family (Family 14 of the OMPdb database, **Table 1**). In each of the examined strains of *Campylobacter* and *Helicobacter*, with the exception of *H. trogontum*, 1 orthologous Imp/OstA protein was found. Orthologous proteins were absent in *E. coli*, however the latter species harbored an OstA-like protein (Family X18, **Table 2**). The phylogenetic tree of OMP family 14 is shown in **Figure 7**. Again, the Imp/OstA protein of *H. mustelae* clustered in between those of the gastric and enterohepatic helicobacters.

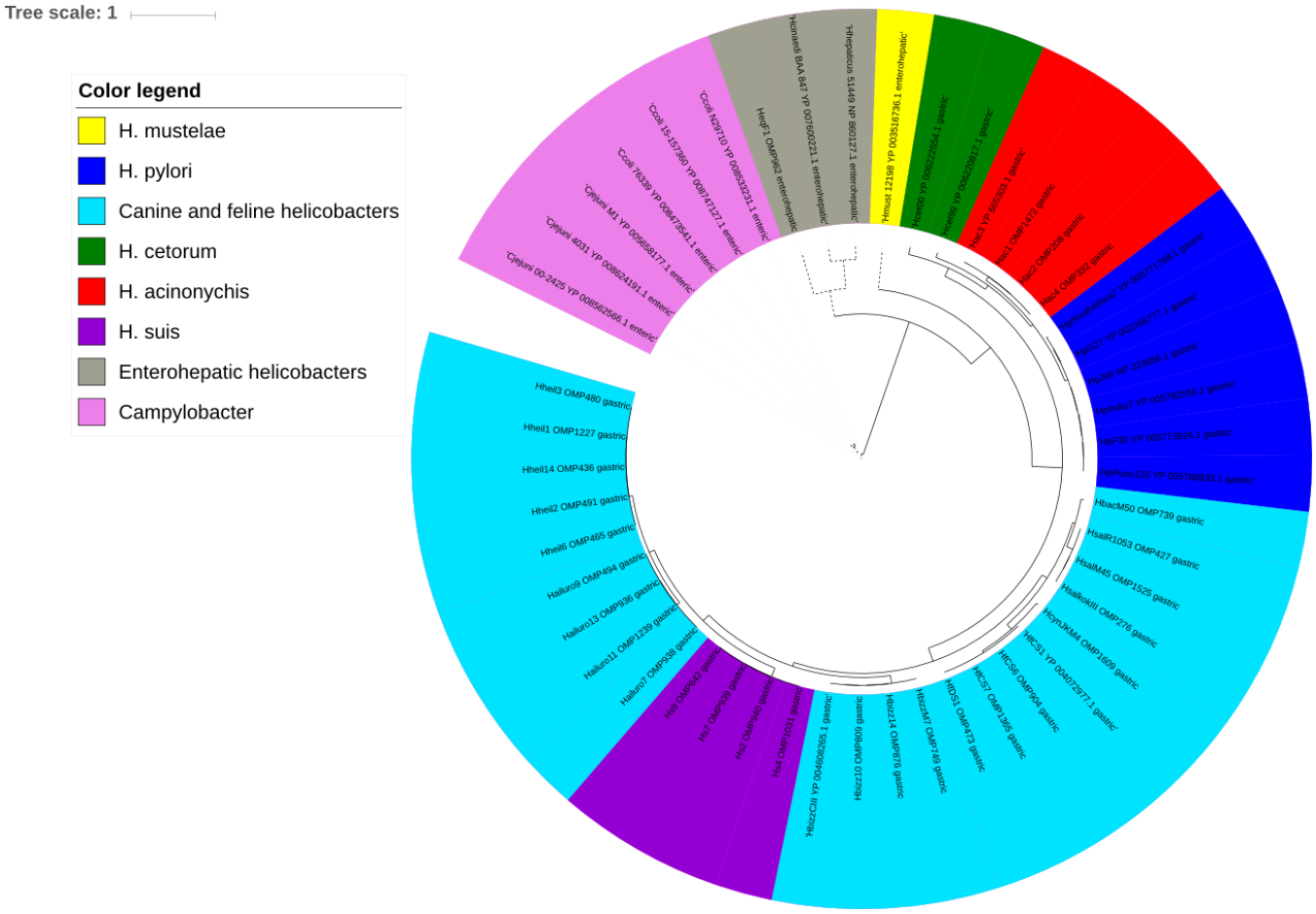

FIGURE 7. Phylogenetic tree of Family 14 – Imp/OstA. One orthologous OMP is present in each of the examined strains of *Campylobacter* and *Helicobacter*, with the exception of *H. trogontum*, and no orthologous OMPs are detected in *E. coli*. The Imp/OstA OMP of *H. mustelae* (yellow) clusters in between those of gastric and enterohepatic helicobacters (grey). OMPs of *H. mustelae*, enterohepatic helicobacters and *Campylobacter* species are indicated by dashed clade lines.

Family 42 - Outer membrane factor (OMF)

Gram-negative bacteria possess energy-dependent transport systems to export proteins, carbohydrates, drugs and heavy metals across the two membranes of the cell envelope (Paulsen et al., 1997). These transport systems commonly consist of a cytoplasmic membrane export system, a membrane fusion protein (MFP) and an outer membrane factor (OMF) and have been shown to play a role in producing both intrinsic and elevated multidrug resistance (Nikaido et al., 2009; Paulsen et al., 1997).

In total, 219 orthologous proteins were identified to belong to the OMF family (Family 42 of the OMPdb database, **Table 1**). Different members of the OMF family were present in all the analyzed *E. coli*, *Campylobacter* and *Helicobacter* strains. The number of orthologs in each strain is indicated in superscript after the strain names in **Table 1**. With protein BLAST, several different OMPs subgroups could be distinguished in the OMF family (see **Table S3**). These different subgroups are indicated in the phylogenetic tree of the OMF family (**Figure 8**). Also here, orthologs of the OMF family of *H. mustelae* clustered with enterohepatic helicobacters rather than with gastric *Helicobacter* species.

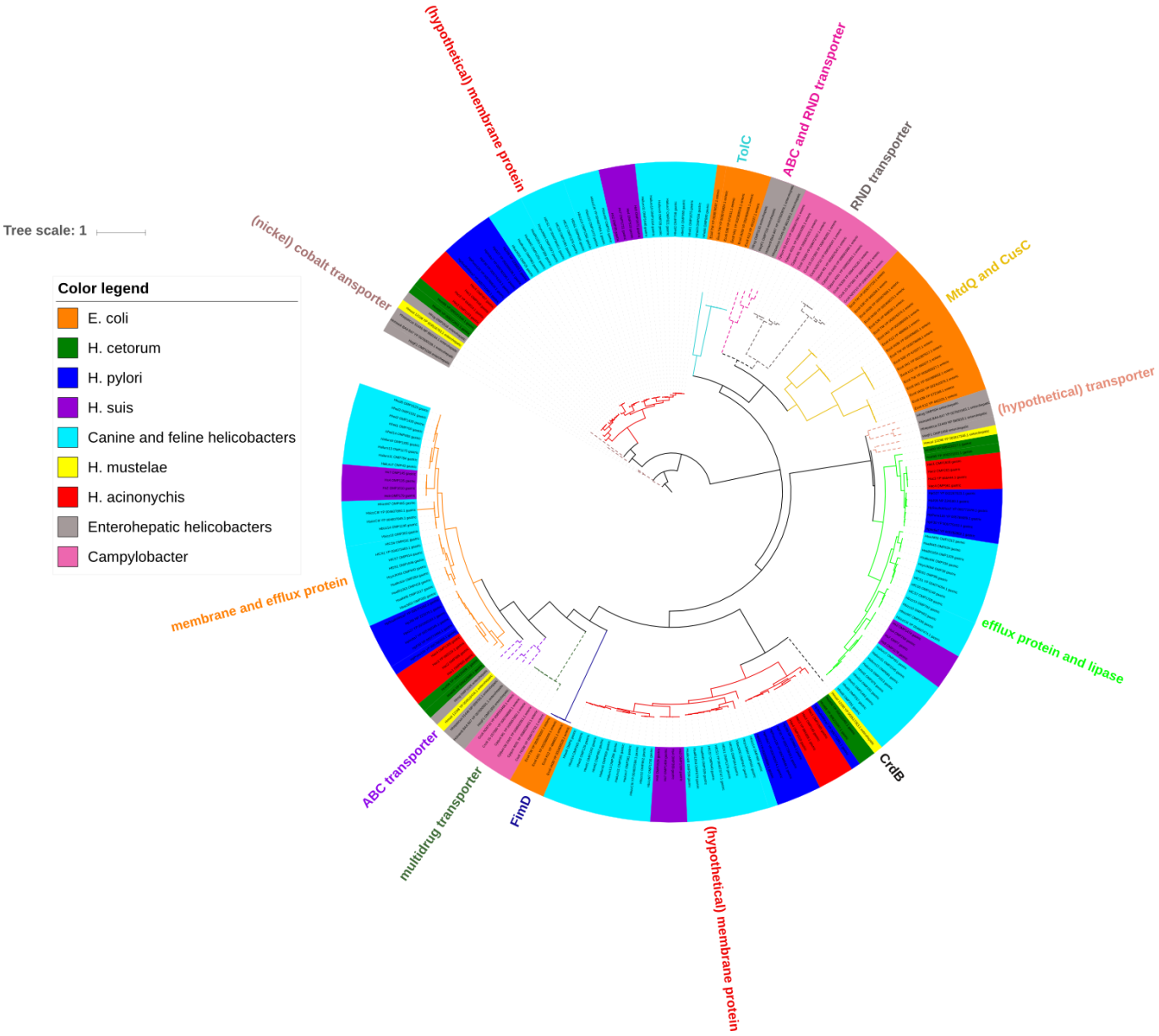

FIGURE 8. Phylogenetic tree of Family 42 – Outer membrane factor (OMF). Orthologous OMPs are present in all examined strains of *E. coli, Campylobacter* and *Helicobacter*. Several different subgroups are indicated. OMPs of *H. mustelae* (yellow) cluster together with enterohepatic helicobacters (grey). OMPs of *H. mustelae*, enterohepatic helicobacters and *Campylobacter* species are indicated by dashed clade lines.

4) OMPs involved in immune evasion

Family 36 - Systemic factor protein A (SfpA/LpxR)

The prototype of this family is the systemic factor protein A (SfpA) of *Yersinia enterocolitica*. Lipid A deacylase (LpxR), a homologous outer membrane protein of SfpA, has been identified in *Salmonella* Typhimurium and has been shown to be important for immune evasion (Petrone et al., 2014). LpxR removes the 3'-acyloxyacyl group of the hydrophobic anchor lipid A of

lipopolysaccharide (LPS) (Reynolds et al., 2006). By this modification, *Salmonella* Typhimurium is able to evade the innate immune response (Kawasaki et al., 2012) and to survive within macrophages (Kawano et al., 2010).

In total, 57 proteins were identified to belong to the SfpA/LpxR family (Family 36 of the OMPdb database, **Table 1**). Orthologs of SfpA/LpxR were present in the examined strains of *Helicobacter* with the exception of *H. cinaedi*, *H. equorum* and *H. hepaticus*. The number of orthologs in each strain is indicated in superscript after the strain names in **Table 1**. Only one orthologous protein was present in *E. coli* strain TW14359 and there were no members of the SfpA/LpxR family detected in *Campylobacter* and the other *E. coli* strains. As shown in the phylogenetic tree in **Figure 9**, also for this OMP *H. mustelae* clustered closer to enterohepatic helicobacters than to gastric NHPH species.

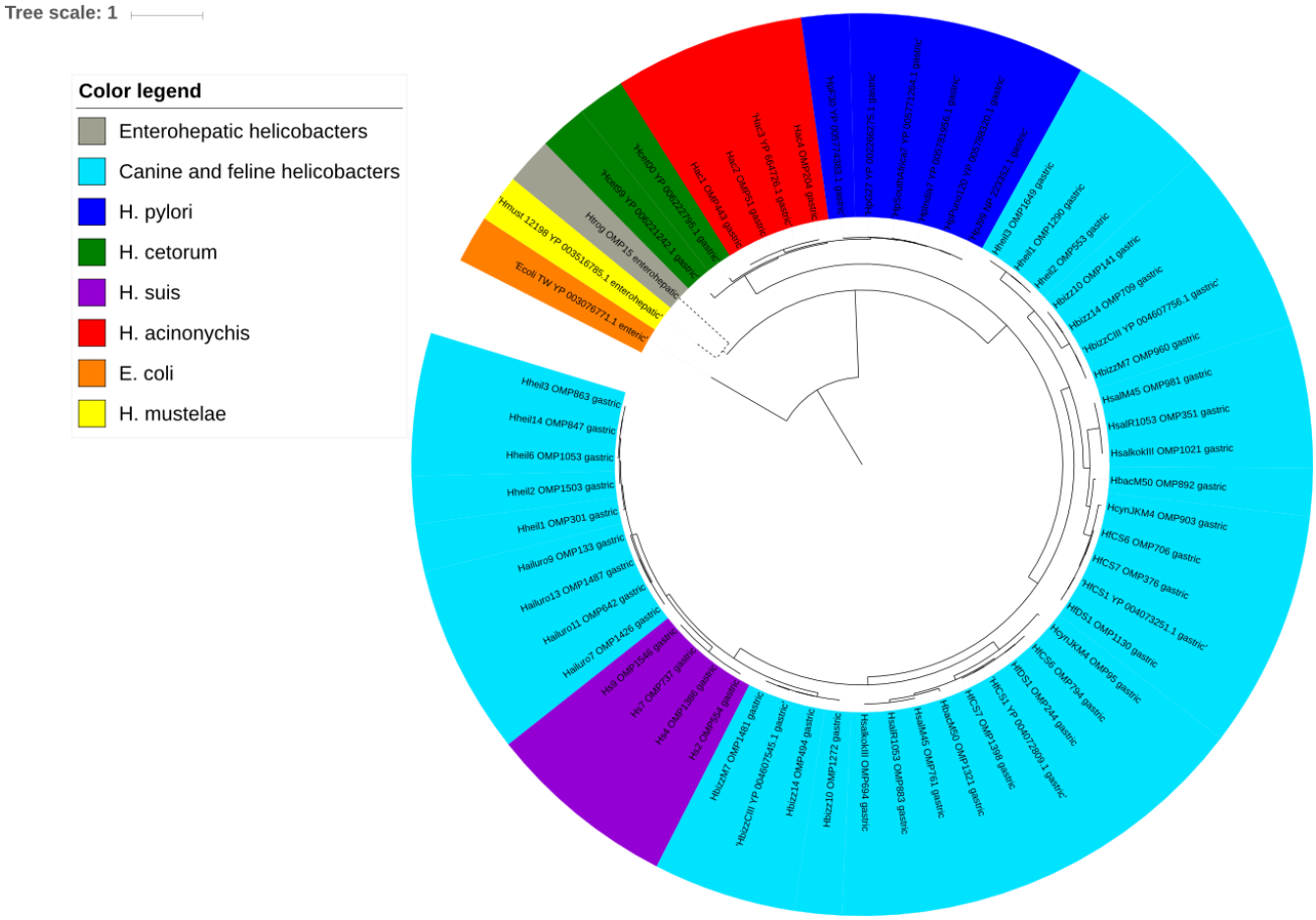

FIGURE 9. Phylogenetic tree of Family 36 – Systemic factor protein A (SfpA/LpxR). Orthologous OMPs are present in the examined strains of *Helicobacter* with the exception of *H. cinaedi*, *H. equorum* and *H. hepaticus*, and only one orthologous OMP is present in *E. coli* strain TW14359 (orange). In the other *E. coli* strains and in *Campylobacter*, no orthologous OMPs are detected. *H. mustelae* (yellow) clusters closer to the enterohepatic species *H. trogontum* (grey) than to gastric NHPH species. OMPs of *H. mustelae* and enterohepatic helicobacters are indicated by dashed clade lines.

5) OMPs that influence colonization capacity and virulence

Family 38 - Outer Membrane Phospholipase (OMPLA)

The outer membrane phospholipase A (OMPLA), encoded by the *pldA* gene which is widespread among Gram-negative bacteria, hydrolyses acyl ester bonds in phospholipids and lysophospholipids (Kingma et al., 2002; Nishijima et al., 1977). OMPLA has been described as a virulence factor. For instance, in *C. coli*, OMPLA was identified as a major hemolytic factor and *H. pylori* OMPLA has been shown to be involved in the colonization and invasion of the human gastric mucosa (Dorrell et al., 1999; Grant et al., 1997; Kingma et al., 2002; Vollan et al., 2012; Ziprin et al., 2001). Moreover, *H. pylori* isolates with high OMPLA activity have been associated with peptic ulcer disease in human patients (Istivan et al., 2006; Tannaes et al., 2005).

In the present study, 53 proteins were classified in the OMPLA family (Family 38 of the OMPdb database, **Table 1**). All *E. coli*, *Campylobacter* and *Helicobacter* strains, with the exception of *H. mustelae*, *H. equorum*, *H. cetorum* MIT 00-7128 and *H. pylori* G27, harbored 1 OMPLA homolog, whereas 4 different homologs were present in *H. trogontum*. Phylogenetically, the OMPLA homologs of all strains clustered separately per genera and per species (**Figure 10**).

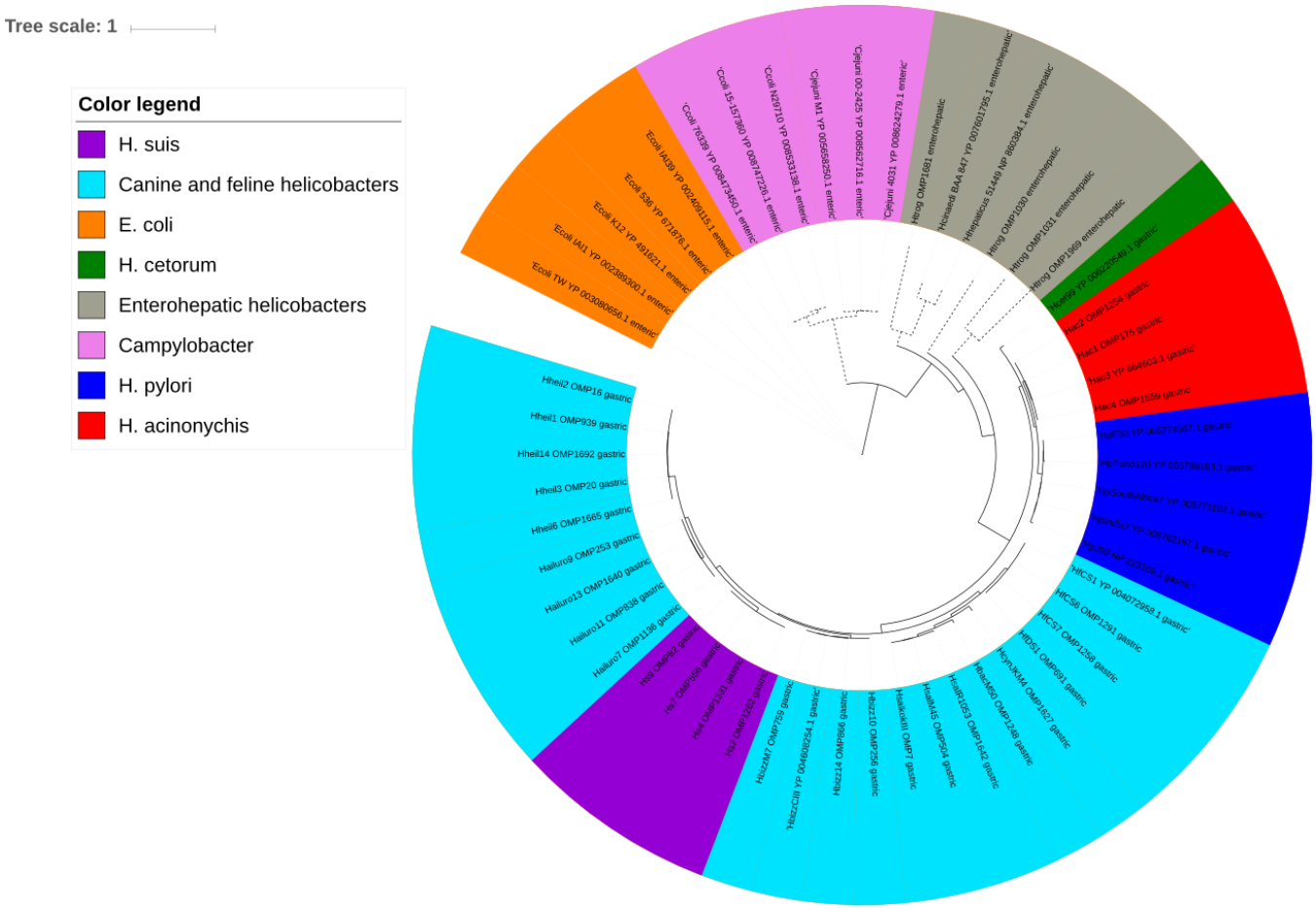

FIGURE 10. Phylogenetic tree of Family 38 – Outer membrane phospholipase (OMPLA). One orthologous OMP is present in each of the examined strains of *E. coli*, *Campylobacter* and *Helicobacter* with the exception of *H. mustelae*, *H. equorum*, *H. cetorum* MIT 00-7128 and *H. pylori* G27, in which this OMP is absent. In *H. trogontum*, 4 OMPLA OMPs are present. OMPs of enterohepatic helicobacters and *Campylobacter* species are indicated by dashed clade lines.

Family X1 and X3 – (Putative) vacuolating cytotoxin family

The secreted vacuolating cytotoxin A (VacA), belonging to the autotransporter OMP family, is an important virulence factor of *H. pylori*. After binding to and internalization into host epithelial cells, VacA induces cellular vacuolation and various other responses (Palframan et al., 2012; Sause et al., 2012). However, a homolog of this VacA protein is absent in most NHPH, except for *H. cetorum* and *H. acinonychis*, although the *vacA* gene is fragmented and inactive in the latter species (Joosten et al., 2015; Kersulyte et al., 2013). In the present study, 51 proteins were classified into the vacuolating cytotoxin family (Family X1 of the Pfam database, **Table 2**). All included *H. pylori* and *H. cetorum* strains each harbored one VacA copy. *H. acinonychis* contained more than one VacA copy, but these were inactivated by insertion sequences compared to *H. pylori* and *H. cetorum*.

Besides VacA, *H. pylori* also contains 3 VacA-like autotransporters that each enhance its capacity to colonize the stomach (Radin et al., 2013). Previously, we reported the presence of a *vacA*-like autotransporter gene in canine, feline and porcine gastric *Helicobacter* species. At the protein level, these VacA-like autotransporters showed to be highly divergent between the different *Helicobacter* species (Joosten et al., 2015). In the present study, 55 homologs of the VacA-like protein were classified into the putative vacuolating cytotoxin family (Family X3 of the Pfam database, **Table 2**). Orthologs were present in the examined strains of *Helicobacter* with the exception of *H. cinaedi*, *H. equorum*, *H. hepaticus*, *H. mustelae* and *H. bizzozeronii* 10. The number of orthologs in each strain is indicated in superscript after the strain names in **Table 2**. There were no homologs of this autotransporter present in *Campylobacter* and *E. coli*. As shown in the phylogenetic tree of family X3 (**Figure 11**), the VacA-like OMPs from *H. pylori*, *H. acinonychis* and *H. cetorum*, closely related with each other, clustered together.

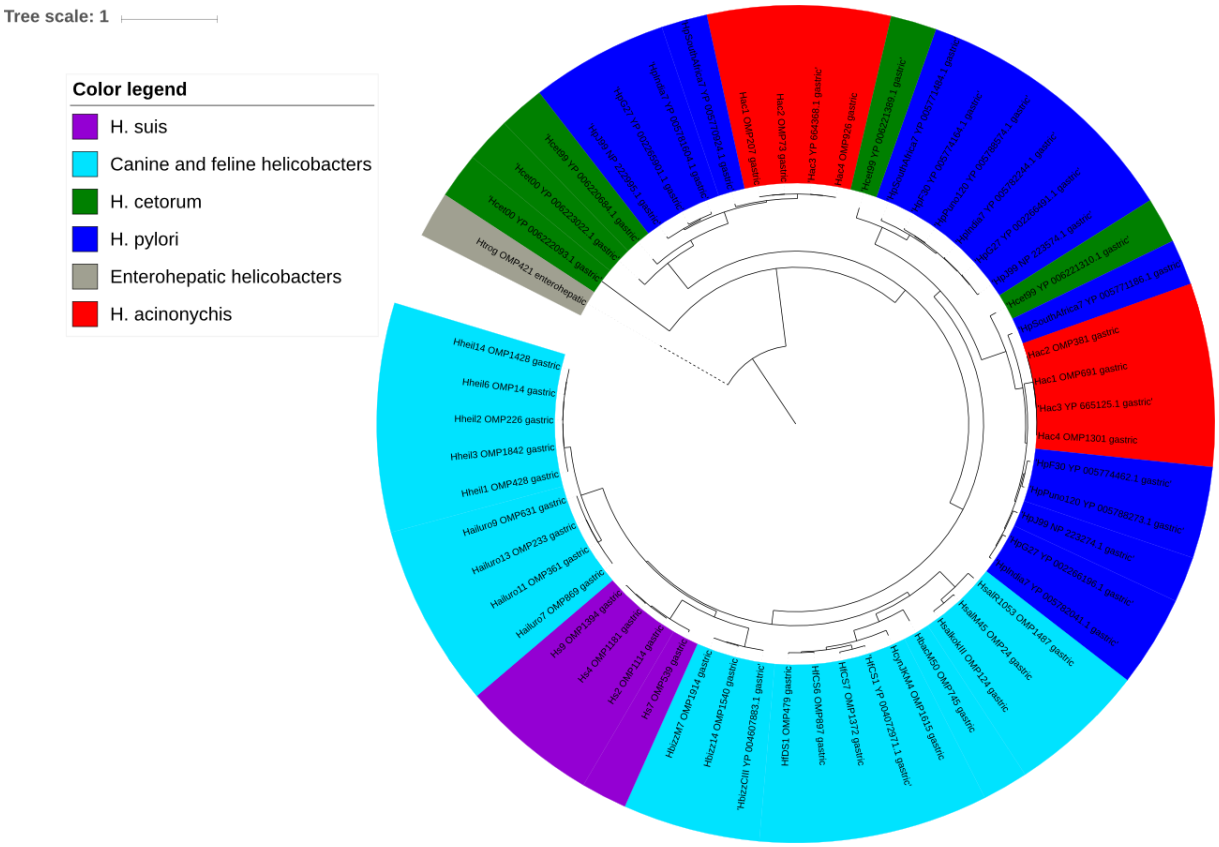

FIGURE 11. Phylogenetic tree of Family X3 – putative vacuolating cytotoxin. Orthologous OMPs are present in the examined strains of *Helicobacter* with the exception of *H. cinaedi*, *H. equorum*, *H. hepaticus*, *H. mustelae* and *H. bizzozeronii* 10. Orthologous OMPs are absent in *E. coli* and *Campylobacter* species. Most *Helicobacter* strains harbor 1 VacA-like protein, whereas *H. acinonychis*, *H. cetorum* MIT 00-7128 and *H. pylori* Puno120 and F30 harbor 2 copies, and *H. cetorum* MIT 99-5656 and *H. pylori* G27, J99, India7 and SouthAfrica harbor 3 copies of the VacA-like OMP. OMPs of enterohepatic helicobacters are indicated by dashed clade lines.

6) OMPs that are possibly involved in virulence of enterohepatic helicobacters and *Campylobacter* species but which are absent in gastric *Helicobacter* spp.

The outer membrane-localized lipid A 3-O-deacylase PagL (Family 1 of the OMPdb database, **Table 1**) was present in the intestinal bacteria *C. coli, E. coli* and *H. hepaticus*. PagL modifies the lipid A portion of LPS by deacylation. For *Salmonella* Typhimurium, it has been demonstrated that lipid A 3-*O*-deacylation by PagL reduces the activity of lipid A in inducing host cellular signaling through TLR4, as a part of the adaptation process to the host environments (Kawasaki et al., 2004).

Secondly, the salt-stress induced outer membrane protein SspA (Family 29 of the OMPdb database, **Table 1**), which plays a role in bacterial growth under salt-stressed conditions (Tsuzuki et al., 2005), was only found in *H. hepaticus* and *E. coli*.

The autotransporter-2 family (Family 44 of the OMPdb database, **Table 1**) was identified in *H. trogontum* and in all examined *E. coli* strains. Several autotransporters of *E. coli* have been shown to play a relevant role in its pathogenesis (Navarro-Garcia and Elias, 2011).

Finally, a secretin family (Family 45 of the OMPdb database, **Table 1**) was present in all analyzed *C. coli, C. jejuni* and *E. coli* strains, as well as in enterohepatic *Helicobacter* species. Secretins are involved in transporting large molecules across the outer membrane and thus can potentially be part of secretion pathways of virulence-associated factors (Gauthier et al., 2003).

7) OMPs from the Y-family without known name or function

Besides the characterized OMP families from the OMPdb and the Pfam database, the last clustering with CD-HIT resulted in 75 OMP families without known name or function and for which the role in colonization and virulence thus remains unknown (Y-families, **Table 3**). These Y-families, consisting of primarily short OMP sequences, were mainly present in gastric *Helicobacter* species (53 out of 75 families) and especially in helicobacters from cats and dogs (22 out of 53 families), including *H. ailurogastricus* (OMPs in 2 Y-families), *H. baculiformis* (OMPs in 3 Y-families), *H. bizzozeronii* (OMPs in 9 Y-families), *H. cynogastricus* (OMPs in 1 Y-family), *H. felis* (OMPs in 4 Y-families), *H. heilmannii* (OMPs in 8 Y-families) and *H. salomonis* (OMPs in 2 Y-families). Remarkably, OMPs from *H. acinonychis*, the gastric *Helicobacter* species from wild felines, were found in 21 Y-families, but in most cases these OMP sequences were fragmented.

Only 3 Y-families were specific for the enterohepatic helicobacters tested here (Y27, Y41 and Y75, **Table 3**) and 2 Y-families were shared between the enterohepatic *H. trogontum* and *C. coli* or *C. jejuni* (family Y4 and family Y56 respectively, **Table 3**).

Furthermore, it should be noted that most Y-families were composed of OMPs from only one species as shown in **Figure 1D**.

Discussion

In this study, we have analyzed the genome sequences from a total of 54 different strains of *E. coli* and the *Helicobacter* and *Campylobacter* genera to cluster their orthologous outer membrane proteins (OMPs) in families. The *E. coli* strains harbored an average of 100 OMPs, whereas among the gastric *Helicobacter* species, an average of 66 OMPs was found. The smallest number of OMPs was found in the enterohepatic *Helicobacter* species and *Campylobacter* species included in the study, with an average of 33 and 24 OMPs, respectively (**Table S2**). Taken into account the genome length and the total protein count, it is clear that gastric *Helicobacter* species harbor proportionally the most OMPs. Based on the NCBI database, a median genome length of 5.17 Mb with a median protein count of 4,931 is found for *E. coli*, whereas for gastric helicobacters, these numbers are much smaller. For instance, *H. pylori* has a median genome length of only 1.63 Mb with a median protein count of 1,451. Thus, ~4% of its total protein count constitutes of OMPs, compared to only ~2% for *E. coli*. These large numbers of OMPs might result from an adaptation to the harsh gastric environment and might be needed to survive here (Alm et al., 2000). Among the gastric *Helicobacter* species, the total OMP number was the highest for the analyzed *H. acinonychis* (a mean of 97) and *H. cetorum* strains (a mean of 110), but it should be noted that these species contain multiple fragmented OMPs. Among enterohepatic helicobacters, *H. trogontum* harbors remarkably more OMPs than *H. cinaedi*, *H. equorum* and *H. hepaticus* (55 versus 29, 24 and 28 OMPs, see **Table S2**). This can be explained by the highly variable genome sizes of enterohepatic helicobacters, with the largest genome size of 2.66 Mb for *H. trogontum*.

The main function of the outer membrane of Gram-negative bacteria is forming a barrier against damaging substances from the environment such as enzymes, detergents and hydrophobic antimicrobials. Furthermore, the outer membrane also interacts with the environment via its OMPs (Alm et al., 2000). The permeability of the outer membrane is determined by the presence of OMPs that function as porins. They contain transmembrane diffusion channels through which small hydrophilic molecules, nutrients and small antibiotics can be transported across the outer membrane (Alm et al., 2000; Buchanan et al., 1999). In our study, several conserved porin families were identified among *E. coli* and the *Campylobacter* and *Helicobacter* genera. The Imp/OstA family (Family 14, **Table 1**) is a porin family with a

function in membrane permeability and resistance to antibiotics (Chiu et al., 2007). This family is absent in *E. coli* and might thus be more specific for the Campylobacterales order. The ample OMPs of the OMF family (Family 42, **Table 1**) that are present in all analyzed species and strains, are part of porin transport systems that are involved in the development of multidrug resistance. They function in conjunction with a cytoplasmic membrane transporter and a membrane fusion protein, and this complex allows the export of various substances across the Gram-negative bacterial cell envelope (Nikaido et al., 2009; Paulsen et al., 1997). The last two porin families found in this study are Families 13 and 33 (**Table 1**). Both families mainly contain OMP orthologs from the genus *Helicobacter* albeit with a greater extent in the gastric helicobacters than in the enterohepatic ones. *C. coli* and *C. jejuni* only harbor one OMP belonging to Family 13, which might be explained by the fact that *Campylobacter* species harbor much less OMPs than gastric helicobacters (see **Table S2**). In *E. coli*, no OMPs were identified in Families 13 and 33. Both these outer membrane porin families can thus be seen as *Helicobacter*-specific OMP families and were probably acquired by *Helicobacter* after splitting-off from a last common ancestor. The *H. pylori* Hop, Hor and Hom proteins belong to Family 13, whereas Family 33 contains the *H. pylori* Hof proteins. Genes encoding Hof proteins are also present in the canine, feline and porcine gastric NHPH species, but they share only few homologs of the Hor and Hom OMPs. Moreover, most gastric NHPH species lack all *H. pylori* Hop adhesins, suggesting that these OMPs were acquired after *H. pylori* speciation (Arnold et al., 2011; Joosten et al., 2015; Schott et al., 2011; Vermoote et al., 2011). Only in the species *H. cetorum* from dolphins and whales and in *H. acinonychis* from wild felines, orthologs of the Hop OMPs were found. Not surprisingly, these two species are the closest related to *H. pylori*. It has been described that *H. acinonychis* originates from *H. pylori* after a host jump from humans to wild felines (Eppinger et al., 2006; Harper et al., 2002). The *H. pylori*-specific Hop porins function as adhesins for gastric epithelial cells or as Lewis B binding adhesins. Using these OMPs as adhesins might be the result of an adaptation to the gastric acidic environment, where any polymeric pilus structure would likely depolymerize (Alm et al., 2000). Also most of the canine, feline and porcine gastric NHPH have been shown to attach to the human gastric mucosa (Joosten et al., 2015; Liu et al., 2016). The absence of the *H. pylori* Hop adhesins in these NHPH species implies that other OMPs function as adhesins in these organisms. Indeed, the HofE and HofF OMPs, belonging to Family 33, have recently been identified as adhesins in *H. heilmannii* (Liu et al., 2016). HofF has also been shown to be important for *H. pylori* colonization, but the function of the other *H. pylori* Hof OMPs remains largely unknown (Kavermann et al., 2003; Liu et al., 2016). Furthermore, the exact role of the other NHPH OMPs

from Families 13 and 33 during colonization and adhesion remains to be further elucidated. In contrast to outer membrane porins, which utilize passive diffusion for solute uptake, outer membrane receptor proteins carry out high-affinity binding and energy-dependent uptake of specific substrates. For the acquisition of iron from the environment, Gram-negative bacteria have several outer membrane receptors at their disposal that transport siderophores into the periplasm, including TonB-dependent receptors that use energy from the proton motive force of the cytoplasmic membrane via the TonB/ExbB/ExbD membrane proteins (Ferguson and Deisenhofer, 2002). In this study, two TonB-dependent receptor families (Family 3, **Table 1** and Family X2, **Table 2**) were identified that contribute to bacterial virulence (Hu et al., 2012). Members of the X2 family are present in all the examined strains of *Helicobacter* and *E. coli*, but absent in the *C. coli* and *C. jejuni* strains, although they are closer related to *Helicobacter* than *E. coli*. Possibly, these OMPs were acquired by horizontal gene transfer at the origin of *Helicobacter*. On the contrary, TonB-dependent receptors belonging to OMP family 3 are present in *Campylobacter* species, ranging from 1 to 3 copies per strain (see **Table 1**). The explicit strain variation among surface epitopes of OMPs might be favorable for evasion of the host's immune response and can be induced either by antigenic or phase variation (Alm et al., 2000). In addition, these *Campylobacter* TonB-dependent receptors clustered together with those from the tested enterohepatic *Helicobacter* species, which might indicate that these genes were acquired via horizontal gene transfer between these two genera. No members of OMP Family 3 were detected in *H. bizzozeronii* and remarkably, OMPs from both Family 3 and Family X2 of TonB-dependent receptors were lacking in the 3 examined strains of *H. salomonis*. This may suggest that these *H. salomonis* strains have other iron uptake mechanisms at their disposal to maintain their iron homeostasis.

H. pylori has developed a very large repertoire of mechanisms to evade both innate and adaptive immune recognition (Lina et al., 2014). Several of these mechanisms have been described for evasion of the innate immune response, including the avoidance of recognition by pattern recognition receptors, inhibition of phagocytic killing and inhibition of killing by reactive oxygen species and nitric oxide. One way of *H. pylori* to evade recognition by pattern recognition receptors is the avoidance of recognition by Toll-like receptors of its bacterial surface molecules such as LPS and flagellin. For example, the bacterium can modify the negatively charged lipid A portion of the LPS molecule, resulting in the alteration of the net charge of the microbial surface. As a result, cationic antimicrobial peptides can no longer bind to lipid A (Cullen et al., 2011). Here, we identified the SfpA/LpxR OMP family (Family 36, **Table 1**) present in all gastric *Helicobacter* species, which also might be involved in immune

evasion by removing the 3'-acyloxyacyl group of lipid A (Kawasaki et al., 2012; Reynolds et al., 2006). For the enterohepatic *Helicobacter* species tested here, SfpA/LpxR was only detected in *H. trogontum*. This OMP family might be more specific for gastric *Helicobacter* species. Moreover, members of the SfpA/LpxR family are also absent in *C. coli*, *C. jejuni* and *E. coli*, except for *E. coli* strain TW14359. On the contrary, *H. hepaticus*, *C. coli* (15-151360) and all analyzed *E. coli* strains possessed another OMP that was absent in gastric helicobacters, namely PagL (Family 1, **Table 1**) that also modifies the lipid A portion of LPS and reduces the host immune response (Kawasaki et al., 2004).

Another virulence factor that has been shown to influence the colonization capacity and pathogenicity of Gram-negative bacteria, is the outer membrane phospholipase A (OMPLA). The encoding *pldA* gene is described to be widespread among Gram-negative bacteria (Kingma et al., 2002; Nishijima et al., 1977). However, OMPLA homologs could not be found in the genomes of *H. cetorum* strain MIT 00-7128, *H. pylori* strain G27, *H. equorum* and *H. mustelae*. Whether the absence of OMPLA in these strains influences their virulence and colonization capacity remains to be further investigated. The absence of OMPLA in only one of the investigated strains of *H. cetorum* and *H. pylori* might be due to the acquisition and loss of genes via horizontal gene transfer, which creates genetic diversity among strains of one species, in order to maintain an optimal virulence and to evade the host's immune response (Fischer et al., 2010).

A very well-studied outer membrane virulence factor of *H. pylori* is the secreted vacuolating cytotoxin A (VacA; Family X1, **Table 2**) that causes cellular vacuolation (Palframan et al., 2012; Sause et al., 2012). In agreement with previous research (Joosten et al., 2015; Kersulyte et al., 2013), we identified the VacA OMP only in *H. pylori* and *H. cetorum* and short fragments of this protein in *H. acinonychis*, but not in other NHPH species. Finally, we also found a *Helicobacter*-specific putative VacA-like cytotoxin family (Family X3, **Table 2**). The VacA-like autotransporters belonging to this family enhance the bacterium's colonization capacity of the stomach (Radin et al., 2013). The VacA-like OMP is well conserved among the different gastric *Helicobacter* species, although their protein sequences exhibit much variation (Joosten et al., 2015). Here, we also show variation in the number of VacA-like autotransporters, not only between the different species but also at the species level (see **Table 2**). For instance, in *H. bizzozeronii* strain 10, no VacA-like autotransporter could be identified, whereas the other examined strains of *H. bizzozeronii* each contained one copy of this OMP. Again, this underlines the genetic diversity among strains of one species. However, it should be noted that the genomes of most NHPH species that were analyzed in this study, including that of *H.*

bizzozeronii strain 10, are draft genomes that lack approximately 5% of the full genome sequence. Therefore, it cannot be excluded that the gene encoding the VacA-like autotransporter of this *H. bizzozeronii* strain is part of the lacking 5% of its genome sequence. The genomes of enterohepatic *Helicobacter* species, except for *H. trogontum*, lack a VacA-like autotransporter as well. This OMP family may therefore be more specific for gastric helicobacters. However, in the *H. mustelae* strain included in our study, the VacA-like OMP is absent. Also for all other OMP families that were analyzed, this *H. mustelae* strain clusters closer to or together with enterohepatic helicobacters, or separately between enterohepatic and gastric species. Thus, although *H. mustelae* has been described as a gastric *Helicobacter* species that has been associated with gastritis, peptic ulcers, gastric adenocarcinoma and MALT-lymphoma in domestic ferrets (Fox et al., 1990; Fox and Marini, 2001), it might be that this species shares more OMP orthologs with the enterohepatic helicobacters than with the gastric helicobacters. More *H. mustelae* strains should be included in the phylogenetic analyses in order to confirm these results. Based on their 16S rRNA and urease gene sequences, 2 other *H. mustelae* strains (ATCC 43772^T and 91-292-E1A Fox M88156) have also been shown to cluster phylogenetically closer to enterohepatic *Helicobacter* species (Gueneau and Loiseax-De Goer, 2002). Taken together, these findings may suggest that *H. mustelae* is rather a gastro-intestinal *Helicobacter* species than a strictly gastric species, although enteric colonization has not been described in ferrets (Kusters et al., 2006). However, *H. mustelae* has been detected in a high percentage of faeces samples of young ferrets, which may indicate lower bowel colonization, or transit of the organism from its gastric niche (Fox et al., 1992).

In conclusion, several important OMP families, mainly from gastric *Helicobacter* species, were determined by using comparative genomic and phylogenetic analyses. To our knowledge, this is the first report showing a classification of the OMP families of gastric NHPH. We showed that most *Helicobacter* species contain TonB-dependent OMPs with a function in iron uptake, an outer membrane factor (OMF) involved in antimicrobial resistance and an outer membrane phospholipase (OMPLA) that plays a role in colonization capacity. Furthermore, we identified the SfpA/LpxR OMP that functions in immune evasion in gastric *Helicobacter* species. The Imp/OstA OMP, involved in antimicrobial resistance, is present in both gastric and enterohepatic *Helicobacter* species. Two *Helicobacter*-specific outer membrane porin families with probable functions in adhesion, and a *Helicobacter*-specific VacA-like cytotoxin family with a role in colonization capacity, were identified primarily in gastric species. Specific for enterohepatic species, a well conserved secretin family was found which might play a role in the secretion of virulence-associated factors. All these OMPs are potential candidates for further

Helicobacter virulence studies and are especially interesting to further unravel the colonization process of gastric NHPH.

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Additional files

TABLE S1. Overview of the species and strains used in this study.

Species and strain designation	Isolated from	NCBI accession number*	
		Chromosome	Plasmid
Campylobacter coli			
15-157360	human	NC_022660	NC_022656
76339	human	NC_022132	
N29710	chicken	NC_022347	NC_022355 and NC_02234
Campylobacter jejuni			
00-2425	human	NC_022362	
4031	contaminated tap water	NC_022529	
M1	human	NC_017280	
Escherichia coli			
536	human	NC_008253	
IAI1	human	NC_011741	
IAI39	human	NC_011750	
K12-W3110	human	NC_007779	
TW14359	human	NC_013008	NC_013010
Helicobacter acinonychis			
Hacino1	Cheetah	NYD**	
Hacino2	Sumatran tiger	NYD**	
Hacino3	Lion	NC_008229	NC_008230
Hacino4	Bengal tiger	NYD**	
Helicobacter ailurogastricus			
ASB7	cat	CDMG00000000	
ASB9	cat	CDMN00000000	
ASB11	cat	CDML00000000	
ASB13	cat	CDMH00000000	
Helicobacter baculiformis			
M50	cat	NYD**	
Helicobacter bizzozeronii			
10	dog	NYD**	
14	dog	NYD**	
CIII	human	NC_015674	NC_015670
M7	dog	NYD**	
Helicobacter cetorum			
MIT 00-7128	whale	NC_017737	NC_017738
MIT 99-5656	dolphin	NC_017735	NC_017736
Helicobacter cinaedi			
BAA_847	human	NC_020555	
Helicobacter cynogastricus			
JKM4	dog	NYD**	
Helicobacter equorum			
eqF1	horse	NYD**	
Helicobacter felis			
CS1	cat	NC_014810	
CS6	cat	NYD**	
CS7	cat	NYD**	
DS1	dog	NYD**	
Helicobacter heilmannii			
ASB1	cat	CDMK00000000	
ASB2	cat	CDMP00000000	
ASB3	cat	CDMJ00000000	
ASB6	cat	CDMM00000000	
ASB14	cat	CDMI00000000	
Helicobacter hepaticus			
ATCC 51449	mouse	NC_004917	

Helicobacter mustelae			
ATC 12198	ferret	NC_013949	
Helicobacter pylori			
Puno120	human	NC_017378	NC_017377
G27	human	NC_011333	NC_011334
F30	human	NC_017365	NC_017369
J99	human	NC_000921	
India7	human	NC_017372	
SouthAfrica7	human	NC_022130	
Helicobacter salomonis			
M45	dog	NYD**	
R1053	dog	NYD**	
KokIII	dog	NYD**	
Helicobacter suis			
HS2	pig	NYD**	
HS4	pig	NYD**	
HS7	pig	NYD**	
HS9	pig	NYD**	
Helicobacter trogontum			
R3554	rat	NYD**	

*NCBI accession numbers are available for the species and strains with fully annotated genomes. On the basis of these genomes, OMP extraction was started.

**NYD= not yet defined. Genomes from these strains are derived from unpublished studies. Only their OMP sequences were deposited in the EMBL database and are publicly available.

TABLE S2. Overview of the number of outer membrane proteins (OMPs) per species and strain.

Species and strains	Number of OMPs
Campylobacter coli	
15-157360	21
76339	24
N29710	27
Campylobacter jejuni	
00-2425	23
4031	24
M1	22
Escherichia coli	
536	110
IAI1	89
IAI39	97
K12-W3110	88
TW14359	116
Helicobacter acinonychis	
Hacino1	95
Hacino2	92
Hacino3	106
Hacino4	94
Helicobacter ailurogastricus	
ASB7	59
ASB9	60
ASB11	60
ASB13	60
Helicobacter baculiformis	
M50	62
Helicobacter bizzozeronii	
10	69
14	65

CIII	68
M7	70
Helicobacter cetorum	
MIT 00-7128	118
MIT 99-5656	101
Helicobacter cinaedi	
BAA_847	29
Helicobacter cynogastricus JKM4	62
Helicobacter equorum	
eqF1	24
Helicobacter felis	
CS1	65
CS6	66
CS7	67
DS1	61
Helicobacter heilmannii	
ASB1	53
ASB2	55
ASB3	56
ASB6	56
ASB14	55
Helicobacter hepaticus	
ATCC 51449	28
Helicobacter mustelae	
ATC 12198	49
Helicobacter pylori	
Puno120	69
G27	69
F30	72
J99	71
India7	74
SouthAfrica7	69
Helicobacter salomonis	
M45	47
R1053	48
KokIII	49
Helicobacter suis	
HS2	53
HS4	52
HS7	53
HS9	53
Helicobacter trogontum	
R3554	55

TABLE S3. Subgroups of family 42 - the outer membrane factor (OMF) family.

OMF subgroups	Species (strains)
Outer membrane efflux proteins/transporters	*E. coli* (536, IAI39, TW14359) *C. coli* (15-157360, 76339, N29710) *C. jejuni* (00-2425, 4031, M1) *H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13) *H. baculiformis* (M50) *H. bizzozeronii* (10, 14, CIII, M7) *H. cinaedi* (BAA_847) *H. cynogastricus* (JKM4) *H. felis* (CS1, CS6, CS7, DS1) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14)

	H. hepaticus (ATCC 51449) *H. mustelae* (ATC 12198) *H. salomonis* (M45, R1053, KokIII) *H. suis* (HS2, HS4, HS7, HS9)
Lipases	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. bizzozeronii* (10, 14, CIII, M7) *H. cetorum* (MIT 00-7128, MIT 99-5656) *H. pylori (*Puno120, G27, F30, J99, India7, SouthAfrica7) *H. suis* (HS2, HS4, HS7, HS9)
Hypothetical membrane proteins	*H. acinonychis* (Hacino1, Hacino2, Hacino3, Hacino4) *H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13) *H. baculiformis* (M50) *H. bizzozeronii* (10, 14, CIII, M7) *H. cetorum* (MIT 00-7128, MIT 99-5656) *H. cynogastricus* (JKM4) *H. equorum* (eqF1) *H. felis* (CS1, CS6, CS7, DS1) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14) *H. mustelae* (ATC 12198) *H. pylori (*Puno120, G27, F30, J99, India7, SouthAfrica7) *H. salomonis* (M45, R1053, KokIII) *H. suis* (HS2, HS4, HS7, HS9) *H. trogontum* (R3554)
Copper resistance determinant proteins CrdB	*H. bizzozeronii* (14) *H. felis* (CS1, CS6, CS7, DS1) *H. mustelae* (ATC 12198) *H. salomonis* (M45, R1053, KokIII)
RND transporters/efflux proteins	*C. coli* (15-157360, 76339, N29710) *C. jejuni* (00-2425, 4031, M1) *E. coli* (536, IAI1, IAI39, K12-W3110, TW14359) *H. ailurogastricus* (ASB7, ASB9, ASB11, ASB13) *H. cinaedi* (BAA_847) *H. equorum* (eqF1) *H. heilmannii* (ASB1, ASB2, ASB3, ASB6, ASB14) *H. hepaticus* (ATCC 51449)
ABC transporter permeases	*H. cinaedi* (BAA_847) *H. equorum* (eqF1) *H. hepaticus* (ATCC 51449) *H. trogontum* (R3554)
(nickel) cobalt transporters/efflux proteins	*H. cinaedi* (BAA_847) *H. equorum* (eqF1) *H. hepaticus* (ATCC 51449) *H. trogontum* (R3554)
Outer membrane channel proteins TolC	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
Multidrug resistance outer membrane proteins MdtQ	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
Cation transporters/efflux systems CusC	*E. coli* (536, IAI1, IAI39, K12-W3110, TW14359)
Outer membrane usher proteins FimD	*E. coli* (IAI1, IAI39, K12-W3110, TW14359)

GENERAL DISCUSSION

At the start of this PhD research, little was known about the pathogenesis of canine and feline non-*H. pylori Helicobacter* species (NHPH) and in particular, it was mostly unknown which virulence-associated factors play a role in their colonization process of the gastric mucosa. Fortunately, the fully annotated genome sequences of all gastric NHPH have recently become available, thus allowing phylogenetic characterization and comparison of their virulence-associated genes and proteins.

1. New insights into the pathogenesis of *H. heilmannii* s.s. infections

In contrast to *H. pylori*, little research has been performed on virulence and colonization mechanisms of canine and feline NHPH. This is mainly due to their fastidious nature and, as a result, the availability of only a limited number of *in vitro* isolates worldwide. Moreover, *H. heilmannii* s.s. had only recently been isolated and cultured *in vitro* (Smet et al., 2012), and no experimental infection studies using pure cultures of this bacterium had been performed so far. In **chapter one** of this thesis, the bacterium-host interactions of 9 different feline *H. heilmannii* s.s. strains, identified on the basis of their 16S rRNA and *ureAB* genes, were studied in an *in vivo* Mongolian gerbil model, in order to obtain better insights into the pathogenesis of human gastric disease associated with this microorganism.

We showed clear differences in colonization capacity and virulence between the 9 isolates after 9 weeks of infection. This indicates that strain-specific virulence factors might play a role in the capacity of *H. heilmannii* to colonize the stomach and to induce inflammation and gastric lesions. Such strain differences in the virulence factor repertoire have often been described for *H. pylori* (Armitano et al., 2013; Dussombekova et al., 2006; Fischer et al., 2010; Franco et al., 2008; Markovska et al., 2011; Yamaoka et al., 2002; Yamaoka et al., 2006). For the virulent *H. heilmannii* isolates, the colonization and inflammation levels were the highest in the antrum of the stomach of the gerbils, which is in agreement with NHPH infections in humans (Debongnie et al., 1998; Haesebrouck et al., 2009; Morgner et al., 2000). This indicates that Mongolian gerbils are a suitable model to study the pathogenesis of *H. heilmannii*-related human gastric disease, as has also been shown for infections with other NHPH (Court et al., 2002; Flahou et al., 2010). Besides Mongolian gerbils, also mouse models are frequently used to study *Helicobacter*-related gastric pathology. Recently, a first experimental infection study with pure cultures of *H. heilmannii* s.s. has been performed in BALB/c mice (Liu et al., 2014). In comparison to our study in Mongolian gerbils, the colonization density was also the highest in the antrum, but inflammation was rather observed in the fundus of the mouse stomach, which highlights that host-differences may play a role in the disease outcome of an *H. heilmannii*

infection. In cats and dogs, the pathogenesis of *H. heilmannii* infections remains to be further elucidated, since no experimental infection studies with pure cultures of this microorganism have been performed in these animals so far. In general, *Helicobacter* species are mainly found in the fundus of the stomach of cats and dogs, although they are able to colonize all stomach regions (Diker et al., 2002; Gombač et al., 2010; Happonen et al., 1996; Simpson et al., 1999). The antrum-dominant chronic active gastritis caused by virulent *H. heilmannii* strains in the gerbil stomach was characterized by a massive lymphocyte infiltration in the lamina propria and submucosa, resulting in the formation of lymphoid follicles. Furthermore, a loss of parietal cells in the antrum was detected and an antral epithelial cell proliferation was observed. Similar findings have been described in the stomach of gerbils experimentally infected with *H. felis* and *H. bizzozeronii* (Court et al., 2002; De Bock et al., 2006a, De Bock et al., 2006b). These gastric lesions thus seem to be characteristic for canine and feline NHPH infections in gerbils.

No inflammation was seen in the fundus of the stomach of the *H. heilmannii*-infected gerbils. In contrast, in *H. pylori*-infected gerbils, inflammation and gastric lesions can occur both in the fundus and the antrum of the stomach. The fundus-dominant gastritis after *H. pylori* infection in Mongolian gerbils has been shown to be dependent on a functional *cag* PAI (Rieder et al., 2005; Wiedemann et al., 2009). Therefore, the lack of *cag* PAI in the genomes of NHPH might explain the absence of inflammation in the fundus of the gerbil stomach. However, it should be noted that the role of *cag* PAI in a fundus-dominant gastritis seems to be host-related, since *H. heilmannii* infection in mice mainly causes inflammation in the fundus of the stomach (Liu et al., 2014).

Although gastritis associated with NHPH infections is usually milder than gastritis associated with *H. pylori*, the risk of developing gastric MALT lymphoma is considered to be higher in NHPH-infected patients, compared to those infected with *H. pylori* (Morgner et al., 2000; Stolte et al., 2002). This finding has also been confirmed in several rodent models. For instance, in the stomach of Mongolian gerbils colonized with the porcine *H. suis* for 8 months, MALT lymphoma-like lesions have been demonstrated (Flahou et al., 2010). Also longterm *H. felis* and *H. heilmannii* infections in BALB/c mice can lead to the development of gastric MALT lymphoma-like lesions (Enno et al., 1995; Liu et al., 2014). MALT lymphoma is typically characterized by an extensive proliferation of B-lymphocytes in the center of large lymphoid aggregates of mononuclear and/or polymorphonuclear cells (Flahou et al., 2010). In our study, MALT lymphoma-like lesions were not observed, but lymphoid aggregates were formed in the antrum of the stomach at 9 weeks after infection with virulent *H. heilmannii* strains. It remains

to be determined if these lymphoid follicles would evolve into MALT lymphoma-like lesions after long-term colonization with *H. heilmannii*.

The innate immune response towards an *H. pylori* infection is characterized by the secretion of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α by gastric epithelial cells, which leads to the recruitment of neutrophils, dendritic cells and monocytes/macrophages (Robinson et al., 2007). Activated macrophages and dendritic cells in their turn produce IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α and activate naïve T-helper (Th) cells to differentiate into Th1 cells (Amedei et al., 2006; Gobert et al., 2004; Kim et al., 2013; Maeda et al., 2001; Mandell et al., 2004; Rad et al., 2007; Rad et al., 2009). Upregulation of IL-1β and TNF-α mRNA has recently been demonstrated in BALB/c mice after an infection with *H. heilmannii* (Liu et al., 2016). This is in agreement with our study in Mongolian gerbils, in which an increase of IL-1β expression was shown after 9 weeks of *H. heilmannii* infection. Also experimental *H. suis* infection in Mongolian gerbils is accompanied by elevated expression levels of IL-1β (Bosschem et al., 2016; Liang et al., 2015; Zhang et al., 2015). This pro-inflammatory cytokine thus seems to play a key role in the innate immune response towards a gastric *Helicobacter* infection.

The adaptive immune response towards an *H. pylori* infection is typically dominated by a mixed Th1/Th17 phenotype, and these T helper cells produce IFN-γ and IL-17, respectively (Shi et al., 2010). In Mongolian gerbil models of *H. pylori* infection, an upregulation of IFN-γ and IL-17 indeed has been confirmed (Sugimoto et al., 2009; Wiedemann et al., 2009; Yamaoka et al., 2005). On the contrary, the immune response following a NHPH infection remains to be further characterized and the available information is still scarce. Since MALT lymphoma is a typical feature of NHPH infections and the proliferation of B-lymphocytes is dependent on Th2-cytokines (Greiner et al. 1997; Knörr et al., 1999), it is likely that a Th2-response plays a role in the pathogenesis of NHPH infections. Indeed, in different mouse models of *H. suis* infection, a Th2/Th17-polarized response has been described, in contrast to the Th1/Th17 response following an *H. pylori* infection (Flahou et al., 2012; Vermoote et al., 2012). Based on the findings from a recent report, it is also suggested that infection with *H. heilmannii* in mice triggers a Th2 response rather than a Th1 response (Liu et al., 2016). On the contrary, an infection with another feline species, *H. felis*, has been shown to be more similar to an *H. pylori* infection and causes a predominant Th1/Th17 immune response in mice (Ding et al., 2013; Hitzler et al., 2012). Remarkably, recent *H. suis* infection studies in Mongolian gerbils, using different *H. suis* isolates from pigs and non-human primates, showed increased expression levels of IFN-γ and IL-17 and the absence of upregulated expression of Th2 cytokines in

animals with MALT lymphoma-like lesions. These latter studies rather indicate a mixed Th1/Th17 phenotype following *H. suis* infection, and thus an adaptive immune response similar to that induced by *H. pylori* (Bosschem et al., 2016; Liang et al., 2015; Zhang et al., 2015). These findings are clearly in contrast to what has been described in the murine model for *H. suis* infections. Taken together, the roles of Th1-, Th2- and Th17-responses in the development of gastric MALT lymphoma after NHPH infection seem to be both host-dependent as well as dependent on the characteristics of the infecting species and strains.

Strain variation among the *H. heilmannii* species might also be relevant for human infections with this pathogen and for infections in its natural hosts as well. Indeed, the pathogenic significance of *Helicobacter* species in cats and dogs is assumed to be related to differences in virulence of the colonizing *Helicobacter* strains (Haesebrouck et al., 2009).

2. New insights into phylogeny and virulence-associated factors of feline *Helicobacter* isolates via genome-sequencing-based approaches

Nowadays, more and more studies are focusing on genome analysis to provide new insights into the colonization- and virulence factors of pathogenic bacteria. The knowledge of an organism's complete genetic make-up allows the identification of potential vaccine and antimicrobial targets, as well as candidate genes for diagnostic purposes (Weinstock, 2000). By annotating the finished genome sequences, the open reading frames (ORFs) are predicted, that possibly encode proteins. Using the BLAST program (Basic Local Alignment Search Tool), the predicted coding sequences can be compared to sequences in databases such as GenBank, in order to identify matches to known genes (Altschul et al., 1990; Altschul et al., 1997). Moreover, by using comparative genomics, the genomic variability and plasticity as well as the phylogenetic relationships of different strains and/or species can be mapped. Genome comparison of two or more closely related species results in lists of genes that are common or different between these species. In addition, comparing two or more different strains of the same species can provide insight into the presence (or absence) of strain-specific virulence genes (Alm et al., 1999; Weinstock, 2000).

In **chapter 2** of this thesis, we used a genome-sequencing-based approach to determine if the variation in virulence between the 9 different feline *H. heilmannii* s.s. strains in the *in vivo* Mongolian gerbil model (**chapter 1**) might be related to the presence or absence of specific virulence-associated genes. The genomes of the 9 isolates were sequenced and their phylogenetic relationship was investigated. Via phylogenetic analysis and the use of several

bio-informatics tools, we showed that the 4 isolates with a lower virulence in Mongolian gerbils actually do not belong to the *H. heilmannii* species, but to a new species which we named *H. ailurogastricus*. Morphologically these two closely related *Helicobacter* species are very similar. Using TEM, we detected only slight differences in the number of spiral turns and in the number of bipolar flagella. Also the biochemical characteristics of *H. ailurogastricus* are very similar to those of *H. heilmannii*, the only difference being that this new species presents alkaline phosphatase activity, which is absent in *H. heilmannii*. The phenotypical similarity contributes to the difficulty of distinguishing between the two species. Sequence analyses of the 16S and 23S rRNA genes have frequently been used to discriminate between the porcine *H. suis* and the group of canine and feline NHPH (Dewhirst et al., 2005; O'Rourke et al., 2004). The canine and feline NHPH, including *H. felis*, *H. bizzozeronii*, *H. salomonis* and *H. heilmannii*, however, share more than 98% sequence similarity among the 16S and 23S rRNA genes and thus cannot be distinguished using these genes (Hänninen et al., 1996; Jalava et al., 1997; O'Rourke et al., 2004; Paster et al., 1991). For discrimination between these feline and canine species, sequencing of the HSP60 gene, the urease A and B genes, and the *gyrB* gene are used (Hannula and Hänninen, 2007; Mikkonen et al., 2004; Neiger et al., 1998; O'Rourke et al., 2004). However, we showed that the discriminatory capacity of the urease A and B genes is not high enough to distinguish between *H. heilmannii* and the newly identified species *H. ailurogastricus*, indicating that these gene sequences should not be used for differentiation between closely related canine and feline NHPH. Therefore, whole genome sequencing should be recommended for studying phylogeny, since this gives a better resolution for distinguishing between closely related bacteria (Vandamme et al., 2014).

The annotated genomes of the *H. heilmannii* and *H. ailurogastricus* strains were compared in order to identify virulence-associated genes that are specific for *H. heilmannii*. The presence or absence of these genes in *H. pylori* and other gastric helicobacters was verified as well.

The *H. pylori* IceA1, HrgA and jhp0562-glycosyltransferase proteins have been reported to be involved in its pathogenesis (Ando et al., 2002; Pohl et al., 2012; Shiota et al., 2012; Xu et al., 2002). We showed that genes encoding homologs of these proteins are present in *H. heilmannii*, but absent in *H. ailurogastricus*. The absence of these virulence-associated factors might contribute to the lower virulence of *H. ailurogastricus*. However, it remains to be elucidated if these proteins, similar as for *H. pylori*, actually play a role in the disease outcome of an *H. heilmannii* infection. An experimental infection study in rodents with gene-deletion mutants, created via chromosomal insertion mutagenesis, might offer more insights into the contribution of these factors to the pathogenesis of infection caused by *H. heilmannii*. It should also be taken

into account that some of these virulence-associated factors exhibit only a limited sequence identity among the different *H. heilmannii* strains, and that this strain-diversity might lead to different disease outcomes (Suerbaum and Josenhans, 2007).

The *H. pylori* genome contains a 64 well-annotated outer membrane proteins (OMPs) (Alm et al., 2000). Most of its OMPs belong to the Hop and Hor family and function as porins or as adhesins that promote adhesion to the gastric epithelium (Oleastro and Ménard, 2013). Similar to *H. pylori*, we identified a large OMP repertoire in the *H. heilmannii* and *H. ailurogastricus* isolates, namely an average of 55 and 60 OMP-encoding genes, respectively. A striking finding was that both *H. heilmannii* and *H. ailurogastricus* genomes share only few homologs of the *H. pylori* Hop, Hor and Hom OMPs. Moreover, the well-studied *H. pylori* adhesins BabA, BabB, SabA, AlpA, AlpB, OipA, HopZ and HopQ are absent in *H. heilmannii* and *H. ailurogastricus*. The lack of these important *H. pylori* Hop adhesins has also been described in *H. bizzozeronii*, *H. felis* and *H. suis*, and our study thus confirms the absence of these OMPs in canine, feline and porcine NHPH. Only in *H. acinonychis* and *H. cetorum*, a few homologs of the *H. pylori* Hop adhesins are found (Arnold et al., 2011; Eppinger et al., 2006; Kersulyte et al., 2013; Schott et al, 2011; Vermoote et al., 2011). Genes encoding Hof OMPs are present in *H. heilmannii* and *H. ailurogastricus*, indicating that these proteins might play a role in adherence to the gastric mucosa. Indeed, a recent *in vitro* binding study of *H. heilmannii* to human gastric mucins resulted in an upregulated expression of all its *hof* genes, and also exposure of this bacterium to a neutral pH7 leads to an increased expression of *hofE*, *hofF* and *hofG*. These findings suggest a modified expression of the *H. heilmannii hof* genes as a response to contact with mucins and to the increasing pH conditions when the bacterium moves away from the acidic lumen of the stomach towards the gastric mucosa (Liu et al., 2016; Skoog et al., 2012). In addition, an experimental infection study with *H. heilmannii* deletion mutants in BALB/c mice clearly showed a role for HofE and HofF in adherence to the gastric mucosa (Liu et al., 2016). HofF is also involved in *H. pylori* colonization, but the function of the other *H. pylori* Hof OMPs remains to be investigated (Kavermann et al., 2003). In contrast to *H. pylori*, we and others found that the *hof* genes of canine, feline and porcine gastric NHPH are located in a ~ 10-kb locus that seems to be unique for these bacteria (Schott et al., 2011).

OMPs that are present in *H. heilmannii* but absent in *H. ailurogastricus* are of particular interest for studying the variation in colonization capacity between these 2 species. Our analysis predicted 6 unique putative OMPs in the *H. heilmannii* genome. Further studies are needed to characterize these proteins, to determine if they are expressed during an infection and if they

play a role in *H. heilmannii* colonization. Transcriptome studies and *in vitro* binding assays might offer more insight into the importance of these unique *H. heilmannii* OMPs.

The pore-forming outer membrane vacuolating cytotoxin A (VacA) is the most important secreted virulence factor of *H. pylori*, which is internalized into the host cell and leads to host cell apoptosis (Palframan et al., 2012). The *H. pylori* VacA cytotoxin belongs to an OMP family of autotransporters (Sause et al., 2012). This important cytotoxin is absent in most gastric NHPH species. An intact *vacA* homolog is only present in *H. cetorum* from dolphin and whales, and fragmented pseudogenes of *vacA* are found in *H. acinonychis* from wild felines (Dailidiene et al., 2004; Eppinger et al., 2006; Kersulyte et al., 2013). In addition, the *H. pylori* genome contains 3 *vacA*-like genes encoding large OMPs of 260-348 kDA, namely the immune-modulating autotransporter (ImaA), the flagella-associated autotransporter (FaaA) and the VacA-like protein C (VlpC). The C-terminal regions of these proteins show homology to the C-terminal region of VacA, which is a β-barrel domain that is required for secretion of VacA via an autotransporter pathway. Therefore, these 3 OMPs are predicted to be autotransporters as well (Fisher et al., 2001). In experimental mouse models, the expression levels of *imaA*, *faaA* and *vlpC* have been shown to be upregulated in the stomach during *H. pylori* colonization. Moreover, these autotransporters each enhance the capacity of *H. pylori* to colonize the mouse stomach (Radin et al., 2013; Sause et al., 2012). We showed that the genomes of *H. heilmannii* and *H. ailurogastricus* also contain a *vacA*-like gene which is also present among other canine, feline and porcine gastric NHPH. Autotransporter proteins of Gram-negative bacteria typically consist of 3 domains: an N-terminal signal peptide, which is required for secretion across the inner membrane, a passenger domain, and a C-terminal β-barrel domain, which facilitates translocation of the passenger domain across the outer membrane (Dautin et al., 2007; Radin et al, 2013). The passenger domain represents the surface-exposed component of the protein. Its structure is well-conserved among Gram-negative bacteria and consists of a right-handed parallel β-helical fold (Benz and Schmidt, 2011, Sause et al., 2012). On the other hand, passenger domains are extremely diverse in both sequence and function. A wide variety of functions related to pathogenesis have been described for the passenger domains of the autotransporters of Gram-negative bacteria, including adhesion, auto-aggregation, invasion, biofilm formation and cytotoxicity (Dautin et al., 2007; Henderson et al., 2004; Radin et al., 2013). In *H. pylori*, the passenger domain of the VacA-like autotransporters contains 3 VacA2 regions that show low similarity to the VacA toxin, but that do not correspond to a functional portion of VacA (Sause et al., 2012). Our *in silico* analysis showed a similar typical construction for both the VacA-like autotransporters of *H. heilmannii* and *H. ailurogastricus*, with the

exception that no known signal sequence was predicted in the N-terminal cytoplasmic tail. Similar to *H. pylori*, the passenger domain of the *H. heilmannii* VacA-like autotransporter contains 3 VacA2 regions. In contrast, the passenger domain of *H. ailurogastricus* was remarkably larger than that of *H. heilmannii* and contains 4 VacA2 regions. Since *H. ailurogastricus* has a lower colonization capacity in Mongolian gerbils (**chapter 1**), the size of the passenger domain possibly plays a role in gastric colonization. Since we were not able to predict a known N-terminal signal sequence, which is required for secretion across the inner membrane, it remains to be investigated if the VacA-like autotransporters of both species are functional and if they are expressed during colonization.

Whole genome sequencing and genome comparison of *H. heilmannii* and *H. ailurogastricus* enabled the detection of genes homologous to *H. pylori* virulence-associated genes. Studying the genetic diversity of different isolates belonging to one species might offer more insights whether certain genotypes are more often associated with disease than others. Therefore, it would be interesting to extend these comparative studies to all known gastric NHPH and to more isolates of each species.

3. New insights into the adhesion capacity of *H. heilmannii* s.s. and *H. ailurogastricus* to the gastric mucosa

In **chapter 2** of this thesis, we further investigated if the differences in colonization capacity and pathogenicity between *H. heilmannii* and *H. ailurogastricus*, as described in our *in vivo* study in Mongolian gerbils in **chapter 1**, are also reflected in their capacity to adhere to the gastric mucosa. Therefore, several *in vitro* binding assays were performed.

The mucus layer covering the human gastric mucosa is the first barrier that gastric pathogens encounter. It mainly consists of secreted mucins and trefoil factors. The secreted mucins are continuously produced in small amounts by gastric epithelial cells at the mucosal surface and in the glands. In response to pathogenic stimuli, the production of mucins is elevated (Lindén et al., 2002; McGuckin et al., 2011). Besides these secreted mucins, also membrane-associated mucins, expressed by surface and glandular epithelial cells, provide a barrier against pathogens. In the stomach of humans, cats and dogs, the membrane-associated MUC1 and the secreted MUC5AC and MUC6 are the most common mucins. MUC1 and MUC5AC are expressed and secreted by the surface epithelium, respectively, whereas MUC6 is secreted by the gastric glands (Atuma et al., 2001; Lacunza et al., 2009; Lindén et al., 2002; McGuckin et al., 2011). These gastric mucins carry on the order of 100 different carbohydrate structures that can function as receptors for microorganisms (Klein et al., 1993). *H. pylori* is able to interact with

epithelial cells that produce MUC1 and MUC5AC, suggesting that these 2 mucins play a role in colonization of the gastric mucosa (Lindén et al., 2002; Lindén et al., 2009). Interestingly, the expression of gastric mucins and their distribution in the stomach alter during the disease progress of an *H. pylori* infection (Lindén et al., 2002; Schmitz et al., 2009; Wang and Fang, 2003). In a recent experimental infection study in BALB/c mice, a clear upregulation of the murine Muc6 was detected in the stomach of both *H. heilmannii*- and *H. pylori*-infected animals in the first 9 weeks post infection (Liu et al., 2014). In addition, Muc5B was expressed at 9 weeks post infection and Muc13 expression was increased already from day 1 until 9 weeks post infection. In humans, MUC5B is normally not expressed in a healthy stomach and MUC13 only at a very low level. On the contrary, the presence of MUC13 and MUC5B have been described in gastric cancer, indicating a role for these proteins in the *Helicobacter*-induced pathology (Babu et al., 2006; Pinto-de-Sousa et al., 2004; Sheng et al., 2011). In our study, we tested the *in vitro* binding of *H. heilmannii* and *H. ailurogastricus* isolates to 2 different human gastric mucin samples, one derived from a healthy stomach and one derived from a patient with a gastric tumor. These samples are thus expected to express different mucins. We observed similar binding capacities to both mucin samples, but in general, the binding capacities of *H. heilmannii* and *H. ailurogastricus* were low. A possible explanation is that the mucin samples used lacked specific carbohydrate receptors for *H. heilmannii* and *H. ailurogastricus*. Nevertheless, we noted a trend toward higher binding of *H. heilmannii* than *H. ailurogastricus* to the gastric mucins and a significantly higher binding capacity of *H. heilmannii* s.s. to DNA (a marker for negative charge). In addition, binding to DNA was 10-fold higher at pH2 than at pH7, indicating a possible role for a charge/low-pH-dependent binding mechanism of *H. heilmannii* s.s. to mucins, a phenomenon that also has been described for *H. pylori* (Lindén et al., 2008).

Once in the mucus layer, gastric helicobacters attach to the gastric epithelium (Odenbreit, 2005). This initial pathogen-host interaction is crucial for colonization of the stomach (Galdiero et al., 2012). We tested the *in vitro* binding capacities of *H. heilmannii* and *H. ailurogastricus* to paraffin-embedded gastric tissue samples from Mongolian gerbils and cats. A clear difference in binding specificity between both species was demonstrated. *H. heilmannii* bound mainly to the glandular cells of both the antrum and the corpus of the stomach. As described above, MUC6, which is secreted by the gastric glands, is changed in expression during *H. heilmannii* infection and might thus play a role in the early colonization phase of *H. heilmannii* (Liu et al., 2014). In contrast, *H. ailurogastricus* had a higher binding capacity to the surface epithelial cells lining the gastric mucosa, which is more similar to binding of *H. pylori* (Lindén

et al., 2002). Based on our *in vitro* binding experiments, *H. heilmannii* also had a higher binding capacity to epithelial cells than *H. ailurogastricus*. It can thus be suggested that *H. heilmannii* binds more easily to gastric epithelial cells and glandular cells than *H. ailurogastricus*, with a preference for glandular cells when both cell types are present. The level of binding of *H. heilmannii* s.s. bacteria to gastric epithelial cells was higher at pH7 than at pH2. This is in agreement with the physiological pH gradient in the stomach, which is approximately pH2 in the gastric lumen and pH7 at the epithelial cell surface (Allen and Flemstrom, 2005; Bhaskar et al., 1992; O'Toole et al., 2005). Gastric helicobacters actively move away from the acidic lumen of the stomach towards the neutral pH environment of the gastric epithelium. As a response to this neutral pH, their motility decreases, which possibly optimizes the attachment to the gastric epithelial cells (Croxen et al., 2006).

Taken together, our *in vitro* binding assays confirm the results from our *in vivo* study in Mongolian gerbils (**chapter 1**) and underline the higher colonization capacity of *H. heilmannii* compared to *H. ailurogastricus*.

4. New insights into the outer membrane protein repertoire of *H. heilmannii* s.s., *H. ailurogastricus* and other NHPH species

Our comparative genomic studies (**chapter 2**) and other recent research highlight that the Hop OMPs of *H. pylori*, that function as adhesins during colonization, are commonly absent in NHPH. In addition, these species also share only few homologs of the *H. pylori* Hor and Hom OMP family (Arnold et al., 2011; Schott et al., 2011; Smet et al., 2013; Vermoote et al., 2011). These findings indicate that NHPH harbor a different OMP repertoire than *H. pylori* and the mechanisms by which these NHPH colonize the gastric mucosa are largely unknown. In **chapter 3** of this thesis, we screened the genomes from all gastric NHPH known so far for the presence of genes encoding OMPs. For comparison, the genomes of 4 enterohepatic *Helicobacter* species, 2 *Campylobacter* species and *Escherichia coli* were included. The identified OMPs were classified into families based on their sequence homology and their phylogenetic relationships were investigated.

A first remarkable finding was that all gastric helicobacters harbor proportionally more OMPs than the other Gram-negative bacteria included in our study. Three to 4 % of the *Helicobacter* genomes encode for OMPs. Most OMPs of *H. pylori* function as porins or as adhesins that promote binding to the gastric epithelium (Oleastro and Ménard, 2013). However, Gram-negative bacteria commonly use pili for adhesion to host cells. Despite the diversity in structure and biogenesis, pili are typically formed by non-covalent homopolymerization of pilins, the

subunit proteins of the pili (Proft and Baker, 2009). In an acidic environment, such as in the stomach, a polymeric pilus structure would likely depolymerize. This is a possible explanation for the large OMP repertoire of gastric helicobacters and their use of OMPs as adhesins (Alm et al., 2000). The only pilus structure, described for *H. pylori*, is the type IV secretion system (T4SS). T4SS is encoded by the *cag* PAI and delivers the CagA oncoprotein into gastric epithelial cells. T4SS forms a needle-like pilus that is assembled by multiple protein-protein interactions and various pilus-associated factors (Backert et al., 2015). However, this pilus structure is only induced after the first contact with the host epithelium, where a neutral pH prevails (Allen and Flemstrom, 2005).

We identified several well-conserved OMPs with a possible function in colonization or bacterial virulence among the *Helicobacter* and *Campylobacter* genera and *E. coli*. These include TonB-dependent receptors with a function in iron uptake, an outer membrane factor involved in antimicrobial resistance and an outer membrane phospholipase that plays a role in colonization. Imp/OstA, a porin involved in antimicrobial resistance, was found only in *Campylobacter* and *Helicobacter*.

Specific for the genus *Helicobacter* and primarily in gastric species, we identified SfpA/LpxR that functions in immune evasion, two *Helicobacter*-specific outer membrane porin families with probable functions in adhesion, and a *Helicobacter*-specific VacA-like cytotoxin with a possible role in colonization as highlighted above. The two *Helicobacter*-specific outer membrane porin families are of great value for gastric *Helicobacter* research. A significant part of all OMPs of gastric helicobacters clustered into these families. The first *Helicobacter* outer membrane porin family contained the largest number of OMPs, including the *H. pylori* Hop adhesins BabA (HopS), SabA (HopP), AlpA (HopC) and AlpB (HopB), OipA (HopH), HopZ, HopQ, LabA (HopD). We here confirmed the absence of these adhesins in almost all NHPH. Orthologs of some of these Hop adhesins were only found in *H. acinonychis* and *H. cetorum*, the only 2 species that also contain the VacA cytotoxin. This is not surprising since they are the closest relatives of *H. pylori* (Eppinger et al., 2006; Harper et al., 2002). The *H. pylori* HorA, HopK, HopJ, HorC, HorH, HorI, HorB, HorJ, HorF, HorK, HopE, HorE, HorM, HorD, HorG, HomA, HomB, HomC and HomD OMPs also belong to this porin family. HorB and HomB might also function as adhesins, but the function of these other OMPs remain largely unknown. For most of these proteins, we identified homologous putative OMPs in *H. acinonychis*, *H. cetorum* and in all porcine, feline and canine NHPH, indicating that they are well-conserved and probably important for the genus *Helicobacter*. A last subgroup belonging to the first porin family contained putative OMPs that were specific for canine, feline and porcine NHPH. The

finding of a unique porin repertoire, together with the absence of the *H. pylori* Hop adhesins, suggests that canine, feline and porcine NHPH use other OMPs to colonize the gastric mucosa and that their pathogenesis is different from *H. pylori*.

Further research is needed to determine the importance of these unique putative porins during NHPH colonization. First, transcriptomic studies are needed to examine if the putative porins are expressed. Next, *in vitro* binding assays, followed by RNA extraction and reverse transcription quantitative PCR can be used to investigate if the expression levels of these OMPs are elevated during the adhesion process. A last valuable approach would be to study the individual roles of these porins by creating gene-deletion mutants that can be used in *in vitro* binding experiments and in *in vivo* infection studies in rodents.

The second *Helicobacter*-specific porin family is composed of the *H. pylori* HofA, HofB, HofC, HofD, HofE, HofF, HofG and HofH proteins. We showed that these 8 Hof OMPs were highly conserved among canine, feline and porcine NHPH, *H. acinonychis* and *H. cetorum*. The functions of most of these Hof proteins are currently unknown with only HofE and HofF being involved in gastric colonization (Kavermann et al., 2003; Liu et al., 2016). However, *Helicobacter*-specific OMPs that are well-conserved among gastric species, such as the Hof proteins, might be of great interest for vaccine development studies. OMPs are considered effective antigens to stimulate immune responses because they are exposed on the bacterial surface and easily recognized by the host immune system (Okamura et al., 2012). Immunization using a well-conserved OMP thus might offer a broad range of protection against infections with gastric *Helicobacter* species. The ultimate goal of such vaccination trials would be to reach protection against all gastric *Helicobacter* species by the use of one single vaccine.

A final interesting finding of our third study was the identification of various species-specific OMP families in only canine and feline NHPH. As described above, they also contain a unique putative porin repertoire with only homologs in the porcine *H. suis*. Although the function and the importance of all these putative proteins remain to be elucidated, it is clear that helicobacters from cats and dogs harbor several OMPs that are absent in other *Helicobacter* species. One might speculate that these unique OMPs are involved in the colonization of their 3 specific hosts; cats, dogs and humans.

5. General conclusion

Gastric NHPH bacteria that infect the stomach of animals and humans have been associated with several gastric pathologies in humans. We showed that *H. heilmannii*, originating from cats and dogs, colonizes the stomach and induces an antrum-dominant chronic active gastritis

in Mongolian gerbils, an *in vivo* model for human gastric disease. The access to bacterial genomes together with the availability of increasing numbers of *in vitro* isolates allows significant advances in the understanding of species-specific bacteria-host interactions involved in disease pathogenesis and will be essential for the future development of strategies to prevent and treat *Helicobacter* infections. Via comparative genomics, we identified several possible virulence-associated factors in *H. heilmannii*. In addition, we described the new feline species *H. ailurogastricus*, that is both *in vitro* and *in vivo* less virulent than its closest relative *H. heilmannii*. Finally, we characterized the OMP repertoire of all gastric NHPH, which will be useful for future *in vitro* and *in vivo* colonization studies.

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SUMMARY

Helicobacter (H.) pylori is the most prevalent *Helicobacter* species colonizing the gastric mucosa of humans and has been associated with gastritis, peptic ulcer disease and gastric cancer. This slightly-curved, Gram-negative and microaerophilic gastric bacterium is highly adapted to the human host and has succeeded to colonize more than 50% of the human population worldwide. Besides *H. pylori*, other spiral-shaped helicobacters, naturally colonizing the stomach of animals, have also been detected in the stomach of humans. These non-*H. pylori Helicobacter* (NHPH) species have been associated with gastritis, gastric and duodenal ulcers and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma in humans. Moreover, the risk of developing MALT lymphoma is considered to be higher after infection with NHPH than with *H. pylori*. NHPH represents a group of closely related but distinct *Helicobacter* species that are found in different animal species, such as *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. heilmannii* sensu stricto (s.s.), *H. cynogastricus* and *H. baculiformis* in cats and dogs and *H. suis* in pigs. NHPH bacteria have an extremely fastidious nature, which so far has resulted in a limited number of *in vitro* isolates available worldwide.
In 2011, our research group was the first to successfully isolate *H. heilmannii* s.s. from the feline stomach and cultivate this species *in vitro*. The general aims of this PhD research were to obtain better insights into the pathogenesis of human gastric disease associated with *H. heilmannii* s.s. infection, to determine the possible virulence-associated factors of this microorganism, and to compare its outer membrane protein repertoire with other NHPH.

In **chapter one,** we used an *in vivo* Mongolian gerbil model to study the bacterium-host interactions of 9 different feline *H. heilmannii* s.s. strains, identified on the basis of their 16S rRNA and *ureAB* genes. At 9 weeks after experimental infection, the colonization levels in the stomach, the intensity of the induced gastritis, the rate of gastric epithelial cell alterations and the expression level of the peptide hormone gastrin were determined. In addition, the immune response following *H. heilmannii* s.s. infection was characterized by measuring the expression levels of various inflammatory cytokines in the stomach.
We showed the induction of an antrum-dominant chronic active gastritis with formation of lymphocytic aggregates after infection with 7 out of 9 *H. heilmannii* s.s. strains. In addition, a high number of proliferating epithelial cells was seen in the antrum of the gerbil stomach. After infection with the 2 other strains, no explicit antral inflammation and no increased gastric epithelial cell proliferation were observed. In all *H. heilmannii* s.s.-infected gerbils, only limited signs of inflammation were detected in the fundus of the stomach, and the epithelial cell proliferation rate was not elevated in this stomach region.

With quantitative PCR, a high-level antral colonization was revealed for 4 *H. heilmannii* s.s. strains, while colonization of 4 other strains was more restricted and one strain was not detected in the stomach at 9 weeks post infection. In general, the colonization capacity in the fundus was lower than in the antrum for all strains tested and the lowest number of bacteria was detected in the duodenum. A reduced expression of the gastric hydrogen potassium (H^+/K^+) ATPase, functioning as a proton pump in gastric acid-secreting parietal cells, was detected in the antrum of the stomach after infection with 3 highly colonizing *H. heilmannii* s.s. strains. This indicates a possible loss of parietal cells and a reduced gastric acid secretion. However, no significant change in H^+/K^+ ATPase expression was seen in the fundus, where the majority acid-secreting parietal cells are located. Two highly colonizing strains caused an increased expression of the peptide hormone gastrin in the fundus of the stomach, which may lead to hypergastrinemia. Although the gastrin-producing G-cells are mainly located in the antrum of the stomach, no antral upregulation of gastrin expression was detected.

All *H. heilmannii* s.s. strains that induced a chronic active gastritis, caused an upregulation of IL-1β in the antrum. This pro-inflammatory cytokine is a potent inhibitor of gastric acid secretion and plays a role in the acute phase of inflammation. Remarkably, there was no upregulation of typical Th1 or Th2 cytokines in the stomach of the gerbils at 9 weeks after *H. heilmannii* s.s. infection.

In conclusion, our experimental infection study in Mongolian gerbils indicates diversity in bacterium-host interactions and virulence between 9 different *H. heilmannii* s.s. strains. Based on the differences in colonization capacity and the level of antral inflammation, 5 strains were shown to be highly virulent. The 4 other strains were low virulent. Since the Mongolian gerbil model is considered to be a good model for human *Helicobacter*-induced pathology, this strain variation is most probably also relevant for human infections with this microorganism and might be important for infections in the natural hosts of *H. heilmannii* s.s. as well.

In **chapter 2,** we investigated if the differences in colonization capacity and virulence properties between the 9 *H. heilmannii* strains were related to the presence/absence of specific virulence-associated genes. Therefore, the genomes of the strains were sequenced to investigate their phylogenetic relationships and to define their gene content and diversity. In addition, several *in vitro* binding assays were carried out to determine possible strain differences in the capacity for adhesion to the gastric mucosa.

Via phylogenetic analysis and the use of several bio-informatics tools, we showed that the 4 isolates with a lower virulence in Mongolian gerbils actually do not belong to the *H. heilmannii*

species, but to a new, closely-related species which we named *H. ailurogastricus*. This new *Helicobacter* species cannot be distinguished from *H. heilmannii* by means of its 16S rRNA and *ureAB* gene sequences. In addition, we showed that these feline *Helicobacter* species are morphologically very similar, with the exception that *H. heilmannii* has more spiral turns and bipolar flagella than *H. ailurogastricus*. Also the biochemical characteristics of *H. ailurogastricus* were very similar to those of *H. heilmannii*, with the only difference that this new species presents alkaline phosphatase activity, which is absent in *H. heilmannii*. We found that several homologs of *H. pylori* virulence-associated factors are present in *H. heilmannii* but absent in *H. ailurogastricus*. These include the ulcer-associated protein IceA1; the HrgA protein, which is part of a DNA restriction-modification system in virulent *H. pylori* strains; and a jhp0562-like glycosyltransferase that is involved in immune evasion and peptic ulcer development. Possibly, these factors contribute to the more virulent character of *H. heilmannii* compared to *H. ailurogastricus*.

Similar to *H. pylori*, our analysis revealed a large outer membrane protein (OMP) repertoire in both *H. heilmannii* and *H. ailurogastricus*. However, both feline species shared only a few homologs of the *H. pylori* Hop, Hor, and Hom proteins and lacked all *H. pylori* adhesins described so far. Interestingly, *H. heilmannii* harbored 6 putative OMPs that are absent in *H. ailurogastricus*. These OMPs might play a role in the colonization process of *H. heilmannii*. Furthermore, we identified a homologous gene of the *H. pylori* VacA-like autotransporter, which enhances the capacity to colonize the stomach, in both *H. heilmannii* and *H. ailurogastricus*. In *H. pylori*, the passenger domain of the VacA-like autotransporter, which confers the effector function of this protein, contains three VacA2 regions. The passenger domains of the VacA-like autotransporters of *H. heilmannii* also contained three VacA2 regions, whereas in *H. ailurogastricus*, this domain contained four VacA2 regions. Since *H. ailurogastricus* has a lower *in vivo* colonization ability than *H. heilmannii*, the size of the passenger domain might play a role in gastric colonization.

The differences in *in vivo* colonization capacity between *H. heilmannii* and *H. ailurogastricus* were also reflected in their *in vitro* capacity to adhere to the gastric mucosa. We noted a trend towards higher binding of *H. heilmannii* to human gastric mucins than *H. ailurogastricus*. *H. heilmannii* also had a higher ability to bind human- and mouse-derived gastric epithelial cells. Additionally, *H. heilmannii* bound mainly to the glandular cells of both the antrum and the corpus of the stomach, whereas *H. ailurogastricus* had a higher binding capacity to the surface epithelial cells lining the gastric mucosa.

In conclusion, we described a new feline gastric species *H. ailurogastricus*, which is closely related to *H. heilmannii*. *H. ailurogastricus* lacks several homologs encoding *H. pylori* virulence and colonization factors and has a lower capacity for binding to gastric epithelial cells *in vitro*. This may explain why its virulence is lower than that of *H. heilmannii*.

The outer membrane of *H. pylori* is equipped with a large set of OMPs and several of these proteins are involved in colonization of the human gastric mucosa. In contrast, it is largely unknown which OMPs play a role in the colonization process of *H. heilmannii*, *H. ailurogastricus* and other gastric NHPH. These species lack all important *H. pylori* Hop adhesins, indicating that other OMPs are used for adhesion to the gastric mucosa.

In **chapter 3** of this thesis, we characterized the OMP repertoire of gastric NHPH by using phylogenetic analyses and we identified the OMP families that are possibly involved in their colonization and virulence properties.

Similar to *H. pylori*, we found that gastric NHPH harbor proportionally more OMPs than other Gram-negative bacteria, which might be the result from an adaptation to the harsh gastric environment.

Several well-conserved OMP families, also present in *Campylobacter*, *E. coli* and enterohepatic helicobacters, and with a possible function in colonization or bacterial virulence, were identified in gastric NHPH. These include TonB-dependent receptors with a function in iron uptake, an outer membrane factor involved in antimicrobial resistance and an outer membrane phospholipase that plays a role in colonization. A porin Imp/OstA, involved in antimicrobial resistance, was well-conserved among the *Campylobacter* and *Helicobacter* genera. Interestingly, several OMP families were detected only in *Helicobacter* and primarily in gastric species. These included SfpA/LpxR that functions in immune evasion, two *Helicobacter*-specific outer membrane porin families with probable functions in adhesion, and a *Helicobacter*-specific VacA-like cytotoxin (characterized in **chapter 2**) with a role in colonization capacity. A significant part of all OMPs of gastric NHPH clustered into the two *Helicobacter*-specific porin families. The first porin family contained the largest number of OMPs, including the *H. pylori* Hop adhesins that are absent in NHPH, except for *H. acinonychis* and *H. cetorum*. The *H. pylori* Hor, Hom and other Hop OMPs also belong to this porin family, and a few homologs of these proteins were present in other gastric NHPH. A last subgroup contained putative OMPs that were specific for canine, feline and porcine NHPH. The finding of a unique porin repertoire, together with the absence of the *H. pylori* Hop adhesins, indicates

that gastric NHPH use other OMPs to colonize the gastric mucosa and that their pathogenesis is different from *H. pylori*.

The second *Helicobacter*-specific porin family was composed of the 8 *H. pylori* Hof OMPs that were highly conserved among gastric NHPH. In contrast to *H. pylori*, the *hof* genes of NHPH were located in a large unique locus. The function of most of these Hof proteins are currently unknown with only HofE and HofF being involved in gastric colonization. Interestingly, various species-specific OMP families were found in canine and feline NHPH, that are absent in other *Helicobacter* species. The function and the importance of these putative proteins remain to be elucidated.

In conclusion, we showed that *H. heilmannii* s.s., isolated from the feline stomach, is capable of inducing gastric disease in Mongolian gerbils, an *in vivo* model for human gastric pathology. In addition, we described a new feline species *H. ailurogastricus* that is closely related to *H. heilmannii*. By using both *in vitro* and *in vivo* studies, we showed that *H. ailurogastricus* is less virulent than *H. heilmannii*. We identified several virulence-associated factors in *H. heilmannii* that might be related to the difference in pathogenicity between these two feline *Helicobacter* species. Finally, we characterized the OMP repertoire of all gastric NHPH, which will be of great value for future *in vitro* and *in vivo* colonization studies.

SAMENVATTING

Helicobacter pylori is de meest voorkomende gastrale *Helicobacter* species die van nature de maag van de mens koloniseert en geassocieerd wordt met gastritis, maagulcera en maagkanker. Deze Gram-negatieve, microäerofiele bacterie is zeer goed aangepast aan zijn gastheer en koloniseert de maag van meer dan 50% van de bevolking wereldwijd. Naast *H. pylori*, die er uitziet als een licht gebogen staafje, kunnen er ook langere, spiraalvormige helicobacters aangetroffen worden in de maag van mensen. Deze zogenaamde niet-*H. pylori Helicobacter* species (NHPH) zijn afkomstig van dieren en werden reeds geassocieerd met gastritis, maagulcera en mucosa-geassocieerde-lymfoïd-weefsel (MALT) lymfomen bij de mens. Bovendien is het risico op het ontwikkelen van MALT-lymfomen hoger na een infectie met gastrale NHPH dan met *H. pylori*. NHPH omvatten een groep van nauwverwante maar verschillende *Helicobacter* species die worden teruggevonden in verschillende diersoorten. Zo koloniseren *H. felis, H. salomonis, H. bizzozeronii, H. heilmannii* sensu stricto (s.s.), *H. cynogastricus* en *H. baculiformis* de maag van hond en kat, terwijl *H. suis* voornamelijk wordt teruggevonden in varkens. Gastrale NHPH zijn bijzonder moeilijk te kweken in het laboratorium, waardoor er wereldwijd maar weinig *in vitro* isolaten beschikbaar zijn. *H. heilmannii* s.s. werd in 2011 voor het eerst succesvol geïsoleerd uit kattenmagen en *in vitro* in cultuur gebracht. Dit doctoraatsonderzoek had tot doel om een beter inzicht te verwerven in de pathogenese van maaginfecties met *H. heilmannii* s.s., om de mogelijke virulentiefactoren van *H. heilmannii* s.s. in kaart te brengen en om het repertoire aan buitenste membraanproteïnen van deze kiem te vergelijken met deze van andere gastrale *Helicobacter* species.

In **hoofdstuk 1** werden de kiem-gastheer interacties *in vivo* bestudeerd voor negen verschillende *H. heilmannii* s.s. stammen, die geïdentificeerd werden op basis van de sequentie van hun 16S rRNA en *ureAB* genen. Hierbij werd gebruik gemaakt van een Mongools gerbilmodel. Negen weken na de experimentele infectie werd nagegaan in welke mate deze stammen de maag konden koloniseren. Ook de intensiteit van de geïnduceerde gastritis, de mate van epitheliale celproliferatie en het expressieniveau van het peptide-hormoon gastrine werden bestudeerd. De immuunrespons werd gekarakteriseerd door de expressieniveaus van verschillende inflammatoire cytokines te meten.

Na infectie met zeven van de negen *H. heilmannii* s.s. stammen ontwikkelden de gerbils een chronische, actieve gastritis, die voornamelijk in het antrum van de maag teruggevonden werd. Bij histologisch onderzoek van dit deel van de maag werden lymfocytaire aggregaten en een hoger aantal prolifererende epitheelcellen gedetecteerd. Deze effecten werden niet waargenomen bij de twee overige stammen. In de fundus werden er enkel beperkte tekenen van

inflammatie opgemerkt na een infectie met elk van de negen *H. heilmannii* s.s. stammen. In deze regio van de maag werd er ook geen verhoogde epitheliale celproliferatie waargenomen. Met kwantitatieve PCR werd er een hoge kolonisatiegraad aangetoond in het antrum van de maag na infectie met vier *H. heilmannii* s.s. stammen. De kolonisatiegraad van vier andere stammen was eerder beperkt en één stam kon niet gedetecteerd worden op negen weken na de experimentele infectie. Het kolonisatieniveau was voor alle stammen hoger in het antrum dan in de fundus van de maag en in het duodenum werd het laagste aantal bacteriën gedetecteerd. Een verlaagde expressie van het waterstof-kalium (H^+/K^+) ATPase - een protonpomp in de maagzuur-producerende pariëtale cellen - kon enkel gedetecteerd worden in de antrale regio van de maag na infectie met drie *H. heilmannii* s.s. stammen die de maag in hoge mate koloniseerden. Dit zou kunnen wijzen op een verlies aan pariëtale cellen en een gereduceerde maagzuursecretie. Er werd echter geen verlaagde expressie van het H^+/K^+ ATPase aangetoond in de fundus van de maag, waar de meeste zuur-secreterende pariëtale cellen gelocaliseerd zijn. Daarnaast veroorzaakten twee sterk koloniserende *H. heilmannii* s.s. stammen ook een verhoogde expressie van het peptide-hormoon gastrine in de fundus regio. Dit zou kunnen leiden tot een hypergastrinemie. De gastrine-producerende G-cellen zijn echter hoofdzakelijk gelocaliseerd in het antrale gedeelte van de maag, waar geen verhoogde gastrine-expressie werd gedetecteerd.

De *H. heilmannii* s.s. stammen die geassocieerd werden met een chronische, actieve gastritis, veroorzaakten een stijging van IL-1β in het antrum van de maag. Dit pro-inflammatoire cytokine is een krachtige remmer van de maagzuursecretie en speelt een rol in de acute fase van inflammatie. Er werd geen verhoogde expressie van T helper (Th)1 of Th2 cytokines gedetecteerd in de maag van de gerbils die geïnfecteerd waren met *H. heilmannii* s.s. In deze studie konden er duidelijke verschillen in kiem-gastheer interacties en virulentie worden vastgesteld tussen de negen verschillende *H. heilmannii* s.s. stammen. Op basis van het kolonisatieniveau en de graad van inflammatie in het antrum van de maag, werden vijf stammen geclassificeerd als hoog-virulent en vier stammen als laag-virulent. Aangezien het Mongools gerbilmodel wordt beschouwd als een goed model voor *Helicobacter*-gerelateerde maagpathologie bij de mens, kan er besloten worden dat deze stamverschillen hoogstwaarschijnlijk ook relevant zijn voor humane infecties met *H. heilmannii* s.s. Deze stamverschillen zouden ook van belang kunnen zijn voor infecties bij de natuurlijke gastheer.

In **hoofdstuk 2** werd onderzocht of de verschillen in kolonisatie en virulentie tussen de negen *H. heilmannii* stammen gerelateerd konden worden aan de aanwezigheid of afwezigheid van specifieke virulentie-geassocieerde genen. Hiertoe werden de genomen van alle stammen gesequeneerd en geanalyseerd. Daarnaast werden *in vitro* bindingstesten uitgevoerd om verschillen in bindingscapaciteit aan de maagmucosa in kaart te brengen.

Op basis van fylogenetische analyse werd er aangetoond dat de laag-virulente isolaten niet behoren tot de species *H. heilmannii*, maar tot een nieuwe, nauwverwante species die *H. ailurogastricus* werd genoemd. Deze nieuwe *Helicobacter* species kon niet onderscheiden worden van *H. heilmannii* op basis van de 16S rRNA en *ureAB* gensequenties. Ook morfologisch zijn beide species zeer gelijkend. Het enige verschil is dat *H. heilmannii* een hoger aantal spiraalvormige windingen vertoont en meer bipolaire flagellen bezit dan *H. ailurogastricus*. De biochemische kenmerken zijn eveneens zeer gelijkaardig, met als enige verschil dat *H. ailurogastricus* alkalische fosfatase-activiteit vertoont, terwijl dit afwezig is in *H. heilmannii*.

Verschillende homologen van virulentiefactoren van *H. pylori* werden teruggevonden in *H. heilmannii*, maar niet in *H. ailurogastricus*. Tot deze homologen behoren het ulcer-geassocieerde eiwit IceA1, het HrgA eiwit dat deel uitmaakt van een DNA restrictie-modificatie-systeem en een jhp0562-like glycosyltransferase dat betrokken is bij immuun-evasie en bij de ontwikkeling van maagzweren. Deze factoren dragen mogelijk bij tot het meer virulente karakter van *H. heilmannii* in vergelijking met *H. ailurogastricus*.

Proteomische analyses toonden aan dat zowel *H. heilmannii* als *H. ailurogastricus*, net zoals *H. pylori*, beschikken over een groot repertoire aan buitenste membraanproteïnen (‘outer membrane proteins’, OMPs). Beide species beschikken echter maar over enkele homologen van de Hop, Hor en Hom eiwitten van *H. pylori*. Alle beschreven adhesines van *H. pylori* zijn zelfs afwezig in beide species. *H. heilmannii* beschikt over zes putatieve OMPs die afwezig zijn in *H. ailurogastricus*. Deze OMPs zijn interessant voor verder onderzoek, aangezien ze een potentiële rol kunnen spelen in het kolonisatieproces van *H. heilmannii*.

Daarnaast werd ook een homoloog van de ‘VacA-like’ autotransporter van *H. pylori* teruggevonden in zowel *H. heilmannii* als *H. ailurogastricus*. Deze autotransporter verhoogt de capaciteit om de maag te koloniseren. Het ‘passenger’ domein van de ‘VacA-like’ autotransporter van *H. pylori*, dat de effectorfunctie uitvoert, bevat drie VacA2 regio’s. De ‘passenger’ domeinen van de ‘VacA-like’ autotransporters van de *H. heilmannii* stammen bevatten eveneens drie VacA2 regio’s, terwijl dit domein bij de *H. ailurogastricus* stammen vier VacA2 regio’s omvat. Aangezien *H. ailurogastricus in vivo* een lagere capaciteit vertoont

dan *H. heilmannii* om de maag te koloniseren, zou de grootte van het 'passenger' domein een mogelijke rol kunnen spelen in het kolonisatieproces.
De verschillen tussen *H. heilmannii* en *H. ailurogastricus* met betrekking tot de mogelijkheid tot kolonisatie *in vivo*, reflecteerden zich ook in hun *in vitro* capaciteit om te adhereren aan de maagmucosa. *H. heilmannii* lijkt gemakkelijker te binden aan humane maagmucines dan *H. ailurogastricus* en heeft een duidelijk hogere bindingscapaciteit aan maagepitheelcellen. Daarnaast bindt *H. heilmannii* voornamelijk aan de klieren van zowel het antrum als de fundus van de maag, terwijl *H. ailurogastricus* eerder bindt aan de epitheelcellen die het maagslijmvlies bekleden.
Samengevat beschreven we in de tweede studie de nieuwe gastrale *H. ailurogastricus* species, afkomstig van katten, die nauwverwant is aan *H. heilmannii* s.s. *H. ailurogastricus* heeft echter een lagere *in vitro* capaciteit om te binden aan maagepitheelcellen en mist verschillende factoren die een rol spelen in virulentie en kolonisatie. Dit zou kunnen verklaren waarom deze nieuwe species minder virulent is dan *H. heilmannii*.

De buitenste membraan van *H. pylori* is uitgerust met een groot aantal OMPs en verschillende van deze eiwitten zijn betrokken bij het koloniseren van de humane maagmucosa. Voor *H. heilmannii*, *H. ailurogastricus* en andere gastrale NHPH is er echter weinig gekend over welke OMPs een rol spelen in het kolonisatieproces. Alle belangrijke Hop adhesines van *H. pylori* zijn afwezig in deze species. Dit impliceert dat zij andere OMPs gebruiken om te adhereren aan de maagmucosa.
In **hoofdstuk 3** van deze thesis werd het OMP-repertoire van gastrale NHPH gekarakteriseerd op basis van fylogenetische analyses en werden de OMP-families die mogelijks betrokken zijn in kolonisatie en virulentie geïdentificeerd. We toonden aan dat gastrale NHPH, net zoals *H. pylori*, beschikken over proportioneel meer OMPs dan andere Gram-negatieve bacteriën. Dit zou het gevolg kunnen zijn van een aanpassing aan de zure omgeving in de maag. Er werden verschillende OMP-families met een mogelijke functie in kolonisatie of virulentie geïdentificeerd in gastrale NHPH. Deze families zijn ook aanwezig in *Campylobacter*, *E. coli* en enterohepatische helicobacters en omvatten de 1) TonB-afhankelijke receptoren met een functie in ijzeropname, 2) een buitenste membraan factor betrokken in antimicrobiële resistentie en 3) een buitenste membraan fosfolipase dat een rol speelt in kolonisatie. Een Imp/OstA porine, betrokken in antimicrobiële resistentie, was geconserveerd in de genera *Campylobacter* en *Helicobacter*. Daarnaast werden er enkel in *Helicobacter* verschillende OMP-families gedetecteerd en dit voornamelijk in gastrale species. Deze families omvatten SfpA/LpxR dat

een rol speelt in immuun-evasie, twee *Helicobacter*-specifieke buitenste membraan porine families met mogelijke functies in adhesie, en een *Helicobacter*-specifiek 'VacA-like' cytotoxine (gekarakteriseerd in **hoofdstuk 2**) met een rol in kolonisatie. Het grootste deel van alle OMPs van gastrale NHPH clusterden in de twee *Helicobacter*-specifieke porine families. De eerste porine familie omvatte het grootste aantal OMPs, waaronder ook de Hop adhesines van *H. pylori* die afwezig zijn in NHPH, behalve in *H. acinonychis* en *H. cetorum*. De Hor, Hom en andere Hop OMPs van *H. pylori* behoren ook tot deze porine familie, en een aantal homologen van deze eiwitten waren ook aanwezig in andere gastrale NHPH. Een laatste subgroep van deze familie bevatte putatieve OMPs die specifiek zijn voor NHPH van hond, kat en varken. De combinatie van een uniek repertoire aan porines en de afwezigheid van de *H. pylori* Hop adhesines wijst erop dat gastrale NHPH andere OMPs gebruiken om de maagmucosa te koloniseren en dat hun pathogenese verschillend is van *H. pylori*.
De tweede *Helicobacter*-specifieke porine familie was samengesteld uit de acht Hof OMPs van *H. pylori*. Deze Hof eiwitten zijn sterk geconserveerd in gastrale NHPH. Echter, in tegenstelling tot *H. pylori* zijn de *hof* genen van NHPH gelocaliseerd in één grote unieke genlocus. De functie van de meeste Hof eiwitten zijn op dit moment ongekend. Enkel voor HofE en HofF werd reeds aangetoond dat ze betrokken zijn in kolonisatie van de maag.
Interessant voor ons onderzoek is dat er verschillende species-specifieke OMP-families werden gevonden in NHPH van hond en kat die afwezig zijn in andere *Helicobacter* species. De functie en het belang van deze putatieve eiwitten moet echter nog worden onderzocht.

In deze thesis toonden we aan dat *H. heilmannii* s.s., geïsoleerd uit de maag van katten, in staat is om inflammatie te induceren in de maag van Mongoolse gerbils, een *in vivo* model voor humane maagpathologie. Daarnaast beschreven we de nieuwe species *H. ailurogastricus*, die nauwverwant is aan *H. heilmannii*. Met behulp van zowel *in vitro* als *in vivo* studies toonden we aan dat *H. ailurogastricus* minder virulent is dan *H. heilmannii*. Er werden verschillende virulentie-geassocieerde factoren geïdentificeerd in *H. heilmannii* die mogelijks bijdragen tot het verschil in pathogeniciteit tussen deze twee *Helicobacter* species. Ten slotte werd het OMP-repertoire van alle gastrale NHPH gekarakteriseerd, wat van grote waarde kan zijn voor toekomstige *in vitro* en *in vivo* kolonisatiestudies.

CURRICULUM VITAE

Myrthe Joosten werd geboren op 14 juni 1988 in Turnhout. Na het doorlopen van haar middelbare studies Latijn-Wiskunde aan het Sint-Pietersinstituut in Turnhout, startte zij in 2006 met de opleiding Biomedische Wetenschappen aan de Universiteit Gent. In 2011 studeerde zij af als Master of Science in de Biomedische Wetenschappen, major Immunologie en Infectie, met grote onderscheiding.

Onmiddellijk daarna startte zij als doctoraatsstudente aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten van de Faculteit Diergeneeskunde van de Universiteit Gent. Gedurende het eerste jaar verrichtte zij er, in het kader van een Geconcerteerde Onderzoeksactie (GOA), onderzoek naar de pathogenese van zoönotische *Helicobacter heilmannii* infecties. Eind 2012 diende zij een projectvoorstel in met betrekking tot *Helicobacter heilmannii* infecties bij hond, kat en mens, bij het Instituut voor Innovatie door Wetenschap en Technologie (IWT). Na een succesvolle projectverdediging behaalde zij een persoonsgebonden doctoraatsbeurs met aanvang in 2013, voor een periode van 4 jaar. Dit doctoraatsonderzoek resulteerde in het huidige proefschrift. Daarnaast werd ook de doctoraatsopleiding met succes voltooid.

Myrthe Joosten is auteur en mede-auteur van meerdere publicaties in internationale tijdschriften en zij was meermaals spreker op internationale congressen.

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