“MORPHOLOGIC, GENETIC, AND BIOCHEMICAL CHARACTERISATION OF NOVEL *HELICOBACTER* ISOLATES FROM LABORATORY MICE AND TIGERS”

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Abbreviations

A

a.a.	aminoacid
A, C, G, T	Adenine, Cytosine, Guanine, Thymine
ATP	AdenosinTriPhosphate
ATCC	American Type Culture Collection
Amp^R, Kan^R	Resistance to ampicillin, resistance to kanamycin
AP-PCR	Arbitrarily Primed Polimerase Chain Reaction

B

BHI	Brain Heart Infusion
bp	Base pair
BSA	Bovine Serum Albumin

C

CDT	Cytolethal Distending Toxin
CLSM	Confocal Laser Scanning Microscopy
CPD	Critical Point Drying

D

DNA	Desoxyrribonucleic acid
DTT	Dithiotreitol

E

EDTA	Ethylendiamin tetra acetic acid
e.g.	For example (*exempli gratia*)
EHS	Enterohepatic Helicobacter Species
et al	And others (*et alii*)

F

FBS	Fetal bovine serum
FELASA	Federation of European Laboratory Animal Science Associations
FESEM	Field Emission Scanning Electron Microscopy
FISH	Fluorescence *In Situ* Hybridization

G

GC	Gastric Cancer
G-C	Gentamycin-Chloramphenicol

GHS	Gastric Helicobacter Species
GLO	*Gastrospirullum* - Like Organisms
GNHP	Gastric non-*Helicobacter pylori*
H	
HPLO	*Helicobacter pylori* -like organisms
HZI	Helmholtz-Zentrum für Infektionsforschung
I	
IARC	International Agency Research on Cancer
IBD	Inflammatory Bowel Diseases
IJBS	International Journal of Biomedical Sciences
IL-8	Interleukin 8
IL-10	Interleukin 10
IMMB	Institüt für Medizinische Mikrobiologie, Magdeburg
IPTG	Isopropyl β-D-thiogalactopyranoside
IVS	InterVening Sequences
IU	International Units
K	
kDa	KiloDalton
L	
LB	Lysogenic Broth
LMP	Low Melting Point
M	
M	Molecular weight marker
MALDI-MS	Matrix-Assisted Laser Desorption/Ionization-Mass spectrometry
MALT	Mucosal Associated Lymphoid Tissue
mg	miligrams
MH	Mueller-Hinton
N	
NCBI	National Center for Biotechnology Information
NCTC	National Culture Type Collection
NF-κB	Nuclear factor κB
NHPH	Non-*Helicobacter pylori* Helicobacters

O

OD_{600}	Optical density at λ=600 nm

P

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
PFGE	Pulse Field Gel Electrophoresis
PUC	Peptic Ulcer Disease

R

RAPD	Randomly Amplified Polimorphisms DNA
Rpm	Rounds *per* minute
rRNA	Ribosomal Ribonucleic Acid
RT	Room temperature

S

SCID	Severe Combined Immunodeficiency
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S.O.C.	Super Optimal broth with Catabolite repressor
SPF	Specific Pathogen Free

T

TBS	Tris Buffer Saline
TEM	Transmission Electron Microscopy
TIGR	The Institute for Genome Research

U

U	Unit of enzyme activity (μmol min^{-1} mg $protein^{-1}$)
UCD	University College of Dublin
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra Violet

V

V	Volts
v/v	Volume per volume

W

WHO	World Health Organisation

w/v	Weight per volume
wt	Wild-type
X	
x *g*	x-times acceleration due to gravity (9,81m/s2)
XSP	Xanthogenate-Sodium dodecyl sulfate-Phenol

1. ABSTRACT

„Morphologic, genetic and biochemical characterisation of novel *Helicobacter* isolates from laboratory mice and tigers"

Since the first description of the Helicobacter genus, the human-specific pathogen *Helicobacter pylori*, a highly adapted and long co-evolved gastric bacterium, multiple disciplinary fields have been opened for researchers around the world. Most of the Helicobacter species encompassed within this genus diversified significantly during evolution to be classified in different taxa, and not only as inhabitants of the human stomach mucosa but also from lower intestines and hepatobiliary pathways including non human primates, domestic and wild animals. Currently these bacteria are classified in gastric Helicobacter species (GHS) and enterohepatic Helicobacter species (EHS), in accordance to the particular colonizing niche and the evolutionary process resulting from longtime selection. EHS presence is worldwide reported in mouse colonies kept at Specific Pathogen Free (SPF) facilities, where these infections often remain unrecognized but can cause severe health complications or more subtle host immune perturbations and therefore can confound the results of animal experiments.

In the first part of the present doctoral thesis a new EHS, *Helicobacter magdeburgensis*, has been characterized from SPF mice. Biochemical analysis of enzyme activities (API campy), morphologic investigation (Gram-staining and electron microscopy) and genetic analyses (16SrRNA and 23SrRNA analyses, DNA fingerprinting, restriction fragment polymorphisms, and pulsed-field gel electrophoresis) revealed a spiral-shaped bacterium with lengths of 2.5–6 µm and containing a single monopolar or single bipolar sheathed flagella. The bacteria were growing under anaerobic conditions, preferably on agar plates supplemented with serum or blood. 16S rRNA sequence analysis placed this yet unknown bacterium in the Helicobacter genera, and based on the pattern produced by two restriction enzymes, *Bam*III and *Ksp*I, the genome size was determined to be about 1.7–1.8 Mbp. Finally a PCR assay was developed and can be used to detect and discriminate *H. magdeburgensis* from other Helicobacter species.

In the second part of the thesis, I characterized some novel Helicobacter isolates from a Bengal tiger. The closest relative of *H. pylori* is *H. acinonychis*, a little characterized species which can be specifically isolated from big felines including cheetahs, lions, and tigers. A species jump from humans to big predator cats has been proposed a few hundred thousand years ago. In concordance, *H. acinonychis* strain Sheeba strain possesses an unusual large number of highly fragmented genes many of them coding outer membrane proteins (OMPs) which may have been inactivated upon host adaptation in order to circumvent deleterious responses from the feline host immune system. Here, I isolated *Helicobacte*r spp of a tiger from a zoo in Thailand. Morphological investigation (Gram-staining and electron microscopy) and genetic studies (16SrRNA and 23SrRNA analyses, DNA fingerprinting and restriction fragment polymorphisms) as well as Western blotting were used to characterize the isolated *Helicobacter* spp. Scanning and transmission electron microscopy revealed the presence of spiral-shaped bacteria, varying in length from 2.5 to 6µm and contained up to four monopolar sheathed flagella. The *Helicobacter* spp. were grown under microaerophilic conditions, with best results obtained on Columbia or GC agar plates containing serum or blood. The 16SrRNA, 23SrRNA, genetic and protein expression analyses indicated the identification of a novel *H. acinonychis* isolate closely related to *H. pylori*. I also demonstrated by immunoblotting that the tiger isolates express UreaseA/B, flagellin A, adhesin BabA, neutrophil-activating protein NapA, protease HtrA, γ-glutamyl-transpeptidase GGT, lytic transglycosylase Slt and two DNA transfer relaxase orthologs as known from *H. pylori*, but are negative for the expression of *cag*PAI, CagA, VacA, SabA, DupA and OipA proteins. These results give fresh insights into *H. acinonychis* genetics and the expression of potential pathogenicity-associated factors and their possible pathophysiological relevance in related gastric infections.

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2. ZUSAMMENFASSUNG

„Morphologische, genetische und biochemische Charakterisierung von neuen *Helicobacter* Isolaten aus Labormäusen und Tigern***“***

Seit der Erstbeschreibung von hoch-adaptiven, human-spezifischen *Helicobacter pylori* entwickelten sich eine Reihe von Forschungsgebieten zur Untersuchung dieses bedeutenden Pathogens. Die meisten *Helicobacter* ssp. (Subspezies) in diesem Genus haben sich während der Evolution signifikant in verschiedenen Taxa spezialisiert und kolonisieren nicht nur den humanen Magen sondern auch andere Organe in nicht-humanen Primaten, sowie Nutz- und Wildtieren. Heute sind diese Gram-negativen Bakterien klassifiziert nach ihrem Vorkommen in zwei grosse Gruppen, gastrische *Helicobacter* Spezies (GHS) und enterohepatische *Helicobacter* Spezies (EHS). Die Präsenz von EHS in Mauskolonien wird sehr häufig in der Literatur berichtet, sogar in Versuchtierkolonien der Kategorie „*specific pathogen free animals* (SPF)“, so wie in unserem Fall, wo diese Infektionen oft unentdeckt bleiben. Allerdings können derartige Infektionen auch mit erheblichen gesundheitlichen Problemen einhergehen und die Ergebnisse von Tierversuchen erheblich beeinflussen.

Im ersten Teil der vorliegenden Arbeit wurde eine bisher unbekannte Spezies, *Helicobacter magdeburgensis*, aus infizierten SPF-Labormäusen in einem Tierlabor am Uniklinikum Magdeburg isoliert. Biochemische Analysen von bestimmten Enzymaktivitäten (API-Campy), morphologische Untersuchungen (Gram-Färbung und Elektronenmikroskopie) und genetische Analysen (16SrRNA und 23SrRNA Sequenzierung, DNS-Fingerprinting, RFLP und Pulsfeldgelelektrophorese) zeigten das Vorhandensein von spiral-förmigen Bakterien von etwa 2.5–6 µm Länge mit einzelnen monopolaren oder bipolaren Flagellen. Die Bakterien konnten unter anaerobischen Bedingungen angezogen werden. Die 16S rRNA-Analyse gruppierte das unbekannte Bakterium in den Genus *Helicobacter*. Die Genomgrösse wurde in der Pulsfeldgelelektrophorese mittels der Restriktionsenzyme *Bam*III und *Ksp*I bestimmt und beträgt 1.7–1.8 Mbp. Ein spezifischer PCR-Assay wurde ebenfalls entwickelt, der zwischen *H. magdeburgensis* und anderen *Helicobacter* ssp. unterscheiden kann.

Dieser Assay ist somit anwendbar, um schnell und zuverlässig Infektionen von SPF-Labormäusen nachzuweisen.

Im zweiten Teil der vorliegenden Arbeit habe ich einige neue *Helicobacter* Stämme aus einem Bengalischen Tiger isoliert und charakterisiert. Der nächste bakterielle Verwandte von *H. pylori* ist *Helicobacter acinonychis,* eine wenig untersuchte Spezies, die man vornehmlich in Grosskatzen wie Löwen und Tigern antrifft. Aufgrund von genetischen Analysen wurde postuliert, das sich *H. acinonychis* ursprünglich aus *H. pylori* weiterentwickelten, die den Wirt vor einigen hunderttausend Jahren wechselten. In Übereinstimmng mit dieser Hypothese besitzt *H. acinonychis* viele hochfragmentierte Gene von äusseren Membranproteinen (OMPs), die offenbar während der Anpassung an seinen neuen Wirt inaktiviert wurden, z.B. um das Immunsystem der Grosskatzen zu umgehen. Ich habe in dieser Arbeit mehrere neue *H. acinonychis* Stämme aus einem Tiger in einem Zoo in Thailand isoliert. Morphologische Untersuchungen (Gram-Färbung und Elektronenmikroskopie), genetische Analysen (16SrRNA und 23SrRNA Sequenzierung, DNS-Fingerprinting, RFLP und Pulsfeldgelelektrophorese) und Westernblotuntersuchungen wurden angewandt, um diese Bakterien zu charakterisieren. Hochauflösende Laserelektronenmikroskopie und Transmissionselektronenmikroskopie zeigten das Vorhandensein von spiral-förmigen Bakterien von etwa 2.5–6 µm Länge, häufig mit vier monopolaren Flagellen. Die neuen Isolate wurden unter mikroaerophilen Bedingungen angezogen, bevorzugt auf Columbia- oder GC-Agar-Platten, die Blot oder Serum enthalten. Die 16SrRNA, 23SrRNA, genetische und Proteinexpressionsanalysen zeigten, das unsere *H. acinonychis* Isolate eng mit *H. pylori* verwandt sind. Untersuchungen zur Expression von Pathogenitäts- und Virulenzfaktoren mittels Westernblot zeigten, das bestimmte Proteine wie UreaseA/B, Flagellin A, Adhesin BabA, Neutrophil-aktivierendes Protein NapA, Protease HtrA, γ-Glutamyl-Transpeptidase GGT, lytische Transglycosylase Slt und zwei DNA-Transfer Relaxasen in *H. acinonychis* exprimiert sind. Dagegen scheinen andere bekannte Virulenzfaktoren wie die *cag* Pathogenitätsinsel, CagA, VacA, SabA, DupA und OipA Proteine zu fehlen. Diese Ergebnisse geben somit ebenfalls wichtige neue Hinweise für das Pathogenitätspotential dieser *Helicobacter* ssp. mit hoher Relevanz für gastrische Infektionen.

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3. INTRODUCTION

3.1 Genus Helicobacter

The genus *Helicobacter* (Goodwin *et al.*, 1989) comprises a rapidly expanding and heterogeneous group of Gram-negative spiral bacteria colonising gastrointestinal and other environments of different mammalian hosts ranging from domestic and wild animals, non-human primates to humans (Fox, 2002; Haesebrouck *et al*., 2009) as well as birds (Dewhirst *et al.*, 1994). Currently there are 32 validated *Helicobacter* species and 225 other isolated candidates have been described (Haesebrouck *et al*., 2009). So far, the most important and best described species within this genus is *Helicobacter pylori*, a human gastric pathogen which infects 50% of the world population (Correa & Piazuelo, 2011).

3.2 Morphological characteristics

The essential property of all Helicobacters is a remarkable cork-screw-like, darting or slower wave-like motility within the viscous gastric mucosa due to spiral morphology and the presence of tufts of terminal bulbs flagella in a variety of numbers and localisations which are sheathed in almost all of them. Many Helicobacter species possess strong ureolytic ability, a hallmark of those associated with gastric mucosa (Sachs *et al*., 2006), and also exhibit considerable diversity in cell morphology with respect to cell length and presence of periplasmic fibrils. In common, Helicobacters are non-spore-forming bacteria. The cellular morphology may be curved, spiral or fusiform, typically 0.2 to 1.2 μm in diameter and 1.5 to 10.0 μm long (Owen, 1998). The spiral wavelength may vary with the age of culture, growth conditions and the given species, and in stationary-growth cultures or those exposed to adverse conditions, cells may gradually undergo to a process and finally become coccoid, a morphological stage of diverse interpretation (Küsters *et al.*,1997; Rivas & Hernández, 2004; Chen, 2004).

An electron-dense glycocalyx or capsule-like layer has been observed on the cellular surface of several *Helicobacter* species in liquid culture, including *H. pylori* (Goodwin *et al.*, 1989; Lee *et al.*, 1992; Paster *et al.*, 1991; Shen *et al.*, 1997; Owen,

1998). Also electron-dense polyphosphate granules have been observed, which may serve as an alternative energy source for the bacteria (Bode *et al.*, 1993, Shen *et al.*, 1997).

3.3 Macromolecular characteristics

The genomic DNA of *Helicobacter* species is composed of a single circular molecule with a mean size of about 1.1 Mb ranging from 1.40-1.73 Mb, and with a base composition in the range 35-37 mol% G+C. DNA-DNA hybridizations show a high level (1>65%) of sequence homology between strains despite evidence for extensive re-arrangements in gene order and sequence variation within genes (Jiang *et al.*, 1996). *H. pylori* is a prime example and characterised by a high level of genetic diversity due to DNA recombination events and endogenous mutations (Salama *et al.*, 2000). Plasmid DNA is present in about 45% of strains although some strains such as *H. pylori* NCTC 11637 are plasmid free. The number and size of plasmids can vary considerably from strain to strain, but many of them carry a single plasmid with sizes from 1.8-63 kbp. Internal transcribed spacers or intervening sequences (IVS) have been described in the 16S rRNA, with a range length from 187 bp to 235 bp. Similar IVS elements have also been found in the 23S rRNA, with sizes ranging from 93 bp to 377 bp (Linton *et al.*, 1994; Fox *et al.*, 1995; Franklin *et al.*, 1999; Saunders *et al.*, 1999).

3.4 Other Characteristics

Another typical characteristic of the entire *Helicobacter* genus is the fastidious nutritional requirements of the individual species, requiring special skills to isolate and keep these organisms in culture (Vandamme *et al*., 1990). *H. pylori* is a microaerophile, best growing for 3 - 5 days in an atmosphere containing 5% O_2 with 5-10% CO_2 on supplemented (see materials & methods) brain heart infusion (BHI) medium in 5% blood agar; optimally at 37°C. All known strains grow over a relatively narrow temperature range of 33 - 40°C, whereas some grow poorly at 30°C and 42°C, and none at 25°C. On a suitable culture medium, *H. pylori* will grow over a wide pH ranging from 5.5 - 8.5. Antibiotics like polymyxin B can be included as a taxonomic marker,

provided that 95% *H. pylori* strains are resistant (300 IU disk). This has beensuggested as an additional test to discriminate between *Helicobacter* and *Campylobacter.* Nalidixic acid and cephalothin are also important to discriminate between *Campylobacter* and *Helicobacter*, because *ca.* 86% of *H. pylori* are resistant to nalidixic acid (30 mg disk) and susceptible to cephalothin (30 mg disk) (Burnens *&* Nicolet, 1993; Owen, 1998).

3.5 *H. pylori*

In the modern world, *H. pylori* is known as one of the most successful pathogens in humans with a highest prevalence around the world (Eurogast, 1993), even when its exact mode of transmission is still uncertain (Taylor *&* Blaser, 1991). This organism, firstly isolated from the human stomach and most exceptionally from dental plaque, faeces and blood has not been yet consistently isolated from any other niche since then, and thus the mechanism by which it colonizes the human gastric mucosa remains largely unknown (Taylor *&* Blaser, 1991). Robin Warren and Barry Marshall (1982) established *H. pylori* as the etiologic agent of gastritis and peptic ulcer disease (PUD), a discovery that revolutionized current knowledge in gastroenterology. Before them, PUD was considered as an acid-driven disease and the human stomach was believed to represent the sterile area, the so called “acid gastric barrier”, dogmatic obstacles to be overcome with time (Malfertheiner, 2007). Today, a novel unifying knowledge emerged and *H. pylori* is recognized as the most common cause of gastritis, mucosa associated lymphoid tissue (MALT) lymphoma (Parsonnet *et al.*, 1991) which in turn leads to the development of more severe gastrointestinal complications such as gastric cancer (GC), which is usually detected at very late stages (Malfertheiner *et al.*, 2010), and its preventive eradication continues to be a major global health problem (Maltfertheiner *et al.*, 2005).

The overwhelming evidence linking *H. pylori* to GC contributed to the classification of this organism as a class 1 carcinogen, it means “definitive”, by the World Health Organisation, WHO, one of the world’s deadliest cancers (IARC, 1994; Peter *&* Beglinger, 2007).

3.6 HISTORY

Observations of spiral microorganisms in the stomach can be dated back to 1875 (Table No1), when the German bacteriologist Bottcher together with his French collaborator Letoulle, demonstrated and attributed to spiral microorganisms the lesions observed in the floor and margins of human gastric ulcerated mucosa. Later on, another German, a pathologist named Klebs (1881) reported a bacillus-like organism free-living in the lumen of gastric glands and between the cells of the glands and the *tunica propria* associated with interglandular small round cell infiltration. Jaworski (1889), Professor of Medicine at the Jagiellonian University of Cracow, Poland was first to describe the spiral organisms found in the sediment of gastric washings obtained from humans, after observations of spiral microorganisms, he named *Vibrio rugula*, suggesting for the first time its possible pathogenic role in gastric diseases. Doenges (1938) showed a prevalence of 43% of spiral organisms in a comprehensive autopsy study in 242 human stomach specimens, however, he did not associate the presence of the spiral organism with gastric diseases. Early controversy existed from the beginning over the possible role of these spiral microorganisms in human gastric disease. It has been suggested that the bacteria observed in gastric biopsies might represent foodborne bacterial contaminants, a hypothesis that gained support after the publication of an extensive histological study of gastric biopsies from 1,000 subjects by Palmer (1954). However, Steer *et al.* (1975) reported the presence of bacteria deep in the mucus layer of gastric mucosa in patients with gastric ulceration, suggesting that the bacteria might cause a reduction in gastric mucosal resistance via predisposal to ulceration.

The serendipitously first successful cultivation by Marshall & Warren of a Gram-negative pleomorphic spiral bacterial bacillus (Waren & Marshall, 1983, 1984), known today as *Helicobacter pylori*, from human gastric mucosa samples was achieved after an accidentally prolonged Eastern holiday weekend cultivation under microaerophillic conditions in the microbiology laboratory at the Royal Perth Hospital in Australia (Fig. 1, A).

Initially considered to belong to the *Campylobacter* genera, the proper classification attracted considerable attention at the Second International Workshop on *Campylobacter* Infections held in Brussels, Belgium, in September 1983 (Pearson, 1983). The described microorganism resembled *Campylobacter* in several respects, including curved morphology, growth on rich media under microaerophilic conditions, failure to ferment glucose, sensitivity to metronidazole, and a G + C content of about 34%, thus referred as "*pyloric Campylobacter*" (*pylorus*, Greek, gatekeeper, or one who looks both ways) and validated as *Campylobacter pyloridis* (Anonymous validation IJSB, 1985). The specific epithet was revised and amended to *Campylobacter pylori* in 1987 to conform to the correct Latin genitive of the noun *pylorus* (Marshall & Goodwin, 1987).

Table No 1. BRIEF CHRONOLOGY OF *Helicobacter pylori* DISCOVERY

	Year	Observations
1.	**Bottcher, G. & Letulle, M. (1875)**	bacteria located at gastric ulcerated margins
2.	**Klebs, C. (1881)**	bacterial colonisation and interglandular cell infiltration
3.	**Letulle, M (1888)**	experimental induction of acute gastric lesions in guinea pigs using . *aureus*
4.	**Jaworski, W. (1889)**	detected spiral organisms in human gastric washings sediments, called "*V. rugula*"
5.	**Bizzozero, G. (1893)**	identified spirochetes in dogs gastric mucosa
6.	**Krienitz, W. (1906)**	confirmed presence of spirochetes in gastric contents of patients with gastric carcinoma
7.	**Turck, F.B. (1908)**	induced gastric ulcers in dogs using *Bacillus* (*Escherichia*) *coli*
8.	**Rosenow, E.C. (1916)**	described streptococcus induced gastric ulcers
9.	**Dragstedt, L.R. (1917)**	identified bacteria in experimental ulcers
10.	**Edkins, J.S. (1921)**	experimental physiology of *Spirilla regaudi* (*H felis*)
11.	**Luck, J.M. (1924)**	discovered gastric mucosal urease
12.	**Hoffman, B. (1925)**	described *Bacillus hoffmani* as a putative ulcerous agent
13.	**Berg, B. (1930)**	partial vagotomy inhibits secondary infections in ulcers
14.	**Doenges, J.L. (1938)**	spirochetes inflammation in Macacus monkey and man
15.	**Freedberg, A.S. & Baron, L. (1940)**	identified spirochetes in man – no etiologic role
16.	**Gorham, F.D. (1940)**	postulated gastric acidophilic bacteria as etiologic agent in ulcer disease
17.	**Palmer, E.D. (1954)**	no spirochetes detected using HE in 1180 suction biopsies
18.	**Steer, H.W. (1975)**	polymorphonuclear migration in ulcers – isolated *P. aeruginosa*
19.	**Warren, J.R. (1983)**	identified *Campylobacter* (*Helicobacter*) *pylori*
20.	**Marshall, B. (1983)**	*H. pylori* isolated and cultured
21.	**Marshall, B & Morris, A. (1985-1987)**	demonstrated *H pylori*'s Koch third posulated
22.	**Goodwin (1989)**	new spiral bacteria officially named *H pylori*

However, early electron micrographic investigations showed multiple sheathed flagella at one pole of the bacterium (Fig. 1, B), in contrast to the single bipolar unsheathed flagellum typical of *Campylobacter* spp. (Fig. 1,C) (Goodwin *et al.*, 1985). Major protein components and fatty acids of *C. pylori* were also markedly different from those of *Campylobacter* species (Goodwin *et al.*, 1985; Pearson *et al.*, 1984), and the subsequent 16S rRNA sequence analysis showed that the distance between *C. pylori* and the true Campylobacters was sufficient to exclude it from the *Campylobacter* genus (Romaniuk *et al.*, 1987), and it was renamed *Helicobacter pylori*, the first member of the new genus *Helicobacter* and creating the genera *Arcobacter* (Fig. 1, D), to relocate *A. frigilis* and *A. butzleri*, two atypical Campylobacters (Goodwin *et al.*, 1989).

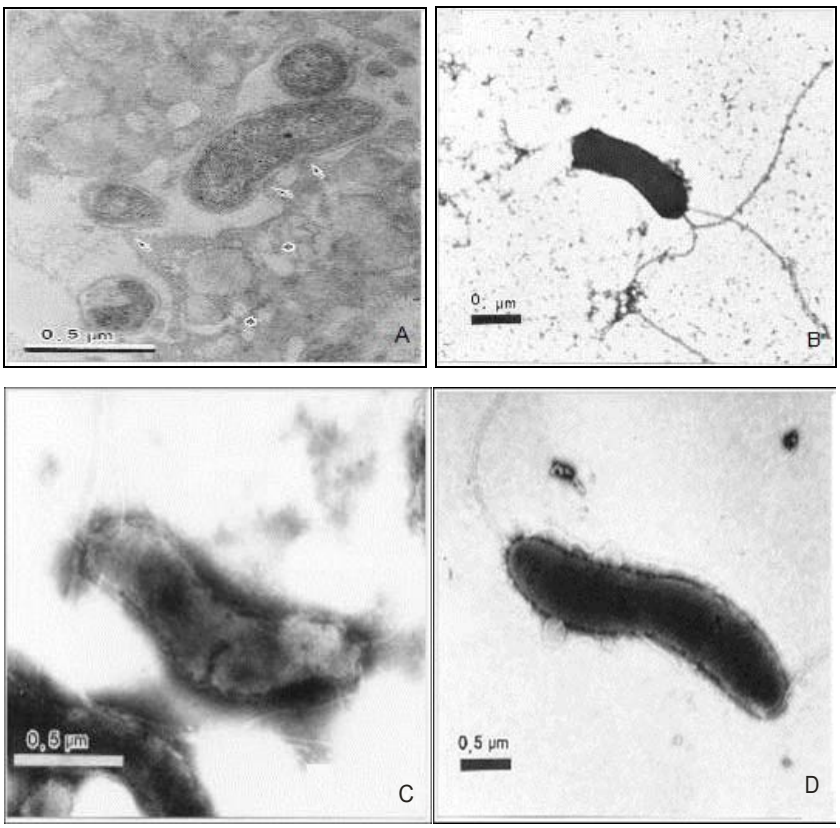


Figure 1. (A) Transmission electron micrographs (TEM) from ultrathin section biopsy from a chronic gastritis patient showing bacteria in a distended celllular space. Arrows showing pedestal-like structures; asterisks indicating degenerated zones compatible with ulcerogenic activity. (B) *Helicobacter pylori* isolated from an antral biopsy from a chronic gastritis patient. Phoshotungstic acid staining evidencing a curved bacteria with blunt ends and a monopolar tuft composed of three sheathed flagella. (C) Transmission electron micrograph showing *Campylobacter jejuni* isolated from a diarrhoeic children exhibiting a bacteria with acute extremes showing a mopolar flagella emerging from a small pore. (D) *Arcobacter butzlerii* bacteria with blunt ends with an emerging flagella (Taken from Rivas & Hernández, 2000).

3.7 Non-*H.pylori* Helicobacter Species (NHPH)

Besides the well known gastric pathogen *H. pylori*, other Helicobacter species with a spiral morphology have also been detected by endoscopy in a minority of human patients associated with gastritis (Debongnie *et al*., 1995), gastric ulcers (Debongnie *et al*.,1998) or gastric cancer (Morgner *et al*., 1995). However, the percentage of patients infected with these species is much lower than those for *H. pylori*, ranging between 0.2 - 6%, difficulting their study and classification. There are currently seven valid named gastric *Helicobacter* species, two *Candidatus* species, and one other species that has been described, but was not yet validated (Haesebrouck *et al*., 2009). Because the very fastidious nature of these Helicobacters makes their *in vitro* isolation extremely difficult, the term “gastric non-*H. pylori* Helicobacters” (GNHP) or non-*H.pylori* Helicobacters (NHPH) has been coined to designate these spiral-shaped bacteria when only results of histopathology or incomplete taxonomic data are available. This could be helpful to reserve true species designations for those situations in which the species has to be defined (Haesebrouck *et al.*, 2009) and the provisional status of *Candidatus* has been implemented for incompletely described prokaryotes (Murray *&* Stackebrandt, 1995). Today, NHPH comprise different species that have been detected in animals such as cats, dogs, pigs and primates (see Table No 2), where the prevalence of similar spiral-shaped microorganism is high (Baele et al, 2009), and recent investigations have opened the possibility that human beings may acquire these infections through animal contact (Jalava *et al*., 2001; De Groote *et al*., 2005; Van den Bulck *et al*., 2005).

Uncultivated bacteria, once referred to as gastrospirillum or as "*Gastrospirillum hominis*" (McNulty *et al*., 1989) were initially identified as Helicobacters when 16S rRNA analysis showed that they clearly belong to the *Helicobacter* genus (Solnick *et al.*, 1993). The bacterium was tentatively designated "*H. heilmannii*," after Konrad Heilmann, a German histopathologist (Heilmann *&* Bourchard, 1991). However, 16S rRNA gene sequences analysis comparisons between clones derived from two patients differed by more than 3% in their nucleotide sequence, suggesting that they might represent different species, which prompted the subclassification of the NHPH into “*H. heilmannii”* type 1 and “*H. heilmannii”* type 2 (Solnick *et al.*, 1993), which was corroborated by 23S rRNA-encoding genes sequencing (Dewhirst *et al.*, 2005).

Table No 2. Characteristics of cultivated Non-*H. pylori* Helicobacters (NHPH)

Taxon	Source	Catalase production	Nitrate reduction	Alcaline phospatase hydrolisis	Urease Production	Iindoxyl acetate hydrolysis	Δ-glutamyl transferase	Growth at 42°C	Growth with 1% glycine	Nalidixic acid 30μ. disk	Cephalotin 30μg disk	Periplasmic fibers	Number of flagella	Distribution of flagella	G+C content (mol %)
1. H. mustelae	ferrets	+	+	+	+	+	+	+	-	R	R	-	4–8	peritrichous	36
2. H . pylori	Humans, rhesus monkey	+	-	+	+	-	+	-	-	R	S	-	4–8	monopolar	39
3. H. bizzozeroni	dogs	+	+	+	+	+	+	+	-	R	S	-	10-20	bipolar	ND
4. H. felis	Cat,dogs	+	+	+	+	-	+	+	-	R	S	+	14-20	bipolar	42
5. H. acinonychis	Cheetah	+	-	+	+	-	+	+	-	R	S	-	2-5	monopolar	30
6. H. nemestrinae	Pig tailed macaque	+	-	+	+	-	ND	-	-	R	S	-	4-8	bipolar	24
7. H. salomonis	Dogs	+	+	+	+	+	ND	-	ND	R	S	-	10-23	bipolar	ND
8. H. suncus	House musk shrew	+	+	+	+	-	+	ND	ND	R	R	-	2	bipolar	ND

+ positive reaction; - negative reaction; ND, not determined; R, resistance; S, sensitive. Adapted from Haesebrouck *et al.*, 2009

Further sequencing of several genes detected in DNA isolated from NHPH-infected tissues has shown that the '*H. heilmannii*' type 2 comprises a group of at least five different Helicobacter species, all of them known to colonize the stomach of animals (Haesebrouck *et al*., 2009).

While "*H. heilmannii*" type 1 was both, morphologically and genetically identical to an uncultured bacterium colonizing the stomachs of pigs (De Groote *et al.*, 1999; O´Rourke *et al.*, 2004), it was originally designated as "*Gastrospirillum suis*" (Mendes *et al.*, 1990; Queiroz *et al.*, 1990). Only after successful *in vitro* culturing, and polyphasic taxonomy approach including 16S rRNA and partially *hsp*60 genes sequencing, fluorescence *in situ* hybridization (FISH) and electron microscopy, it was shown that these previously detected organisms described as "*Candidatus Helicobacter suis"* (De Groote *et al.*, 1999), belonged to the genus *Helicobacter* with sufficient differences from all existing species to constitute a new taxon and the description of *H. suis*, as a new species (Baele *et al*, 2008).

"*H. heilmannii*" type 2, represented a group of species, including three Helicobacters that have been isolated from the stomachs of cats and dogs, namely *H. felis, H. bizzozeronii* and *H. salomonis* and one still non cultured species detected in the stomachs of humans, wild felids, dogs, and cats designated "Candidatus *Helicobacter heilmannii*" (O´Rourke *et al.*, 2004). Two other closely related species, one of which was isolated from a dog and the other from a cat, have been described as *H. cynogastricus* and *H. baculiformis*, respectively (Van den Bulck *et al.*, 2006; Baele *et al.*, 2008). However, to date no information is available about the presence of these bacteria in humans (Haesebrouck *et al.*, 2009).

3.8 ENTEROHEPATIC *HELICOBACTER* SPECIES (EHS)

The identification of *H. pylori* from the human gastric mucosa and the subsequent recognition of its prevalence and clinical significance as a cause of gastric malignancies, attracted considerable research interest focused on similar microbial flora in human gastrointestinal tract of different animal species (Haesebrouck *et al*., 2009).

Many *Helicobacter* species have now been isolated from lower intestines and hepatobiliary niches of various mammals including non-human primates, canines felines, equines, bovines, rodents and birds (Häanninen, 1998), and several of them are responsible for significant degrees disorders and chronic diseases in their hosts. Based on their preferential ecologic niche of colonization and phylogenetic analysis of 16S and 23S rRNA gene sequences, members of the genus *Helicobacter* are now commonly divided into gastric *Helicobacter spp.* (GHS) for those inhabiting the gastric niche, and enterohepatic *Helicobacter spp.* (EHS), to refer to those in lower intestines and hepatobiliary system (Fox, 2009).

EHS are defined as a growing group of microaerobic organisms, also motile by a variety of flagella styles in number and locations, fusiform to curved spiral rod morphology Gram negative bacteria belonging to Helicobacteraceae. These members account for two-thirds of all known *Helicobacter* spp. (Fox, 1997), which in common with the GHS type species such as *H. pylori*, can persistently colonize the stomach

causing chronic inflammation sometimes leading to PUD and development of GC. However, when isolated from lower intestines, hepatobiliary system and diarrhoeic faeces of humans and animals, they seem increasingly associated with epithelial cell hyperproliferation leading to neoplasic disease (Bohr *et al.*, 2004; García *et al.*, 2006).

Table No 3 Enterohepatic *Helicobacter* spp (EHS)

Taxon	source	Catalase production	Nitrate reductase	Alkalyne phosphatase	urease production	Indoxyl acetate hydrolysis	Δ-glutamyl transferase	Growth with 1%	Growth with 1% glycine	Nalidixic acid 30μ disk	Cephalotin 30μg disk	Periplasmic fibers	Distribution of flagella	G+C content (mol %)
1. H. rodentium	Mouse	+	+	-	-	-	-	+	+	R	R	-	bipolar	ND
2. H. pullorum	Chicken, human	+	+	-	-	-	ND	+	ND	R	S	-	monopolar	34-35
3. H. canadensis	Human	+	v	-	-	+	-	+	+	R	R	-	bipolar	ND
4. H. fenellieae	Human	+	-	+	-	+	-	-	+	S	S	+	bipolar	35
5. H. trogontum	Rat	+	+	-	+	ND	+	+	ND	R	R	+	bipolar	ND
6. H. muridarum	Mouse, rat	+	-	+	+	+	+	-	-	R	R	-	bipolar	34
7. H. hepaticus	Mouse	+	+	ND	+	+	ND	-	+	R	R	-	bipolar	ND
8. H. canis	Dog, human	-	-	+	-	+	ND	+	ND	S	I	+	bipolar	48
9. H. bilis	Mouse, dog, human	+	+	ND	+	-	ND	+	+	R	R	-	bipolar	ND
10. H. cinaedi	Human, Hamster	+	+	-	-	-	-	-	+	S	I	+	bipolar	37-38
11. "H. sp. flexispira	Sheep, dog, human, mouse	+	-	-	+	ND	+	+	-	R	R	-	bipolar	34
12. H. cholecystus	Hamster	+	+	+	-	-	-	+	+	I	R	-	monopolar	ND
13. H. typhlonicus	Mouse	+	+	-	-	-	-	-	+	ND	ND	-	bipolar	ND
14. H. mesocricetorum	Hamster	+	+	+	-	ND	-	+	-	S	R	-	bipolar	ND
15. H. pametensis	Bird, swine	+	+	+	-	-	-	+	+	S	S	-	bipolar	38
16. H. westmeadii	Human	+	+	+	-	ND	ND	-	ND	S	R	-	monopolar	ND
17. H. sp. cotton top	Cottontop tamarin	+	+	+	+	-	+	+	+	S	R	-	bipolar	31
18. H. mainz	Human	+	-	-	-	-	ND	-	ND	R	S	ND	ND	ND

+ positive reaction; - negative reaction; ND, not determined; R, resistance; S, sensitive; I, intermediate; V, variable. Adapted from Haesebrouck *et al.*, 2009

The initial isolation of *H. muridarum* (Lee *et al*, 1992) and *Flexispira rappini* (Schauer *et al.*, 1993), now called *H. rappini* (Dewhirst *et al.*, 2000), from the intestinal mucosa of mice and rats with no clinical signs, was rapidly followed by the discovery of *H. bilis* (Fox *et al.*, 1995). *H. bilis* is a colonizer of liver and lower intestines, and has been associated with multifocal chronic hepatitis and inflammatory bowel diseases (IBD) in interleukin 10 deficient (IL-10 $^{-/-}$) mice. In addition, *H. hepaticus* infection induces chronic active hepatitis and hepatocellular carcinoma and typhlocolitis in A/JCr and B6C3F$_1$ mice strains (Ward *et al.*, 1993,1994), AXB recombinant inbred mice and is regarded as responsible of cholesterol gallstones, IBD and colon cancer (Rice *et al.*, 1992). Another species, *H. typhlonius*, is the causing agent of enteric disease as characterized by mucosal hyperplasia, colitis and typhlitis in severe combined immunodeficient (SCID) and IL $^{-/-}$ mice (Franklin *et al.*, 2001). Moreover, *H. trogontum* is able to elicit inflammatory responses in other regions of the gastrointestinal tract, gastric mucosa and the liver of gnotobiotic mice (Moura *et al.*, 1999). *H. rodentium* (Shen *et al.*, 1997) and *H. ganmani* have also been isolated from a colony of SCID laboratory mice (Bronwyn *et al.*, 2001), but even when the pathogenic potential and symptomatology associated with *H. rodentium* remains unknown, it has been implicated in diarrhoea when mice were co-infected with *H. bilis* and augments IBD in immunodeficient mice when co-infected with *H. hepaticus* (Myles *et al.*, 2004) or *H. mastomycrinus* (Shen *et al.*, 2005).

Among them, hepatocarcinogenic *H. hepaticus* (Suerbaum *et al.*, 1997) has been repeatedly used in pure culture to reproduce natural disease fulfilling Koch´s postulates, thus considered the prototype of all EHS. *H. hepaticus* was discovered in 1992, after 2-year carcinogenesis studies were confounded by the presence of hepatocellular tumors and hepatic hemangiosarcoma in control subjects. At that time, researchers at the National Cancer Institute, Frederick Cancer Research and Development Centre barrier-maintained facility, observed by Warthin-Starry staining and electron microscopy the bacteria in the bile canaliculi of male A/JCr mice, serving as saline-injected controls in a long-term chemical carcinogenesis assay, had a higher incidence of liver tumours than expected (Ward *et al.*, 1994). *H. hepaticus* was later also isolated from the same mice livers suffering from acute, focal non suppurative

necrotizing hepatitis which progressed to chronic active hepatitis (Fox *et al.*, 1994, 1997).

Because of the effects of such infections are not limited to the gastrointestinal system but can affect reproduction and remote other organs such as breasts and responses to vaccines assays, and since the lesions caused by EHS often mimic those seen in human IBD, the possible role of a confounding factor in mouse model liver tumorigenesis studies (Fox *et al.*, 1998), the high prevalence in rodents colonies all over the world (Feng *et al.*, 2005), the awareness of possible zoonotic origin of important clinical conditions in humans and the evidence of association with chronic inflammation and epithelial cell hyperproliferation leading to neoplasic disease (Bohr *et al.*, 2004; García *et al.*, 2006), EHS have been considered as potential murine pathogens according to international standards proposed by the Federation of European Laboratory Animal Science Associations (FELASA) (Nicklas *et al.*, 2002) . This attracted the attention of many scientists and clinicians around the world to study these diseases (Fox *et al.*, 2007), as well of the status of mice kept in research and commercial animal houses facilities (Taylor *et al.*, 2007). Moreover, additional EHS have been detected which have not yet been definitely validated as novel *Helicobacter* species (Shomer *et al.*, 2001).

The labs of Prof. Dr. Malfertheiner and Prof. Dr. Backert on campus are interested in the investigation and characterization of novel EHS found in laboratory mice colonies (Bohr *et al.*, 2006; Rivas *et al*., 2010). In contrast to the well-described *H. pylori*, almost nothing is known about potential virulence factors in EHS. It is known, however, that cytolethal distending toxin (CDT), a well-recognized toxin, first described by Johnson *&* Lior (1988), is encoded in the genomes of several Helicobacter species (Ge *et al.*, 2008). To evaluate the prevalence of EHS infections in mouse strains harboured in our specific-pathogen-free (SPF) facilities, 40 mouse lines permanently living in nine colony rooms were tested using a group-specific PCR, which detects all Helicobacter species currently known (Bohr *et al.*, 2006). When Helicobacter-negative and infected mice shared the same cage, transmission of the infection occurred within two weeks at very high frequency (100%). Furthermore, it was found that mice from commercial breeding facilities may carry undetected Helicobacter infections (Bohr *et al.*, 2006). It was also shown that infection with EHS may occur and spread frequently

in mice under SPF conditions, and despite extensive safety precautions. These recent PCR analyses also indicated a high prevalence of rather uncommon *Helicobacter* species, which may be a consequence of current routine procedures for health screening of SPF mice.

3.9 *H. acinonychis*

Eaton *et al*., (1993) isolated a spiral microorganism from the stomach of vomiting captive cheetahs (called *Acynonix jubilatus*) suffering from chronic gastritis and based on four isolates, the group described a novel *Helicobacter* species which was phenotypically similar to *H. pylori*. This species was initially named as *H. acinonyx*, after the host, and subsequently re-named as *H. acinonychis* (corrig. Eaton *et al*., 1993). Morphologically, the bacterial cells were relatively small (0.3 x 1.5 to 2 μm), spiral-shaped Gram-negative rods, without periplasmic fibers and highly motile due to the presence of monopolar tufts composed of two to five sheathed flagella. The bacteria also produced spherical forms ranging from 2 to 4 μm in diameter in stationary cultures.

H. acinonychis strains are growing well under microaerophilic conditions at 37°C but not at 25 or 42°C and produced urease, catalase, oxidase, alkaline phosphatase and δ-glutamyl transpeptidase. No growth was seen on blood agar plates under aerobic or anaerobic conditions and also no growth was observed on brucella agar plates containing 1.0% glycine or 1.5% NaCl. Biochemically and culturably *H. acinonychis* isolates were similar to previously described *Helicobacter* spp. but clearly differentiated from previously described members of the genus *Helicobacter* by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein profiling and percentage of G+C content. The complete 16S rRNA sequences and phylogenetic analysis of two *H. acinonychis* isolates demonstrated their very close relatedness to the human gastric pathogen *H. pylori*.

Clinical symptoms observed in captive cheetahs also occurs in some other big cats, and Jakobs *et al.,* (1997) used immunolabelling and electron microscopy methods to report the presence of *Helicobacter pylori-like* organisms (HPLOs) in the gastric mucosa of tigers and lions; but even when in some cases a second group of non culturable microorganisms. morphologically similar to "*H. heilmannii"* and most often referred to as *Gastrospirillum hominis* or *Gastrospirillum - like* organisms (GLO) have

also been described, their contribution in disease remained uncertain, and possibly playing a commensal role (Eaton *et al.*, 1993).

Later on Schröder *et al.,* (1998) succeeded on the isolation of *H. acinonychis* strains from the stomachs of two aged Sumatran tigers (*Panthera tigris sumatrae*) from a German zoo, with similar histological changes consisting on multiple aggregates of lymphocytes in the glandular and subglandular region of the *lamina propria* plus aggregates varying from small lymphocytic clusters to large lymphoid nodules, often with well defined germinal centres compatible to the previously reported “tiger disease” (Elze, 1978).

Worldwide, a range of conditions can affect the population of captive cheetahs ranging from outbreaks of vomiting episodes, weight loss, failure to thrive, associated with *H. acinonychis* infection (Eaton *et al.*, 1993; Munson *et al.*, 1993; Munson *et al.*, 1999), as well as progressive gastritis and lesions leading to resolution after treatment eradication (Cattoli *et al.*, 2000), conditions rarely seen in wildness despite the presence of abundant HPLOs observed in gastric mucosa (Terio *et al*., 2005). As an example, within the North American captive population of cheetahs, a moderate to severe gastritis was present in greater than 70% of animals died since 1995 after the developing of a serum A protein amyloidosis, secondary to gastritis and renal failure (Papendick *et al.*, 1997); and among the South African captive cheetah population, gastritis was a major cause of death or the reason for humanly euthanasia in 69% of cheetahs (Munson *et al.*, 1999).

Terio *et al*., (2005) histologically compared the stomachs of 23 captive and 10 wild cheetahs infected with bacteria whose ultrastructural morphology and 16S rRNA analysis identified sequences highly resembling *H. pylori* in some cases and *“H. heilmannii”* in others but only 95% similar to *H. acinonychis* irrespective of the severity of gastritis, opening the question about additional still non cultured *Helicobacter* strains contributing to the development of gastritis and supporting the premise that host and environmental factors may account for the differences in disease between captive and wild cheetah populations as well (Munson *et al.*, 1999).

Based on genome sequencing and other reports, the closest relative of *H. pylori* is *H. acinonychis* (Eaton *et al*., 1993; Dailidiene *et al*., 2004; Eppinger *et al.*, 2006). Complete sequencing of *H. acinonychis* strain Sheeba and alignment comparisons to European and African *H. pylori* strains exhibited similar core genes as well as some particular features including an unusually high number of fragmented genes for the vacuolating cytotoxin VacA and outer membrane proteins (OMPs). Consistently, gene products that become unimportant or even deleterious after a host jump might be uniformly lost, inactivated or alternatively acquired by horizontal gene transfer within the new evolved species as a mechanism of host adaptation, proposing a host jump from early humans to large felines, which probably happened about 200,000 years ago (Eppinger *et al*., 2006).

However, current knowledge on non-pylori *Helicobacter* spp. such as *H. acinonychis* is still widely incomplete. In fact, there are only very few Genbank database entries for *H. acinonychis* isolates, as illustrated by a total of only two deposited 16S rRNA sequences (accession numbers AM260522.1 and AF057163.1). Our group is very interested in identifying novel *Helicobacters*, and to characterise their genetics, bacterial pathogenicity factors and gastric disease-associated processes (Rivas *et al.,* 2010; Tegtmeyer *et al*., 2011).

Beyond the discovery of oncogenic *H. pylori*, considerable research has now focused on novel *Helicobacter* spp. isolated from humans and animals as testified by the growing number of related papers available in PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Moreover, altogether with the complete annotated genome information for *H. pylori* 26695 (Tomb *et al*., 1997) and more than a dozen other *H. pylori* strains, full sequences for *H. hepaticus* (Suerbaum *et al*., 2003), *H. mustelae* (O'Toole *et al*., 2010), *H. acinonychis* (Eppinger *et al*., 2006) and *H. felis* (Arnold *et al.*, 2011) are also accessible. The identification and characterization of novel *Helicobacter* spp. and closely related species as well as their possible association with clinical disease in their natural host and zoonotic environment with accompanied genomic studies, will provide multiple new opportunities of research that will no doubt shed light on critical genes and associated molecular events that could be targets in diagnosis, preventing and treatment of diseases in the future.

3.10 Aims of the study

Among the gastric Helicobacter species, *H. pylori* constitutes the most frequent diagnosed infection with *ca*. 50% of world population infected associated with diverse clinical outcomes. Within the Helicobacter genus there is also an emerging group of important pathogens, the enterohepatic Helicobacter species (EHS), frequently isolated from lower intestines and hepatobiliary pathways of humans and a diverse group of animals, whose extent in terms of diversity and evolutionary association is not completely explored.

The first aim of this study was to isolate and characterise morphologically, biochemically and genetically a novel EHS from the intestine of laboratory mice in a specific pathogen free (SPF) animal facility on campus at University Magdeburg. In addition, a PCR assay should be developed in order to provide a rapid test for the presence of these bacteria in mice or other animals.

The second aim of this study was to isolate and characterise new Helicobacter strains from a bengal tiger and to compare these strains morphologically, biochemically and genetically with known *H. pylori* and *H. acinonychis* species.

The results of this study should provide novel information about the prevalence and pathophysiologic relevance of EHS infections in SPF facilities, and to further characterise Helicobacters isolated from big felines, which are closely related to *H. pylori* in humans.

4. MATERIALS & METHODS

4.1 CULTURE MEDIA AND PLATES

4.2 Lysogeny broth (LB) medium (per liter)

tryptone ……10.0 g

yeast extract …….5.0 g

NaCl …….10.0 g

Note: previously to autoclave adjust pH to 7.5 or 8 with 1M NaOH

4.3 LB plates (per liter)

tryptone ……10.0 g

yeast extract …….5.0 g

NaCl …….10.0 g

Agar……7.0g

Note: previously to autoclave adjust pH to 7.5 or 8 with 1M NaOH

4.4 S.O.C. medium Final concentration

Tryptone…….. 2.0%

yeast extract…… 0.5%

NaCl…….10.0 mM

KCl……... 2.5 mM

$MgCl_2$……10.0 mM

$MgSO_4$ ……10.0 mM

glucose ……...20.0 mM

Note: adjust pH to 7.0 with 10N NaOH, autoclave to sterilize, add 20 ml of sterile 1 M glucose immediately before use.

4.5 MUELLER – HINTON (MH) (per liter)

Beef Extract ..... 2.0 g

Acid Hydrolysate of Casein ..... 17.5 g

Starch ..... 1.5 g

Agar ..... 17.0 g

Note: Final pH 7.3 ± 0.1 at 25°C

4.6 Campylobacter selective plates (per liter)

Nutrient Broth No. 2 ……25.0 g

Bacteriological charcoal…….. 4.0 g

Casein hydrolysate…….3.0 g

Sodium desoxycholate……...1.0 g

Ferrous sulphate…….0.25 g

Sodium pyruvate…….0.25 g

Cefoperazone……32.0 mg

Amphotericin B……..10.0 mg

Agar ……12.0 g

4.7 Columbia agar blood plates

Enzymatic Digest of Casein ..... 5.0 g

Enzymatic Digest of Animal Tissue ..... 8.0 g

Yeast Enriched Peptone ..... 10.0 g

Corn Starch ..... 1.0 g

Sodium Chloride ..... 5.0 g

Sheep blood.....5%

Agar ..... 14.0 g

Note: Final pH: 7.3 ± 0.2 at 25°C

4.8 *Helicobacter pylori*-selective supplements (DENT)

Vancomycin……10.0mg

Trimethoprim……5.0mg

Cefsulodin…….……5.0mg

Amphotericin B…….……5.0mg

4.9 Brain Heart Infusion BHI medium (per liter)

BHI……37.0 g

FCS……..10.0%

Vitamin-mix……...1.0%

Vancomycin……10.0mg/l

4.10 Vitamin – Mix

100 g/l Dextrose; 10g/l L-Glutamin; 26g/l L-cystein; 0,1 g/l Cocarboxylase; 0,02 g/l $Fe(NO_3)_3$; 0,003 g/l Thiamin-HCl; 0,013 g/l p-benzoic acid; 0,250 g/l NAD^+; 0,010 g/l Vitamin B_{12}.

4.11 Antibiotics **Final concentration**

Ampicillin……..……...100 µg/ml

Kanamycin……30 µg/ml

4.12 Western blot

Gel running buffer pH 8.3	**Transfer buffer pH 8.4**	**10xTBS buffer pH 7.4**
Tris .....3.2 g	Tris..... 3.0 g	Tris.....24.2 g
Glicine .....18.8 g	Glicine.....14.5g	NaCl…….. 80.0 g
10% (v/v) SDS……10 ml	SDS ……..10% (v/v)	
	Methanol……200 ml	

TBS-T buffer pH 7.4	**Coomassie stain**	**Coomassie de-staining sol.**
10xTBS pH 7.4 …100 ml	Coomassie	Methanol……. 300 ml
Tween 20……..1 ml	Briliant Blue….2.5g	Acetic acid ……...100 ml
	Methanol…..450 ml	
	Acetic acid .100 ml	

Stripping buffer pH 6.7

per 100 ml

1M Tris…… ……6.25 ml

10% (v/v) SDS…… 20.0 ml

ß-mercaptoethanol….. 0.833 ml

4.13 PCR OLIGONUCLEOTIDES

C-97 (1.2 kb)	5'-CgT ATg ACg ggT ATC C-3
CO5	5'-ACT TCA CCC CAg TCg CgT-3'
C70 (1.6 kb)	5´-AgA gTT TgA TYM Tgg C-3´
B37	5´-TAC ggY TAC CTT gTT ACg A-3´
	(Fox *et al.*, 1998)
O68 (1.5kb)	5´- Agg CgA TgA Agg ACg TA-3´
M86	5´- CYA CCT gTg WCR gTT T-3´
M93 (1.5 kb)	5´- WgC gTA AYA gCT CAC-3´
P46	5´- Cgg TCC TCT CgT ACT Ag-3´
	(Dewhirst *et al.*, 2005)
538F-*H.magdeburg*	5'-ATG CCG CCC TTG CAT CTG TC-3'
538R-*H.magdeburg*	5'-GGC GTA AAA ACT GAT GAA GCG AT-3'
	(Rivas *et al.*, 2010)

4.14 STANDARD FINGERPRINTING (RAPD) PCR PRIMERS

D-8635(RAPD)	5´-gAg Cgg CCA AAg ggA gCA gAC-3´
D-9355(RAPD)	5´-CCg gAT CCg TgA TgC ggT gCg-3´
D-14307(RAPD)	5´-ggT Tgg gTg AgA ATT gCA Cg-3´
	(Akopyanz *et al.*, 1992)
1254 (RAPD)	5´-CCg CAg CCA A-3´
1281 (RAPD)	5´-AAC gCg CAA C-3´
1283 (RAPD)	5´-gCg ATC CCC A-3´
1290 (RAPD)	5´-gTg gAT gCg A-3´
	(Dailidiene *et al.*, 2004)

4.15 *H. PYLORI* TYPING OLIGONUCLEOTIDES

*cag*A28F	5´-TTC TCA AAg gAg CAA TTg GC-3´
*cag*A-P1C	5´- gTC CTg CTT TCT TTT TAT TAA CTT KAg C-3´
*cag*A-P2TA	5-´TTT AgC AAC TTg AgT ATA AAT ggg -3´
*cag*A-P3E	5´- ATC AAT TgT AgC gTA AAT ggg-3´ (Argent *et al.*, 2005)

346F-*vac*A-S1/2 (176/203 bp)	5´-Atg gAA ATA CAA CAA ACA CAC-3´
346R-*vac*A-S1/2	5´-CCT gAR ACC gTT OCT ACA gC-3´

347F-vacA-ml/2 (401/476 bp)	5´- CAC AgC CAC TTT CAA TAA CgA-3´
347R-*vac*A-ml/2	5´-CgT CAA AAT AAT TCC AAg gg-3´

348F-*vac*A-m1/2 (570/645bp)	5´-CAA TCT gTC CAA TCA AgC gAg-3´
348R-*vac*A-m1/2	5´-gCg TCT AAA TAA TTC CAA gg-3´ (van Doorn *et al.*, 1998)

41 F-RT*vir*D4a	5´- TCA TCA ATT gCA ATA TTT ACA ggg-3
41 R-RT*vir*D4a	5´-Tag ATg AAT TTg TgC gTT TTg g- 3 '

42F-RT*vir*D4b	5´-Tag ATg AAT TTg TgC gTT TTg g- 3 '
42R-RT*vir*D4b	5 ´-TCA ATATTCATA ATATCTTgCgCg - 3 ´

43F-RT*vir*B4	5 ´-ggg TAA Tgg TgATATTACgCTAgg - 3 ´
43R-RT*vir*B4	5´-AtgTggAAgTTCAgATAA AAAggC - 3 ´

44F-RT*vir*B7	5´-ATT CATggTgCA AgTgAAgTTg-3´
44R-RT*vir*B7	5´-TCATAgCCATgCAAgTTTTTTg-3´

45F-RT*vir*B9	5´- AAg AgCgTgCAA AAA ATAgAgC-3´
45R-RT*vir*B9	5´- ACA AgA TTA gTT CTC CAA gCg C - 3´

45F1-RT*vir*B9	5´- CCA AATggA ACg ACT AgA ggA C – 3 ´
45R1-RT*vir*B9	5´- CTC TTC AAT AAC gCT AgA gAC TTC g – 3´
46F-RT*vir*B10	5´- TgC CTT Tgg AAg ATA AAA gCT C- 3´
46R-RT*vir*B10	5´- TTC AgT TTT ggC ATT TTT gAT g – 3 ´
47F-RT*vir*B11	5´- CCC CTA AAA TgA TTC TAT CAg gC - 3 ´
47R-RT*vir*B11	5´- AgCAAA ACA ACCTATCCTCATAgC- 3´
48F-RT*cag*A	5´- AAA ggA TTg TCC CTA CAA gAA gC-3´
48R-RTcagA	5´- gTA AgC gAT TgC TCT TgCATC-3´
49F-RTvacA	5´- CTT CTT CAA CCC CAg AAA ACC- 3´
49R-RTvacA	5´-ATTgCCATA ACCgATgATTTTC-3´
vacA cysS-F2*b* (530bp)	5´-TgA Tgg ACA CCC CAC AAg g-3´
va1-R	5´-CTg CTT gAA TgC gCC AAA C-3´
2579-F2*b*(1,580bp)	5´-Agg TgT CgC TTC AAg AAC AgC Cgg-3´
2579-R2	5´-gAg CAT TTT CCC gCA CTC ATA CCA Tg-3´
2579-F3*b* (1,000bp)	5´-gTg Tgg ATg ggC CgT TTg CAA TAT-3´
2579-R2	5´-gAg CAT TTT CCC gCA CTC ATA CCA Tg-3´
Vam-F*b* (706bp)	5´-ggC CCC AAT gCA gTC AgT gAT -3´
Vam-R	5´-gCT gTTA gTg CCT AAA gAA gCA T-3´
vac6-F*b* (790bp)	5´-TAA TAg AgC AAT TCA AAg AgC gCC-3´
vac6-R	5´- CCA AAH CCD CCY ACA ATR gCT T-3´
vac7-F*b* (950bp)	5´-CCA ATg TTT ggg CTA ACg CTA TTg g-3´
vac7-R	5´-gCR Ygg KTT TAA gAC Cgg TAT TT-3´

vacAs va1-F (*s1*,259)	5´-ATg gAA ATA CAA CAA ACA CAC-3´
va1-R(*s2*,286)	5´-CTg CTT GAA TgC gCC AAA C-3´
vacAs vac5-F*c* (100 bp)	5´-gTg TCg CTT CAA gAA CAg C-3´
vac5-R	5´-CCC AAC CCT AAT CTC YTT g-3´
vacAmd vam-F (706 bp)	5´-ggC CCC AAT gCA gTC AgT gAT -3´
vam-R	5´-gCT gTT AgT gCC TAA AgA AgC AT-3´
vacAm2e va4-F (352 bp)	5´-ggA gCC CCA ggA AAC ATT g-3´
va4-R	5´-CAT AAC TAg CgC CTT gCA C-3´
flaA flaA-F (870 bp)	5´-AAG AAT TYC AAg TDg gKg CTT ATT YTA AC-3´
flaA-R	5´-TTT TTg CAC AgA ACC YAA RTC AgA KCg SAC-3´
flaB flaB-F (675 bp)	5´-TTT WCT AAY AAA gAA TTT CAA ATY ggH gCg-3´
flaB-R	5´-CTg AAR TTC ACV CCg CTC ACR ATR ATg TC-3´
	(Dailidiene *et al.*, 2004)

4.16 STANDARD SEQUENCING PRIMERS

SP6	5'-CAT TTA ggT gAC ACT ATA G-3'
T7	5'-TAA TAC gAC TCA CTA TAg gg-3'
T3-Primer	5'-AAT TAA CCC TCA CTA AAg GG-3'
T7	5'-gTA ATA CgA CTC ACT ATA ggg C-3'
C97-20:	5´- ggC TAT gAC ggg TAT CCg gC-3´
H5A:	5´-CgC gTg gAg gAT GAA gg-3´
C98:	5´-gAT TTT ACC CCT ACA CCA-3´
H2:	5´-TCg CAA TgA gTA TTC CTC TT-3´
H3A-20:	5´-gCC gTg CAg CAC CTg TTT TC-3´
	(Bohr *et al.*, 2004)

4.17 DATA BANK

For literature searching and sequence analysis the following links were consulted

NCBI (National Center for Biotechnology Information): http://www.ncbi.nlm.nih.gov

Pubmed: http://www.ncbi.nlm.nih.gov/sites/entrez

16S rRNA sequence data were entered and aligned using the program RNA, which is set for data entry, editing, sequencing alignment, secondary structure comparison, similarity matrix generation, and dendrogram construction and is written in Microsoft QuickBASIC®. Similarity matrices were constructed from the aligned sequences using only those base positions for which data were available for 90% of the strains and were corrected for multiple base changes by the method of Jukes and Cantor.

A phylogenetic tree from the distance matrix was created with growtree using the UPGMA method.

4.18 BACTERIAL CULTURE

4.19 Bacterial isolation

Colonies of *Helicobacter* strains were isolated from mice intestines kept at Animal facilities, Otto von Guericke University, Germany, and from diarrhoeic faeces of a bengal tiger (*Panthera tigris tigris*) suffering from gastritis in a zoo in Thailand. Samples were incubated with brain heart infusion (BHI) medium (5 mL per gram material), shaken for 20 min at 37°C in 50mL Falcon tubes at 1,000 x *g*. The mixture was then centrifuged for 10 min at 2,000 x *g* to remove larger particles and non-digested material. The supernatant was removed and passed through sterile filter paper (Whatman, GE Healthcare, UK limited Amersham Place, UK) to further remove debris. Bacteria were then cultured in different amounts (100, 50, 25 or 5 µL) on different agar plates (*H. pylori* selective agar plates, Gentamycin-Chloramphenicol (GC) agar plates with 10% horse serum, *Campylobacter* selective plates, Müller-Hinton (MH) agar plates, and Columbia agar plates containing 5% sheep blood). These plates were incubated for 2, 3, 4, and 7 days, respectively. The gas generating systems Campygen, Anaerogen (both from Oxoid/Fisher Scientific, Germany), Anaerocult (Merck, Darmstadt, Germany), and an anaerobic chamber with a mix of N_2, CO_2 and H_2 (90%, 5% and 5%, respectively) were used for incubation at 37°C. Single bacterial colonies (called HM001, HM002, HM003, HM004, HM005, HM006 and HM007 from

mice; SB1, SB2, SB3, SB4 and SB5 from tiger) were picked and grown for further analysis.

4.20 BACTERIAL GROWTH

H. pylori strains were grown in a gas-controlled incubator under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C, usually on Columbia agar (Difco) supplemented with 5% horse blood, 0.4% Iso-VitaleX, and the antibiotics amphotericin B (8 µg per ml), trimethoprim (5 µg per ml), and vancomycin (6 µg per ml).

4.21 BACTERIAL CELLS

***Helicobacter* species**	**strains**	**source**
H. pylori	26695	Strauss-Ayali *et al*. 2001
H. pylori	J99	Alm *et al*. 1999
H. pylori	Shi470	Kersulyte *et al*. 2010
H. pylori	Cuz20	Kersulyte *et al*. 2010
H. aurati	MIT 97-5075	J. Fox
H. bilis	ATCC43879	ATCC
H. bizzozeronii	NL07-2005	N Lehn, unpublished
H. canis	ATCC51401	ATCC
H. cinaedi	DSM5359, DSMZ	Braunschweig, Germany
H. felis	ATCC49179	ATCC
H. fennelliae	ATCC35684	ATCC
H. hepaticus	1549/00	IMMB, Magdeburg
H. mustelae	NL03-2004	N. Lehn, unpublished
H. magdeburgensis	HM-007	Rivas Traverso *et al*. 2010
H. salomonis	NL07-2005	N. Lehn, unpublished
H. acinonychis	uncharacterised	Schröder *et al*. 1998
H. acinonychis	uncharacterised	Schröder *et al*. 1998
H. acinonychis	ATCC51101T	ATCC
H. acinonychis	89-624	D. Berg
H. acinonychis	89-2579	D. Berg
H. acinonychis	India	D. Berg
H. acinonychis	Sheena	D. Berg
H. acinonychis	Sheeba	D. Berg
H. acinonychis	Tiger 1	N. Lehn
H. acinonychis	Tiger 2	N. Lehn
H. acinonychis	Tiger Thailand SB-1 to SB-5	S. Backert

4.22 DNA EXTRACTION

Bacteria were harvested using a cotton swab, resuspended in sterile distilled water and centrifugated using a benchtop centrifuge 5 min x 5,000 rpm. The pellet was mixed with 200 µl of lysis buffer (50 mM EDTA, pH 8.0, 1 % SDS, 0.1 mg/ml proteinase K) and kept at 55°C for 1-2 hrs to ensure complete cell lysis. Subsequently, one-tenth volume of 3 M sodium acetate pH 5.5 was added. To remove proteins, solution was extracted with a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) and then with chloroform to get pure DNA. The DNA-containing aqueous phase was separated by centrifuging (13,000 x *g*, 10 min, RT). DNA was precipitated with 2.5 volumes of absolute ethanol and collected by centrifuging (13,000 x *g*, 30 min, 4°C). Co-precipitated salts were removed by washing with 70% (v/v) ethanol. Ethanol was discarded and the pellet allowed to dry for 15 minutes at 37°C and resuspended in 30 µl of distilled water to which 1 µl of RNase was added and incubated 30 minutes at 37°. Finally the tubes were stored at -20°C. The purified DNA was used as template for PCR amplification.

4.23 MOLECULAR BIOLOGY METHODS

4.24 Preparation of *E. coli* chemically competent cells

For preparation of *E. coli* chemically competent cells, 10 ml LB medium was inoculated with a single colony of TOP10 (Invitrogen) from a fresh overnight plate and incubated overnight at 37°C, at 200 rpm. Subsequently, 100 ml LB medium was inoculated with 1 ml of this preculture and further grown to an OD600 of 0.45-0.55. The culture was chilled on ice and centrifuged (4,000 x *g,* 10 min, 4°C). The pellet was re-suspended in 50 ml of cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. Cells were centrifuged again and re-suspended in 5 ml of cold 0.1 M $CaCl_2$/10% (v/v) glycerol. 100 µl aliquots were shock frozen on dry ice and stored at -80°C.

4.25 Transformation of chemically competent *E. coli*

Transformation was performed according to standard protocols (Hanahan *et al.,* 1983). For this purpose, 100 µl of chemically competent *E. coli* TOP10 cells were thawed on ice and mixed with 0.25 µg plasmid DNA or the total ligation sample and incubated 15 min at RT. After 10 min on ice, cells were heat-shocked for 30 s at 42°C for DNA incorporation and incubated further 10 min on ice. Afterwards, 1 ml of SOC medium

was added and cells were regenerated for 1 hr at 37°C under shaking at 200 rpm and plated on LB ampicillin/X-gal and IPTG selective plates before incubation for 18-24 hrs.

4.26 Isolation of plasmid DNA

Plasmid DNA isolation from *E. coli* was carried out with the modified alkaline/SDS lysis (Birnboim & Doly, 1979) and anion exchange adsorption method using JETSTAR plasmid purification system (Genomed, Löhne, Germany), according to the manufacturer's instructions. Briefly, *E. coli* cells were prepared by alkaline/SDS lysis and after neutralization, applied onto JETSTAR Columns (Mini, Midi or Maxi). The plasmid DNA, selectively bound to the anion exchange resin, was washed to remove impurities. Finally, the purified plasmid DNA was eluted from the column and concentrated by isopropanol precipitation. The precipitated DNA was re-dissolved in double distilled sterile water.

4.27 PLASMID PREPARATION AND PLASMID LIBRARY CONSTRUCTION FOR DNA SEQUENCING AND PRIMER DESIGN

Plasmid DNA was extracted and purified using the XSP buffer method described by De Ungria *et al*., (2002). Briefly, cell pellets were resuspended in 100 µl of TES (10mM Tris-HCl [pH 7.4], 1 mM EDTA [pH 8.0], 15 mM NaCl) by adding 1.5 ml of prewarmed (65°C) XSP buffer. XSP buffer contains (1% potassium ethyl xanthogenate, 100 mM Tris-HCl [7.4], 20 mM EDTA [pH 8.0], 1% sodium dodecyl sulfate [SDS], and 800 mM ammonium acetate) with and equal volume of phenol. The resuspended pellets were incubated for 30 min at 65°C. After a short vortexing, incubation on ice for 5 min, and centrifugation at 14,000 x g for 15 min the aqueous phase was transferred to a fresh Eppendorf. The samples were purified with two phenol-chloroform-isoamyl alcohol (25:24:1) extractions, and RNA was digested with RNase A for 30 min at 37°C. One volume of isopropanol and 0.1 volume of 3 M sodium acetate, pH 4.8, were used to precipitate the DNA, which was washed with 70% ethanol once. After being dried the plasmid DNA was resuspended in 50µl of distilled water. Chromosomal DNA was removed by digestion with and exonuclease (Plasmid-Safe ATP dependent DNase; Epicentre). Purified plasmid was digested using Sau3AI for 3 hours and fragments were cloned into pBluescript II SK (+/-) and used to transform *Escherichia coli* Top 10 to create a DNA library.

4.28 Determination of DNA concentration and quality

DNA concentration and quality was determined spectro-photometrically with use of Biophotometer and UVette cuvettes (Eppendorf, Hamburg, Germany), according to Sambrook *et al.,* (1989). The concentration of DNA in aqueous solutions can be estimated by adjusting the A_{260} measurement for turbidity (measured by absorbance at A_{320}), multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 = 50 µg/ml pure DNA. DNA concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50 µg/ml. By additional measurement at λ=280 nm, the purity of DNA can be estimated.

4.29 DNA Sequencing

Sequencing was done automatically with the Big Dye v 3.1 Terminator cycle sequencing kit (PE Applied Biosystems, Foster City, Calif. USA).

4.30 FINGERPRINTING (RAPD)-PCR

The RAPD (or AP-PCR) fingerprinting method (Akopyanz *et al*., 1992) was used to compare and the diversity of the DNA sequences. This method uses arbitrarily chosen oligonucleotides to prime DNA synthesis from genomic sites to which they are fortuitously matched, or almost matched. We used 20 ng from genomic DNA, 3.0mM $MgCl_2$, 20 pmol each primer (D8635, D9355, D14307), 1U Taq DNA-polymerase (Qiagen, Hilden) and 250 µM from each of dCTP, dGTP, dATP, and dTTP in 10 mM Tris-HCl pH 8.3, 50 mM KCl and 0.001% gelatin. A Perkin-Elmer thermal cycler model 9700 for amplification was used. The cycling program when using 10-nt primers was 4 cycles of [94°C, 5 min; 36°C, 5 min; and 72°C, 5 min], 30 cycles of [94°C, 1 min; 36°C, 1 min; and 72°C, 2 min], and then 72°C, 10min. The cycling program when using longer (≥ 17 nt) primers was four cycles of [94°C, 5 min; 40°C, 5 min; and 72°C, 5 min; low stringency amplification], and a final incubation at 72°C for 10 min. After PCR 20µl aliquots of products were electrophoresed in 2 % agarose gels containing 0.5 µg/ml ethidium bromide in the gel and 1x Tris acetate running buffer, and photographed under UV light. The 1 kb DNA ladder (Fermentas) was used as a size marker (M) in all gels.

4.31 PCR AMPLIFICATION OF *HELICOBACTER* GENUS SPECIFIC 16sRNA PRODUCTS

One set of primer sequences chosen for PCR amplification recognize a region of the 16S rRNA of the *Helicobacter* genus. This set of primers produces an amplified product of 1.2 kb and PCR was achieved by the method of Fox *et al.,* (1998). The reaction mixture (50 µl) contained 1x Platinum Taq DNA (Invitrogen Corporation) polymerase buffer supplied by the manufacturer but supplemented with $MgCl_2$ to a final concentration of 1.5 mM, 0.5 µM each of the two primers, 200 µM of each deoxyribonucleotide.

4.32 AMPLIFICATION AND SEQUENCING OF A 1.6-kb PCR PRODUCT OF THE 16S rRNA GENE

For amplification of the complete 16S rRNA gene, primers pair C70 forward and B37 reverse, were used (Bohr *et al.*, 2004). Amplicons were then purified and sequenced directly by using the amplification primers C70 and B37, as well as internal primers C97-20 and H5A (forward); and C98, H2, H3A-20 (reverse) (Fox *et al.*, 1998).

4.33 AMPLIFICATION AND SEQUENCING OF PCR PRODUCTS OF THE 23S rRNA GENE

Using primers O68 (forward), M86 (reverse), M93 (forward), and P46 (reverse), a 2.258-bp segment of the 23S rRNA gene was amplified as described (Dewhirst *et al.*, 2005) and sequenced using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). The consensus sequence was deposited in GenBank (accession number HM222564).

4.34 MICROSCOPY METHODS

4.35 Gram-staining

Grown bacterial colonies were screened by standard Gram-staining (Crystal violet, Gram's iodine solution, acetone/ethanol (50:50 vol/vol), 0.1% basic fuchsin solution). This method was applied as an initial step to investigate the morphology, homogenicity and culture purity of the isolated bacterial microorganisms.

4.36 Field emission scanning electron microscopy (FESEM)

Bacterial cells were harvested and fixed in a sterile solution containing 5% formaldehyde, 2% glutaraldehyde in cacodylate buffer (0.1 mM cacodylate, 0.01 mM $CaCl_2$, 0.01 mM $MgCl_2$, 0.09 mM sucrose, pH 6.9) for 1 hour on ice. The solution was centrifuged and passed through a sterile filter. After several washes with cacodylate buffer and TE buffer (20 mM Tris, 1 mM EDTA, pH 6.9), samples were dehydrated in serial dilutions of acetone (10%, 30%, 50%, 70%, 90%, and 100%) on ice for 15 min each step. Samples were then allowed to reach room temperature before another change of 100% acetone, after which they were subjected to critical-point drying with liquid CO_2 (CPD030; Bal-Tec, now Leica, Wetzlar, Germany). Samples were finally covered with a *ca.* 10.0 nm thick gold film by sputter coating (SCD500; Bal-Tec) and examined in a field emission scanning electron microscope (Zeiss DSM 982 Gemini) using an Everhart Thornley SE detector and in-lens detector in a 50:50 ratio at an acceleration voltage of 5.0 kV. All microscopic methods were done in close collaboration with Prof. Dr. M Rohde (HZI, Braunschweig)

4.37 Electron microscopic analysis by negative staining

For negative staining, thin carbon support films were prepared by indirect sublimation of carbon on freshly cleaved mica. Samples were then absorbed to the carbon film and negatively stained with 1% (wt⁄vol) aqueous uranyl acetate (pH 4.5). After air drying, samples were examined by transmission electron microscopy (TEM) in a Zeiss TEM 910 at an acceleration voltage of 80 kV and at calibrated magnifications using a line grid replica. Images were recorded digitally with a Slow-Scan CCD-Camera (ProScan, 1024x1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany).

4.38 Cleavage of DNA with restriction enzymes

Restriction enzymes were used according to the manufacturer's instructions (New England Biolabs GmbH, Frankfurt am Main, Germany). For analytic restriction, 20 µl reaction mixes containing: 0.5 µg of DNA and 5 U of enzyme were prepared. For preparative restriction, 30 µl reaction mixes containing 2.5 µg of DNA and 15 U of enzyme were used. Reaction mixes were incubated 2 hrs at the optimal temperature for the chosen enzymes.

4.39 Polymerase chain reaction (PCR)

PCR was used for amplification of DNA fragments, cloning and verification of bacterial strains. Reactions were carried out in the PTC-225 Peltier Thermal Cycler (MJ Research, Waltham, USA). DNA was amplified with pairs of specific primers (MWG-Biotech, Ebersberg, Germany) and Taq DNA polymerase (Qiagen) in PCR reaction mix. *Pfu* DNA Polymerase (Fermentas) was used for high fidelity amplification. The PCR programs included: one denaturation step (94°C, 5 min) and 35-40 times repeated cycles consisting of denaturation, oligonucleotide hybridization (primer annealing) and DNA synthesis. The annealing temperature used was 4°C lower than the Tm value of the shorter primer. The Tm value of primers depends on oligonucleotides' base composition and can be calculated with the following formula: Tm=2*S(AT) + 4*S(GC). The elongation times used were depending on the length of DNA fragments which were to be amplified and were calculated as follows: 1 min per 1000 bp.

4.40 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separation and size determination of DNA fragments. Agarose gels 0.8% (w/v) with 0.1 µg/ml ethidium bromide and 0.5 x TBE-buffer (44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA, pH 8.0), as running buffer were utilized. Before application on the gel, samples were mixed with 1/5 volume of 6x loading buffer (0.09% (w/v) bromophenol blue, 0.09% (w/v) xylene cyanol, 60% (v/v) glycerol, 60 mM EDTA) (Fermentas). For determination of DNA fragment sizes, GeneRuler 1 kb-DNA Ladder (Fermentas) was applied. Ultraviolet-induced fluorescence, emitted by ethidium bromide molecules intercalated into DNA, was detected with use of the Lumi-Imager F1 (Roche).

4.41 Purification of DNA fragments and extraction from agarose gel

Purification of DNA fragments was performed by agarose gel electrophoresis followed by gel extraction using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA bands of interest were excised from the 0.8% (w/v) agarose gels stained with 0.04% (w/v) methylene blue and solubilized by addition of three volumes of QG buffer and incubation at 50°C for 10 min. Subsequently, sample was applied to the QIAquick column. DNA selectively bound to

the resin was washed with PE buffer and eluted with 30 µl of water. Extracted DNA was verified by standard agarose gel electrophoresis.

4.42 Preparation of Genomic DNA in Low Melting Point (LMP) Agarose Plugs

After 24–48 hours of growth on corresponding agar plates, bacterial colonies were suspended in TE buffer (50 mm Tris, 5 mm EDTA, pH 8.0) and embedded in low melting point (LMP) agarose (Mo Bio Laboratories, Inc., Carlsbaad, CA, USA), which were subsequently placed in lysis solution containing 250 mM EDTA (pH 9.0), 0.5% lauroyl sarcosyl, and 0.5 mg of proteinase K per ml, as described previously (Chang *et al.*, 1990). One millimeter slices of the LMP agarose blocks were washed with phenylmethylsulfonyl fluoride solution (PMSF, 0.175 mg⁄mL) for 15 min, at least three times, and then washed three times with TE buffer. The agarose plugs were stored in TE buffer at 4° C until further analyses.

4.43 Restriction Digests and Pulsed-field Gel Electrophoresis (PFGE)

The LMP agarose plugs containing genomic DNA were preincubated with 100 µL of the appropriate 1x restriction enzyme buffer before digestion was carried out with 50 U of enzyme in fresh 1x buffer. All restriction digests were incubated overnight at the temperature recommended by the manufacturer. Genomic DNA was digested with the following enzymes (Roche, Indianapolis, IN, USA) *Apa*I, *Asc*I, *Bam*HI, *Bgl*II, *Cla*I, *Hind*III, *Kpn*I, *Ksp*I (*Sac*II), *Mlu*I, *Not*I, *Nru*I, *Pac*I, *Sac*I, *Sa*lI, *Sma*I, *Spe*I, *Xba*I, and *Xma*I. Restricted DNA fragments were separated by the contour-clamped homogeneous electric field method (CHEF Mapper, Bio-Rad, Hercules, CA, USA) in 1% SeaKem © Gold Agarose (Lonza, Basel, Switzerland) gels. Three different switch times were used, ranging from 3 to 35.38 seconds. Electrophoresis times varied from 12 to 18 hours at 6V⁄cm to visualize fragments of differing sizes. Two DNA markers were used to determine the sizes of the fragments. A Low Range PFGE Marker (New England Biolabs, Ipswich). The PFGE gels were done in the Lab of Prof. Omar Oyarzábal (Alabama Sate University, Montgomery, U.S.A.).

4.44 BIOCHEMICAL METHODS

4.45 Characterization Using the API Campy® kit

For the biochemical characterization, the APICampy® kit was used according to the recommendations of the manufacturer (bioMerieux, Marcy l'Etoile, France). API Campy® is a standardized system for the identification of enzymatic activities in *Campylobacter*-like bacteria, which uses miniaturized tests, as well as a specially adapted database. Briefly, the API Campy® strip consists of 20 microtubes containing dehydrated substrates which is made up of two parts. The first part of the strip (enzymatic and conventional tests) is inoculated with a dense suspension, which rehydrates the substrates. During incubation (in aerobic conditions), metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The second part of the strip (assimilation or inhibition tests) is inoculated with a minimal medium and incubated in microaerobic conditions. The bacteria grow if they are capable of utilizing the corresponding substrate or if they are resistant to the antibiotic tested. The reactions were read and evaluated in accordance with the manufacturer's identification table. These experiments were done with the help of Dr. Alexandra Clarici (IMMB, Magdeburg).

4.46 Total protein profiling

A previously published method (Megraud *et al*., 1985) was adapted. Briefly, a washed pellet of the strains to be analyzed was suspended in 0.5 mL of sodium dodecyl sulfate (SDS) buffer [(50 mM Tris hydrochloride (pH 6.8), 5% β-mercaptoethanol (vol/vol), 1% sodium dodecyl sulphate (wt/vol), 15% glycerol (vol/vol), and 0.01% bromophenol blue]. The homogenate was heated 99°C for 5 min in a thermal heat block. Insoluble debris was removed by centrifugation at 10,000 x g for 5 min at 20°C. Supernatants were stored at -20°C before testing. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system consisted of a separating gel containing an exponential 15 to 20% acrylamide gradient with an upper stacking 5% acrylamide gel. These experiments were done with the help of Dr. N. Tegtmeyer (UCD, Dublin).

4.47 SDS polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie staining and and Immunoblotting

SDS-PAGE involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportional to the length of the polypeptide chain. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. Following electrophoresis, proteins in a polyacrylamide gel can be visualized by staining (*e.g.* Coomassie staining) or blotted onto a positively charged membrane and probed with protein-specific antibodies. With the semidry electro-blotting method, the gel and membrane are sandwiched between two stacks of filter paper that have been pre-wet with transfer buffer. The membrane is placed near the anode, and the gel is placed near the cathode. Proteins are transferred to the membrane when an electric current is applied. The specificity of the antibody-antigen interaction enables single proteins to be identified in the midst of a complex protein mixture that has been immobilized on a membrane. The membrane is blocked to prevent any non-specific binding of antibodies to its surface and then a primary antibody is added. In order to locate it, a secondary antibody is applied which binds to all IgG antibodies from animal species in which primary antibody was generated. The secondary antibody is chemically coupled to a reporter, *e.g.* to an enzyme that after addition of appropriate substrate produces luminescent reaction products which allows its detection.

Whole bacterial cells harvested from agar plates are lysed in SDS-PAGE buffer (2% SDS, 10 % glycerol, 0.01% bromophenol blue, 62.6 mM Tris-HCl pH 6.8, 0.1 M DTT) (Fermentas, St. Leon-Rot, Germany), boiled at 95°C for 5 min, separated on 6-15 % polyacrylamide gels (depending on the size of the protein of interest), and either stained with Coomassie-Brilliant Blue or blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, USA). As a standard, PageRuler Prestained Protein Ladder Plus (Fermentas) was used. Preparation, running, blotting and staining of the gels was performed according to Sambrook *et al.,* 1989 with use of Mini-Protean 3 gel system, (120 V, 1.5-2 hrs, RT) (Bio-Rad Laboratories, Hercules,

USA), semi-dry blotting apparatus (0.8 mA/cm2, 2 hrs, RT) (Roth, Karlsruhe, Germany).

After blotting, membranes were blocked with TBS-T-5%-BSA or TBS-T-5% non-fat dry milk, either at 4°C overnight or 1-2 hrs at RT. Subsequently, membranes were incubated with primary antibodies overnight at 4°C or 2 hrs at RT rotating, according to the manufacturer's instructions and then washed three times for 10 min with TBS-T. As secondary antibodies, horseradish peroxidase-conjugated a-mouse IgG, a-rabbit IgG or a-goat IgG were applied for 1 h at RT, rotating (Dako Cytomation, Hamburg, Germany), followed by washing three times for 15 min with TBS-T. Immuno-reactive bands were visualized by ECL plus Western Blotting Detection System (Amersham Biosciences).

4.48 ANTIBODIES

The following primary antibodies were used: Mouse monoclonal α-CagA antibody was purchased from Austral Biological (San Ramon, CA, USA). The mouse polyclonal α-urease antibodies and α-CagN antibodies were described elsewhere (Bourzac *et al*., 2005; Kwok *et al*., 2007). Polyclonal rabbit antibodies recognizing a series of other *H. pylori* proteins, were raised against peptides corresponding to the following conserved amino acid (aa) residues derived from TIGR strain 26695 as listed below.

4.49 POLYCLONAL RABBIT ANTIBODIES	**antigen (amino acid sequence)**
α-BabA	(aa 126-140: CGGNANGQESTSSTT)
α-SabA	(aa 172-186: CAMDQTTYDKMKKLA)
α-OipA	(aa 275-282: NYYSDDYGDKLDYK)
α-NapA	(aa 105-118: EFKELSNTAEKEGD)
α-Slt	(aa 492-505: LRRWLESSKRFKEK)
α-HtrA	(aa 90-103:DKIKVTIPGSNKEY)
α-FlaA	(aa 93-106: KVKATQAAQDGQTT)
α-GGT	(aa 175-188: RQAETLKEARERFL)

α-DupA	(aa 551-564: MLNIDSDNQQDNKA)
α-VirB10/CagY	(repeat region: VSRARNEKEKKE)
α-Cagδ	(aa 32-45: IKATKETKETKKEA)
α-Rlx2	(aa 131 – 144: HLVFSIDENSNEKN)

Rabbit α-Rlx1 and α-CagM antibodies were raised against the entire recombinant Rlx1 or CagM proteins, respectively. All antibodies were affinity-purified and prepared according to standard protocols by Biogenes GmbH (Berlin, Germany). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit polyvalent sheep immunglobulin was used as secondary antibody (DAKO Denmark A/S, DK-2600 Glostrup, Denmark). Blots were developed with ECL Plus Western blot reagents (GE Healthcare, UK limited Amersham Place, UK).

5 RESULTS (PART I)

5.1 Isolation of live *Helicobacter* organisms from Mouse Intestines

Recently, the presence of EHS-DNA in 35 of 40 mouse strains harboured at the animal facility of Prof.Dr. P. Malfertheiner in the Dept. of Gastroenterology, Hepatology and Infectious Diseases on campus was detected. Direct sequencing of the PCR amplicons revealed that the mouse strains were infected with different known EHS including *H. ganmani*, *H. hepaticus*, *H. typhlonicus* as well as with novel EHS, which were not yet characterized (Bohr *et al.*, 2006). The following collaborative project was initiated with the group of Prof. Dr. S. Backert at the Dept. of Microbiology in order to identify these putative novel EHS. In order to start with the project, I applied a more direct approach for the identification, isolation, culturing, and identification of putative novel EHS that were found to be present in the intestinal tract of some mice. For this purpose, we screened animals from mouse lines that were potentially harbouring EHS. Altogether 13 mice (named HM001 to HM013) belonging to three mouse lines (BALB／c, C3H, and C57BL／6) were identified to be infected by EHS. Infected mice were then euthanized to prepare the organs and to obtain bacterial cultures. For this purpose, the ileum and the large intestine were cut into small pieces and incubated with prewarmed BHI medium. Suspensions were prepared and then cultured in different dilutions (100, 50, 25 or 5 µL) on multiple agar plates as described in Materials and Methods. *Helicobacter*-selective and GC agar plates with 10% horse serum, Campylobacter selective plates, Müller-Hinton (MH) agar plates, and Columbia agar plates containing 5% sheep blood were used for this purpose. These plates were incubated from 2 to 7 days using Campygen™ (5% O_2, 10% CO_2, and 85% N_2), Anaerogen™ (>1.0% O_2), Anaerocult™ (O_2-deficient, CO_2-enriched) or in an anaerobic chamber (92.5% N_2, 4.5% CO_2 and 3% H_2), respectively. Of the complete microbiologic setting, EHS-like colonies were only identified from a subset of mice and only under anaerobic conditions using either GC agar plates with 10% horse serum or Columbia agar plates with 5% sheep blood. EHS-like bacterial colonies were obtained from HM003 (n = 1), HM004 (n = 1), HM006 (n = 2), HM007 (n = 2), HM009 (n = 4), HM010 (n = 1), and HM013 (n = 1). Gram-staining indicated that all these bacteria were Gram-negative

(data not shown). In six other mice (HM001, HM002, HM005, HM008, HM011, and HM012), EHS-like colonies could not be cultured.

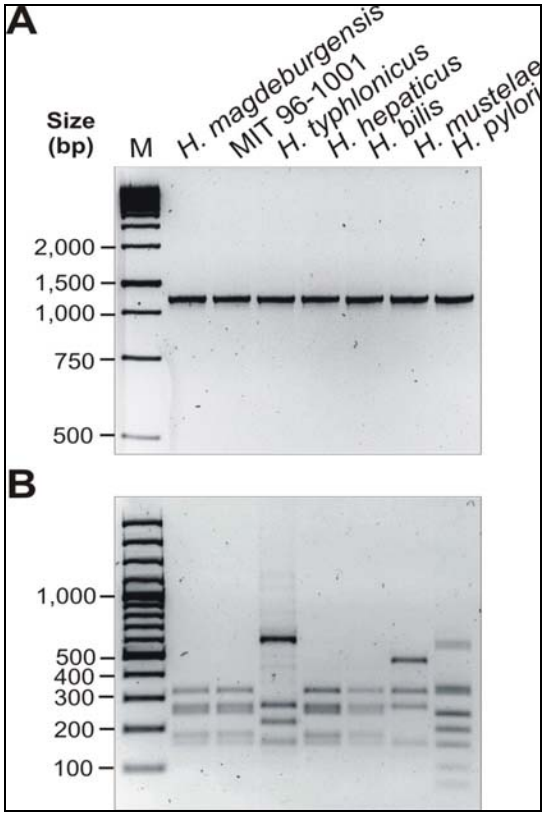


Figure 2. Investigation of 16S rRNA genes of different *Helicobacter* species by PCR and RFLP analyses. (A) DNA isolated from bacteria belonging to the genus Helicobacter (*H. magdeburgensis*, *H. typhlonicus*, *H. hepaticus*, *H. bilis*, *H. mustelae, H. pylori*, and the MIT strain 96-1001) was applied for conventional PCR of the 16S rRNA gene. A conserved 1.2-kb DNA fragment in the genus *Helicobacter* was amplified (Fox *et al.*, 1998) as shown. (B) To confirm the specificity of these fragments, all PCR products were then digested with the restriction endonuclease *Alu*I, which gives raise to a specific band pattern as described (García *et al.*, 2006).

5.2 16S rRNA and 23S rRNA Phylogenetic Analysis

We suspected that the isolated bacteria belonged to the genus Helicobacter. To test this hypothesis, DNA was isolated for conventional PCR of the 16S rRNA gene. For this purpose, a 1.2-kb DNA subfragment of the 16S rRNA gene was amplified, which is highly conserved within the genus *Helicobacter* (Fox *et al.*, 1998). I also included controls of other known *Helicobacter* species such as *H. typhlonicus*, *H. hepaticus*, *H. bilis*, *H. mustelae*, *H. pylori*, and the MIT strain 96-1001. Amplification of the 1.2-kb PCR product was achieved for each of the species as expected (Fig. 2A). To confirm the specificity of these fragments, all PCR products were then digested with the restriction endonuclease *Alu*I, which yields specific band patterns as described (García

et al., 2006). Indeed, the *Alu*I pattern was identical between the new Helicobacter isolates and MIT strain 96-1001, *H. hepaticus*, and *H. bilis* but was different to that of *H. typhlonicus*, *H. mustelae* and *H. pylori* (Fig. 2B). Similar results were obtained using other recommended restriction enzymes such as *Hha*I and *Apa*LI (data not shown).

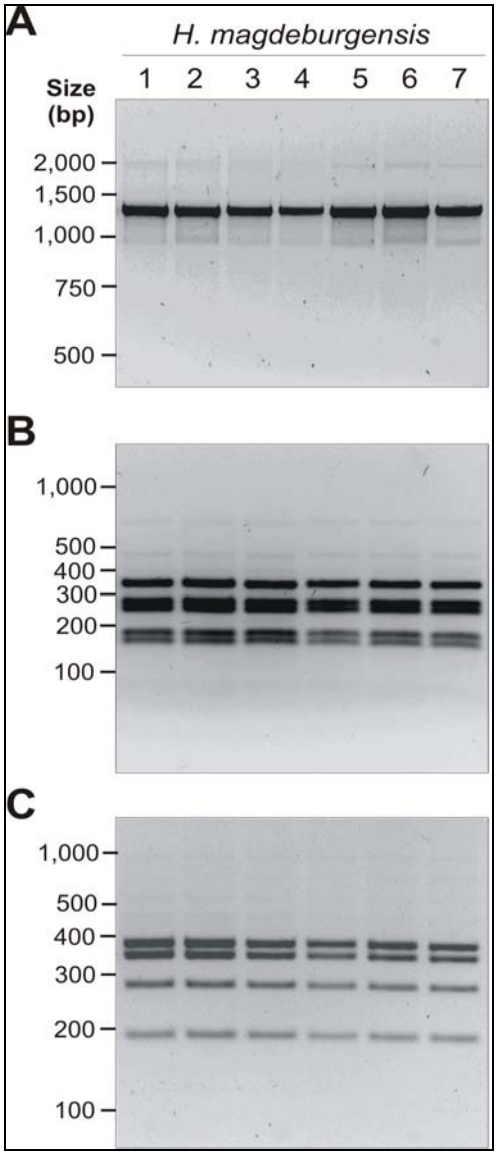


Figure 3. Analysis of the 16S rRNA gene of different *H. magdeburgensis* isolates by PCR and RFLP. (A) DNA isolated from seven individual clones belonging to *H. magdeburgensis* was investigated by conventional PCR of the 16S rRNA gene. A conserved 1.2-kb DNA fragment in the genus *Helicobacter* was amplified (Fox *et al.*, 1998). To confirm the specificity of these fragments, all PCR products were then digested with the restriction endonuclease *Alu*I (B) or *Hha*I (C) gives raise to a specific band pattern as described (García *et al.*, 2005) and was identical among all investigated clones.

Next, the 16S rRNA gene product from six other bacterial isolates from three mice (HM006, HM007, and HM009) was amplified and digested with *Alu*I and *Hha*I, respectively. The results show that the PCR products and restriction fragment sites were identical suggesting the isolation of identical bacterial species from different mice (Fig. 3A–C). Next, the complete 16S rRNA gene sequence of the isolates HM006, HM007, and HM009 was determined by sequencing of a 1,617-bp PCR product as described in Materials and Methods. Respective help was provided by Dr. Bohr. All sequenced 16S rRNA genes from these mice gave rise to completely identical sequences. The 16S rRNA gene sequence of HM007-1, representative for these isolates, was deposited in the NCBI GenBank (accession number EF990624).

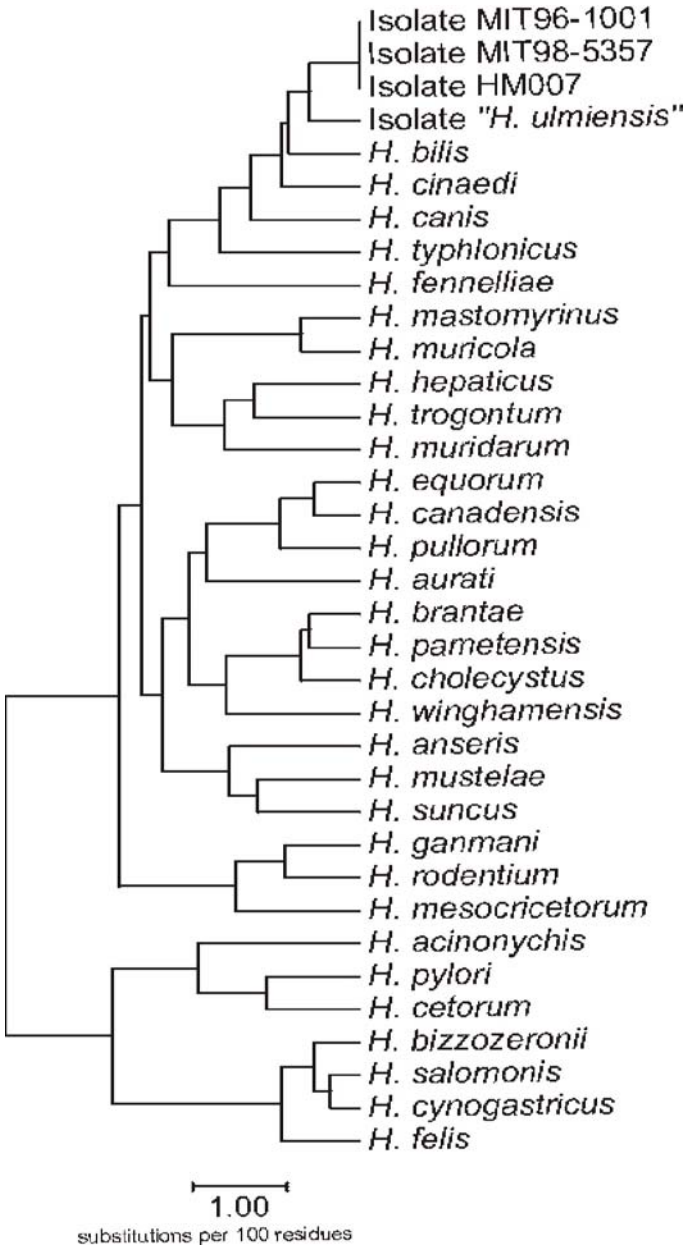


Figure 4. Phylogenetic tree of 16S rRNA sequences of different *Helicobacter* species including *H. magdeburgensis*. This data analysis was done with the kind collaboration of Dr. U.Bohr and Prof. Dr. P. Malfertheiner.

Phylogenetically, the novel *Helicobacter* species isolated from HM006, HM007, and HM009 (hereafter named *Helicobacter magdeburgensis*) belong to a specific 16S rRNA gene cluster, which includes the species *H. bilis*, *H. canis*, *H. cinaedi*, *H. typhlonicus*, and the isolates MIT 96-1001 and MIT 98-5357, and *H. ulmiensis*. The 16S rRNA sequences of *H. magdeburgensis* and that of MIT 96-1001 and MIT 98-5357 were identical but varied clearly in comparison with other *Helicobacter* species as

indicated in the phylogenetic tree (Fig. 4). Interestingly, the 16S rRNA gene of *H. magdeburgensis* contained an IVS of 179 bp. IVSs have also been described in *H. bilis*, *H. typhlonicus*, *H. ulmiensis*, and the EHS isolates MIT 96-1001 and MIT 98-5357. The IVS of *H. magdeburgensis* is identical to that of *H. bilis* and the *Helicobacter* isolates MIT 96-1001 and MIT 98-5357, while *H. typhlonicus* and *H. ulmiensis* have distinct IVS types. The 23S rRNA from HM007-1 was also amplified and sequenced as described in Materials and Methods (accession number HM222564). Sequence analysis of this gene yielded a dendrogram, which was discordant with the dendrogram generated by the analysis of the 16S rRNA gene. But this discordance is not surprising in Helicobacter species because of the possible mosaic molecules in the 16S rRNA gene and the presence of IVS in the 23S rRNA genes as well, which alter or may even produce a loss of phylogenetic information in these genes (Dewhirst *et al.*, 2005).

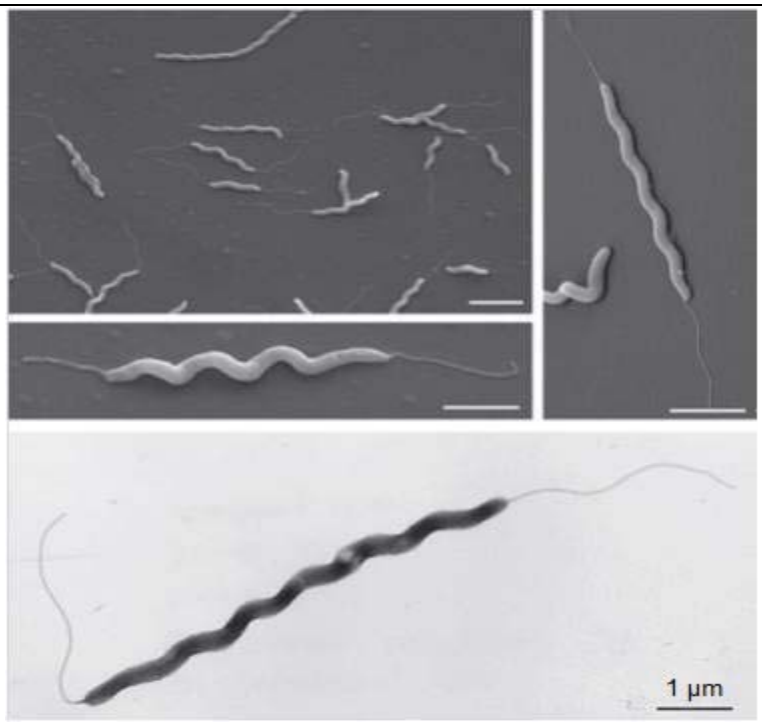


Figure 5. Morphologic analyses of novel *Helicobacter* species by electron microscopy. (A–C) Scanning electron microscopy revealed spiral-shaped bacteria that were about 0.18–0.22 µm in diameter and varied in length from about 2.5–6 µm. The majority of bacteria contained single monopolar or single bipolar flagella. Representative pictures are shown from three preparations. (D) Investigation of the *Helicobacter* species by another method (negative staining) revealed similar results with respect to size and morphology. Each bar corresponds to 1µm. The electron microscopic pictures were taken in the lab of Prof. Dr. M. Rohde at HZI Braunschweig.

5.3 Morphologic Description of the Isolated *Helicobacter* ssp.

Next, the isolated *H. magdeburgensis* from HM006, HM007, and HM009 were visualized. For this purpose, I was growing single bacterial colonies for 2 days on Columbia agar plates containing 5% sheep blood, harvested, fixed and prepared for electron microscopy as described. Scanning electronic microscopic investigation of these samples was done in the lab of Prof. Dr. M. Rohde at the HZI Braunschweig. In all cases, spiral-shaped bacteria were observed (Fig. 5A–C). These bacteria were about 0.18–0.22 µm in diameter and varied in length from 2.5 to 6 µm. The majority of these bacteria contained single monopolar or single bipolar flagella with lengths of about 1.5–2.5 µm. These flagella were commonly sheathed and about 28–32 nm in diameter. The non-sheathed flagella were about 16.5– 17.5 nm in diameter. Preparation of *H. magdeburgensis* by another method (negative staining) revealed similar results, and thus confirmed our findings (example in Fig. 5D).

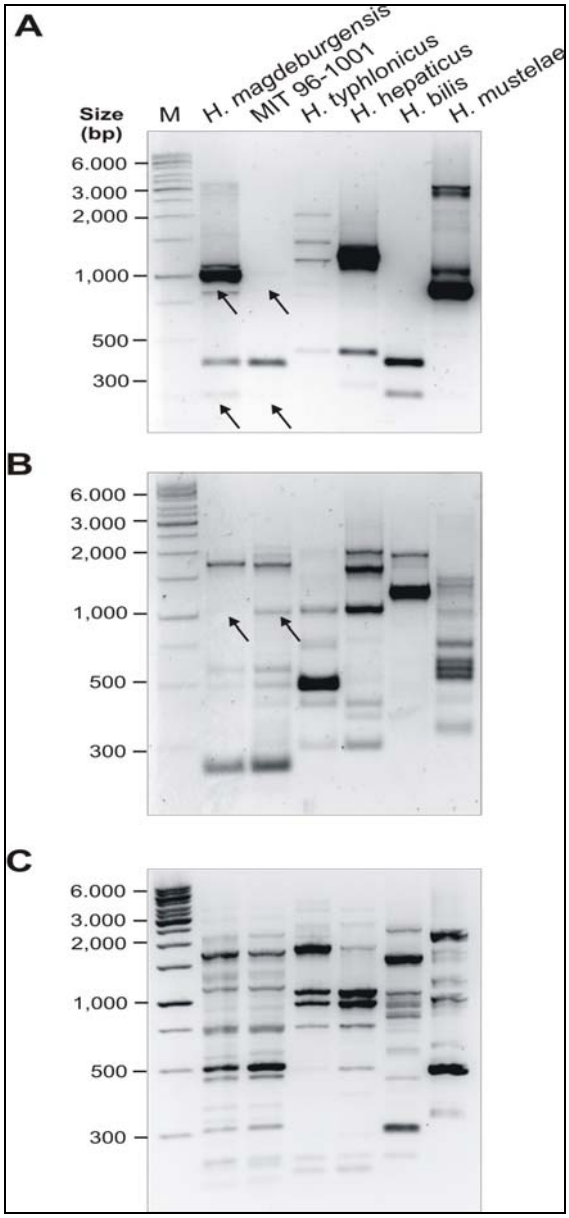


Figure 6. PCR-based randomly amplified polymorphic DNA (RAPD) fingerprinting of *Helicobacter* species. To investigate the genetic relatedness between *H. magdeburgensis* and the closest known relative (MIT 96-1001) and other strains, I performed RAPD analysis as described (Akopyanz *et al.*, 1992). (A–C) This method uses a set of single primers (D14307, D9355 or D8635), which arbitrarily anneal and amplify genomic DNA resulting in strain-specific fingerprinting patterns (Akada *et al.*, 2003). Typical RAPD fingerprinting profiles with each of the three primers are shown. Arrows indicate some bands either present or missing in *H. magdeburgensis* and MIT 96 -1001, respectively.

5.4 RAPD Fingerprinting of *Helicobacter* DNA

To further investigate the genetic relatedness between our strain and the closest known relative, MIT strain 96-1001 and other strains, I performed PCR-based

randomly amplified polymorphic DNA (RAPD) fingerprinting analysis as described elsewhere (Akopyanz *et al.*, 1992). This method uses a set of single primers (D14307, D9355 or D8635), which arbitrarily anneal and amplify genomic DNA
resulting in strain-specific fingerprinting patterns (Akada *et al.*, 2003). Typical RAPD fingerprinting profiles with each of the three primers are shown in Fig. 6A–C. All control strains tested gave different RAPD profiles, indicating that they represent unrelated Helicobacter isolates. In agreement with the 16S rRNA analysis described above, I found that the RAPD pattern of our strain with two primers was very similar to that of MIT 96-1001 (Fig. 6,C). However, using the primer D14307, I obtained a strong band at about 1 kb and a 1 weaker band at 200 bp, which were present in *H. magdeburgensis* and absent in MIT 96-1001 (Fig. 6A, arrows). In addition, a 1.1-kb band present in MIT 96-1001 is absent in our strain using primer D9355 (Fig. 6B, arrow) implying that *H. magdeburgensis* and the MIT 96- 1001 represent different strains.

Table 4. Enzymatic analysis of *Helicobacte*r spp. by API Campy® test

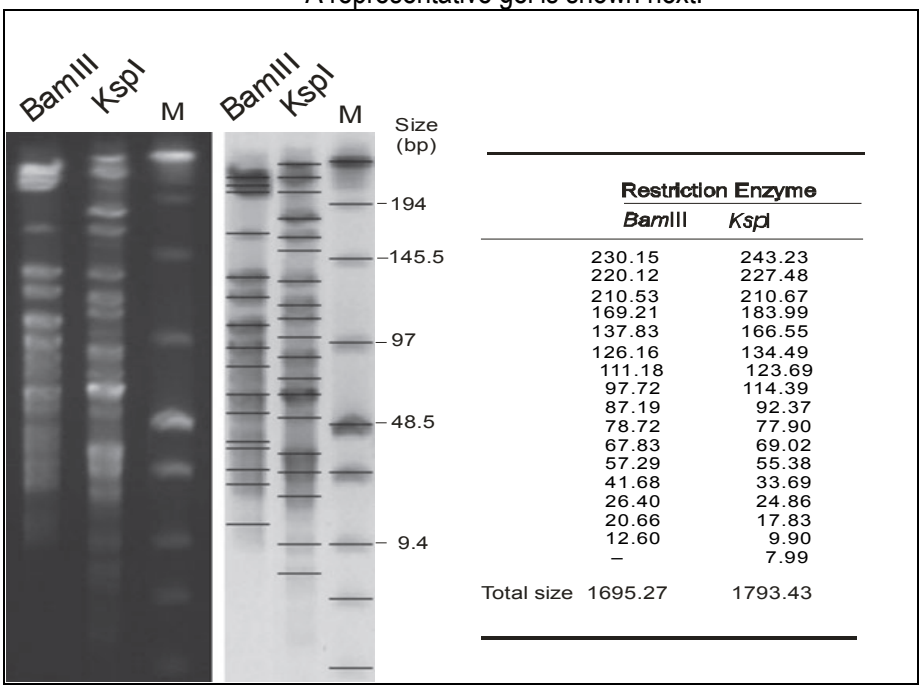
		H. magdeburgensis	MIT 96-1001
URE	UREase	—	—
NIT	Reduction of NITrates	+	—
EST	ESTerase	—	—
HIP	HIPurate	—	—
GGT	Gamma Glutamyl Tranferase	—	—
TTC	Reduction of Triphenyl Tetrazolium Chloride	—	±
PyrA	Pyrrolidonyl Arylamidase	—	—
ArgA	l-Arginine Arylamidase	—	—
AspA	L-Aspartate Arylamidase	—	—
PAL	Alkaline Phosphatase	—	—
H_2S	Production of H2S	—	—
GLU	Assimilation (GLUcose)	—	—
SUT	Assimilation (sodium succinate)	—	—
NAL	Growth inhibition (NALidixic acid)	—	—
CFZ	Growth inhibition (sodium CeFaZoline)	—	—
ACE	Assimilation (sodium ACEtate)	—	—
PROP	Assimilation (PROPionate)	—	—
MLT	Assimilation MaLaTe)	—	—
CIT	Assimilation (trisodium CITrate)	—	—
ERO	Susceptibility – therapeutic prediction (ERYthromycin)	—	—

Abbreviations used: **URE**, urea; **NIT**, potassium nitrate; **EST**, 5-bromo-4-chloro-3-indoxyl acetate; **HIP**, sodium hippurate; **GGT**, γ–L-glutamic acid- b–naphthylamide; **TTC**, triphenyltetrazolium chloride; **PyrA**, pyroglutamic acid ß–naphtilamide; **ArgA**, L-arginine-4-methoxy- ß- naphthylamide; **AspA**, aspartic acid- ß-naphthylamide; **PAL**, 2-naphthyl phosphate; **H_2S**, sodium thiosulfate; **GLU**, D-glucose; SUT, sodium succinate; NAL, nalidixic acid; CFZ, sodium cefazoline; ACE, sodium acetate; **PROP**, propionic acid; **MLT**, malic acid; **CIT**, trisodium citrate; **ERO**, erythromycin.
[a]Negative result, - ; positive result, +; intermediate values, ±

5.5 Biochemical Characterisation

To further characterize our *H. magdeburgensis* isolate, the biochemical activity of specific enzymes using the conventional API Campy® kit was determined, and I compared the data of the representative HM007 isolate with that obtained for the MIT strain 96-1001 (Table 4). *H. magdeburgensis* was urease-negative as most of the EHS strains as assessed with a simple urease test, which is in concordance with our observation that it was found in the intestine of infected mice and does not require urease activity like GHS in the stomach. The major difference identified between the tested strains was that *H. magdeburgensis* was nitrate reductase-positive, while MIT 96-1001 was not. A minor difference was observed in the reductase of tetracoleum that showed weak activity for MIT 96-1001 but strong activity for *H. magdeburgensis* (Table 4). These results further demonstrate that *H. magdeburgensis* and MIT 96-1001 are closely related but not identical.

Table 5. Determination of *Helicobacter magdeburgensis* genome size by Restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) analysis. A representative gel is shown next.



	Restriction Enzyme	
	*Bam*III	*Ksp*I
	230.15	243.23
	220.12	227.48
	210.53	210.67
	169.21	183.99
	137.83	166.55
	126.16	134.49
	111.18	123.69
	97.72	114.39
	87.19	92.37
	78.72	77.90
	67.83	69.02
	57.29	55.38
	41.68	33.69
	26.40	24.86
	20.66	17.83
	12.60	9.90
	–	7.99
Total size	1695.27	1793.43

Figure 7. Pulsed-field gel electrophoresis (PFGE) analysis of *Helicobacter magdeburgensis*. Chromosomal DNA was digested with the restriction endonucleases *Bam*III and *Ksp*I, respectively. Low Range PFGE Marker was used as the DNA size marker (M). BioNumerics software was used to identify bands and to determine band sizes.The values from the genome calculations are summarized in table 5.

5.6 Pulsed-Field Gel Electrophoresis (PFGE) Analysis of Chromosomal DNA and Calculation of Genome Size

In the next set of experiments, *H. magdeburgensis* HM007-1 was analyzed in terms of restriction enzyme digests and estimation of its genome size. For this purpose, I was growing the bacteria for two days on plates as described above, followed by preparation of LMP-agarose plugs including the intact genomic DNA as described in the Materials and Methods section. A series of commonly used restriction enzymes were then tested to determine which ones were adequate for genome mapping using PFGE. Interestingly, *Apa*I, *Asc*I, *Cla*I, *Kpn*I, *Mlu*I, *Not*I, *Pac*I, *Sa*II, *Sma*I, and *Xma*I failed to digest the genome, while *Bgl*II, *Hind*III, *Nru*I, *Sac*I, *Spe*I, *Xba*I yielded a large number of short DNA fragments that were difficult to discriminate by PFGE (Fig. 7). However, the digestion with *Bam*HI resulted in 16 DNA fragments with sizes ranging from 12.86 to 231.98 kbp, and the digestion with *Ksp*I resulted in 17 fragments with sizes between 7.99 and 243.23 kbp (Table 5). These two restriction enzymes proved to be the most suitable for PFGE analysis of the *H. magdeburgensis* genome.

In addition, we used these two enzymes to calculate the approximate genome size of *H. magdeburgensis* (Table 5). Digests with *Bam*HI revealed a total genome size of about 1,695 kb and the restriction with *Ksp*I yielded a size of about 1,793 kb, respectively.

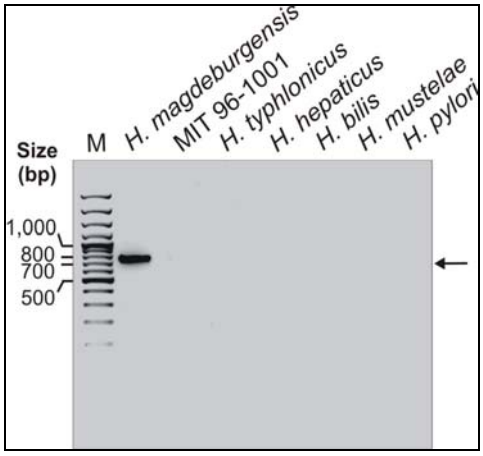


Figure 8. Development of a *H. magdeburgensis*-specific PCR detection assay. I designed specific PCR primers as described in the Materials and Methods section. Using these primers, I developed a PCR assay giving rise to a single *H. magdeburgensis*-specific 750-bp DNA fragment, which is clearly absent in all other Helicobacter isolates tested, even after 35 PCR cycles, including the MIT strain 96-1001.

5.7 Development of a *H. magdeburgensis*-specific PCR Assay

Finally, to discriminate our new isolate from other Helicobacter species, I developed a *H. magdeburgensis*-specific PCR assay. For this purpose, I digested isolated *H. magdeburgensis* genomic DNA with *Sau*3AI, a frequent cutting restriction enzyme, which produced 0.05–10-kb DNA fragments on conventional agarose gels. These fragments were cloned into the pBluescript vector and 25 randomly selected single clones were sequenced as described in the Materials and Methods section. The results showed that 12 fragments had some weak homology to chromosomal DNA from *H. hepaticus*. The majority of the other cloned inserts exhibited weak homology to DNA from other bacteria but were mainly very small in size (< 100 bp) and therefore not useful for a PCR assay. However, one of the clones having the size of about 800 bp was of particular interest because it did not show any homology to known sequences in the NCBI database. Thus, the latter DNA fragment was used to design species-specific PCR primers as described in Materials and Methods. Using these primers, I developed a PCR assay for the detection of a single *H. magdeburgensis*-specific 750-bp DNA fragment. This DNA fragment is clearly absent in all other Helicobacter isolates tested, including the MIT strain 96-1001, *H. typhlonicus*, *H. hepaticus*, *H. bilis*, *H. mustelae* and *H. pylori* (Fig. 8).

5.8 RESULTS (PART II)

5.9 Isolation and identification of *Helicobacter* strains from tigers

In a second parallel project, I investigated some novel *Helicobacter* isolates that were obtained from faecal samples of Bengal tigers from a Zoo in Thailand, as kindly provided by Prof. Dr. S. Backert. To isolate viable *Helicobacter* sp. from Bengal tigers, diarrhoeic faecal samples were prepared as described in the Materials and Methods section. Single *Helicobacter*-like colonies were identified under microaerobic growth conditions using either Columbia agar plates with 5% sheep blood or GC agar plates with 10% horse serum. Five individual colonies (called SB-1 to SB-5) were frozen in 20% glycerol-containing BHI medium for storage and further testing. Gram-staining indicated that these bacteria were Gram-negative (data not shown). We suspected that the isolated bacteria belonged to the genus *Helicobacter*. To test this hypothesis, I

isolated DNA for conventional PCR of the 16S rRNA gene. For this purpose, a 1.2-kb DNA subfragment of the 16S rRNA region which is highly conserved within the genus *Helicobacter* was amplified as described (Fox *et al*., 1998).

All five colonies gave the expected 1.2-Kb 16S rRNA PCR product (Fig. 9A, top). To confirm the specificity of these fragments, all PCR products were then digested with the restriction endonuclease *Hha*I, which yields specific band patterns for all *Helicobacter* species (Garcia *et al*., 2006). Indeed, the *Hha*I pattern revealed four bands of about 380 bp, 350 bp, 280 bp and 190 bp for each of the tiger *Helicobacter* isolates SB-1 to SB-5 (Fig. 9A, bottom). To further investigate the genetic relatedness between the colonies, I performed PCR-based randomly amplified polymorphic DNA (RAPD) fingerprinting analysis as described elsewhere (Akopyanz *et al*., 1992). As described above, this method uses a set of single primers (D14307, D9355 or D8635) which arbitrarily anneal and amplify genomic DNA resulting in strain-specific fingerprinting patterns (Akada *et al*., 2003). Typical RAPD fingerprinting profiles with two of the three primers are shown in Fig. 9B. All strains tested gave the same RAPD profiles, indicating that we have isolated genetically identical *Helicobacter* isolates.

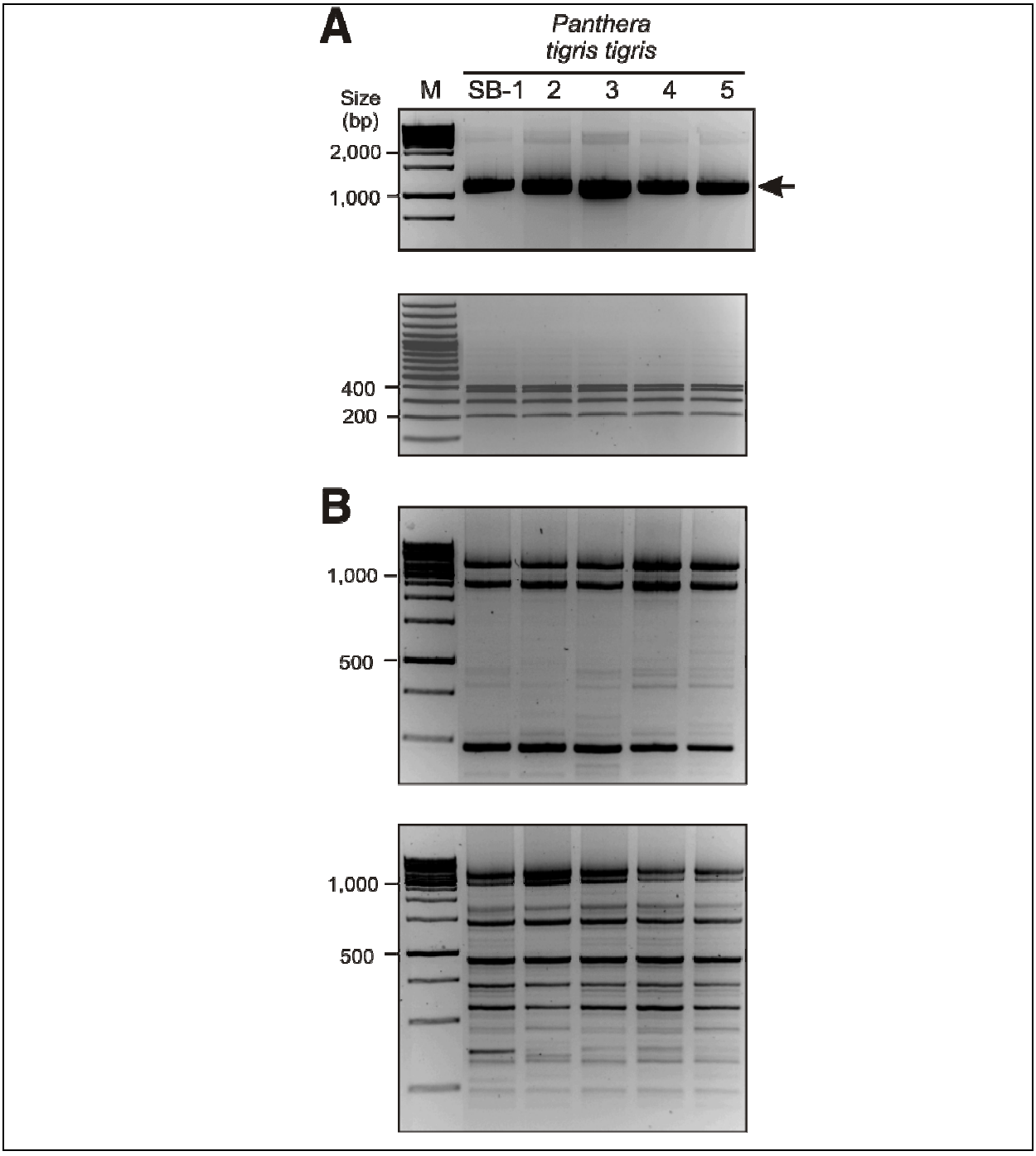


Figure. 9 Analysis of 16S rRNA and RAPD fingerprinting of different *Helicobacter*-like isolates from Bengal tiger, *Panthera tigris tigris,* by PCR. (A) DNA isolated from five individual clones was investigated by conventional PCR of the 16S rRNA gene. A conserved 1.2-kb DNA fragment in the genus *Helicobacter* was amplified (Garcia *et al*., 2006). To confirm the specificity of these fragments, all PCR products were then digested with the restriction endonuclease *Hha*I giving raise to a specific band pattern as described (Garcia *et al*., 2006), and which was identical among all investigated clones. (B) PCR-based randomly amplified polymorphic DNA (RAPD) fingerprinting of the five *Helicobacter* isolates according to a method as described (Akopyanz *et al*., 1992). Typical RAPD fingerprinting profile for primer D9355 is shown and revealed identical patterns among all investigated clones.

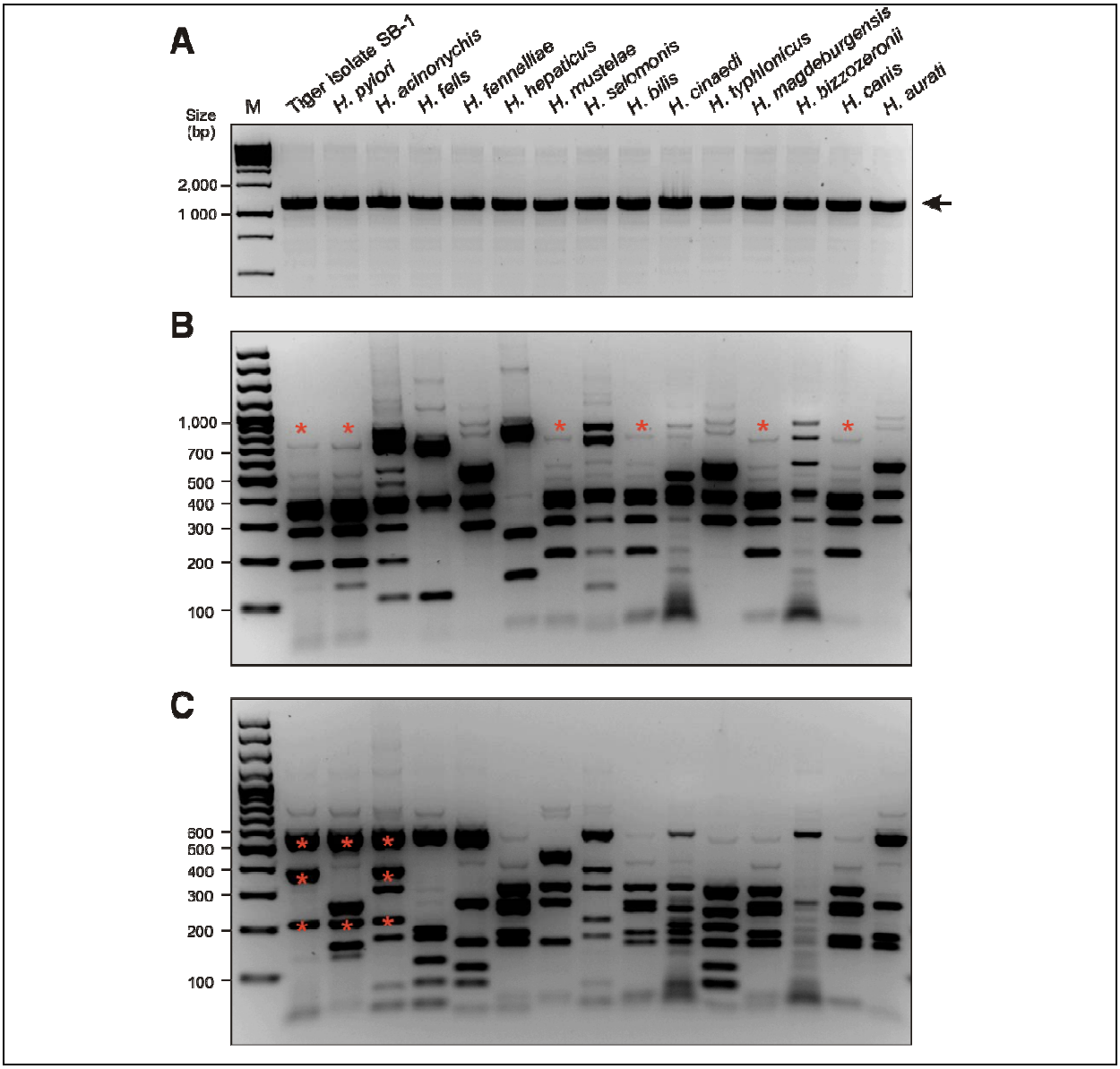


Figure 10. Investigation of 16S rRNA genes of different *Helicobacter* species by PCR and RFLP analyses. (A) DNA isolated from bacteria belonging to the genus *Helicobacter* was applied for conventional PCR of the 16Sr RNA gene as indicated. A conserved 1.2-kb DNA fragment in the genus *Helicobacter* was amplified (Fox *et al*., 1998). To confirm the specificity of these fragments, all PCR products were then digested with the restriction endonuclease *Hha*I (B). Asterisks highlight samples sharing identical RFLP patterns between the tiger isolate SB-1 and five other *Helicobacter* species as indicated. The digest with *Alu*I (C) revealed a more severe RFLP pattern among the species. The *Alu*I pattern of the tiger isolate gave raise two 3 major bands (asterisks) which were unique to all other *Helicobacters*. Some similar bands were only found in the RFLP pattern of *H. pylori* and *H. acinonychis*, indicating their close genetic relatedness.

5.10 16S rRNA and 23S rRNA phylogenetic analysis

Next, I aimed to investigate if the isolates belong to a specific known *Helicobacter* species or respresent a putative novel species. For this purpose, I

amplified a 1.2-kb of the 16S rRNA of well-known *Helicobacter* species including *H. pylori*, *H. acinonychis*, *H. felis*, *H. fennelliae*, *H. hepaticus*, *H. mustelae*, *H. salomonis*, *H. bilis*, *H. cinaedi*, *H. typhlonicus*, *H. magdeburgensis*, *H. bizzozeroni*, *H. canis* and *H. aurati*. Amplification of the 1.2 kb PCR product was achieved for each of the species as expected (Fig. 10A).

All PCR products were then digested with the restriction endonuclease *Hha*I. Interestingly, the *Hha*I pattern was identical between the tiger *Helicobacter* isolate SB-1 and *H. mustelae*, *H. bilis*, *H. magdeburgensis* and *H. canis,* while that of *H. pylori* and *H. acinonychis* revealed the same pattern but with one or more extra bands (Fig. 10B). To further investigate the 16S rRNA PCR products, I digested the 1.2-kb bands using another recommended restriction enzyme, *Alu*I (Garcia *et al*., 2006). The resulting restriction patterns were different among all employed *Helicobacter* species. The *Alu*I pattern of tiger *Helicobacter* isolate SB-1 revealed three bands of 580 bp, 440 bp and 220 bp (Fig. 10C). However, the most similar *Alu*I patterns were observed for *H. pylori* and *H. acinonychis*, suggesting that our tiger isolates are closely related to *H. pylori* and *H. acinonychis*, but not identical.

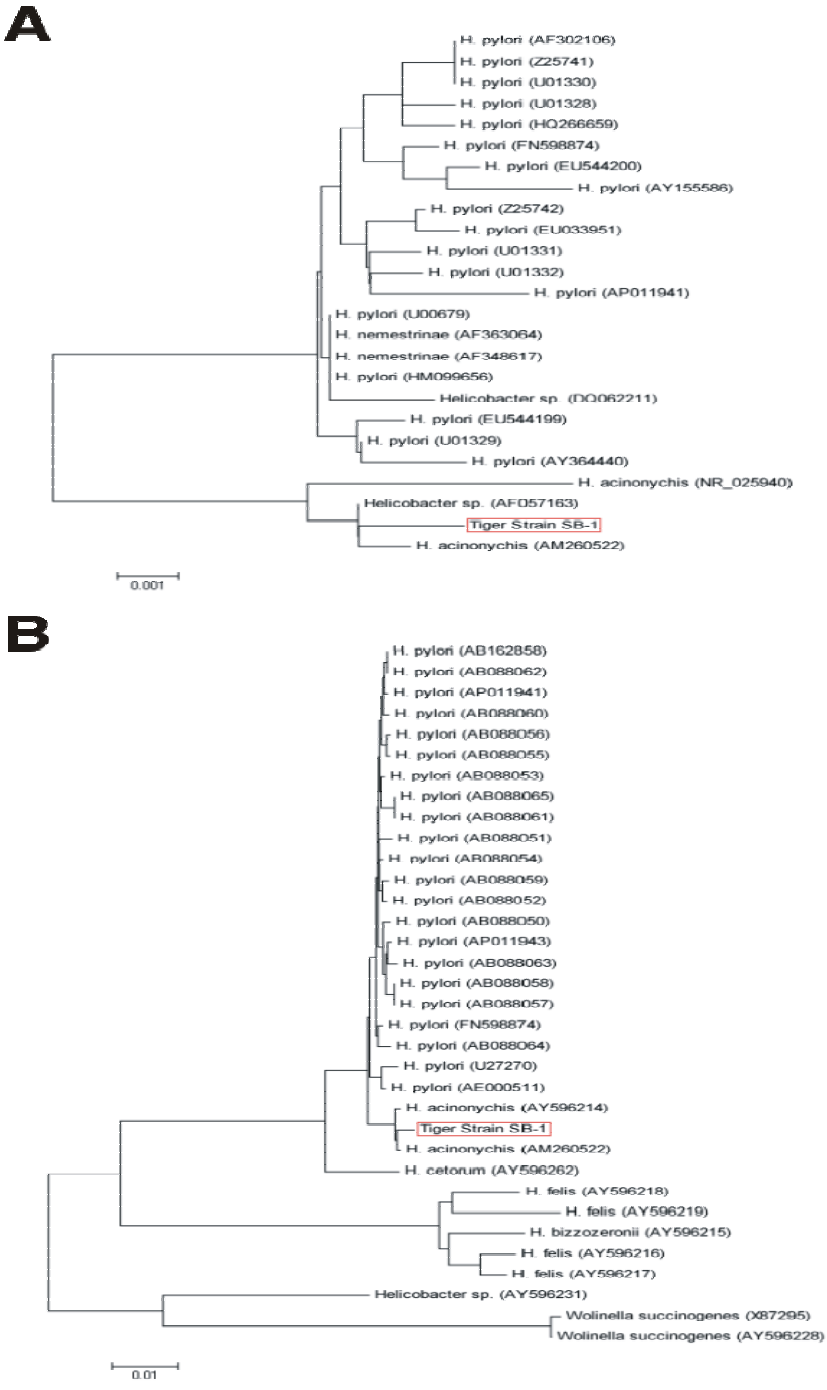


Figure 11. Phylogenetic trees of different *Helicobacter* species including the tiger strain SB-1 based on ribosomal RNA genes. The alignment was done with BioEdit using gap penalties of 10 for gap opening and 5 for gap extension. MEGA5 was used to infer DNA relatedness using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The optimal tree with the sum of branch length was equal to 0.0360 for 16S rRNA (A) and 0.3061 for 23S rRNA (B).The phylogenetic trees for the 16S rRNA (A) and 23S rRNA (B) gene sequences are shown. In both cases our strain branched together with *H. acinonychis* strain AM 260522, thus demonstrating a good relatedness among them. This data analysis was done with the kind support by Prof. Dr. O. Oyarzabal.

Next, the complete 16S rRNA gene sequences of the tiger *Helicobacter* isolate SB-1 to SB-3 were determined by sequencing of a 1.617-bp PCR product as described in Materials and Methods. All sequenced 16S rRNA genes from the tiger gave rise to completely identical sequences. The 16S rRNA gene sequence of SB-1, representative for these isolates, was deposited in the NCBI GenBank (accession number 1463758). In agreement with the results described in Fig. 10C, the isolated tiger *Helicobacter* species from SB-1, SB-2, and SB-3 belong phylogenetically to a specific 16S rRNA gene cluster, which includes isolates of the species *H. acinonychis* and *H. pylori* (Fig. 11A). The 23S rRNA from SB-1 was also amplified and sequenced as described in Materials and Methods. Sequence analysis of this gene yielded a dendrogram which was also in full agreement with that generated by the analysis of the 16S rRNA gene (Fig. 11B).

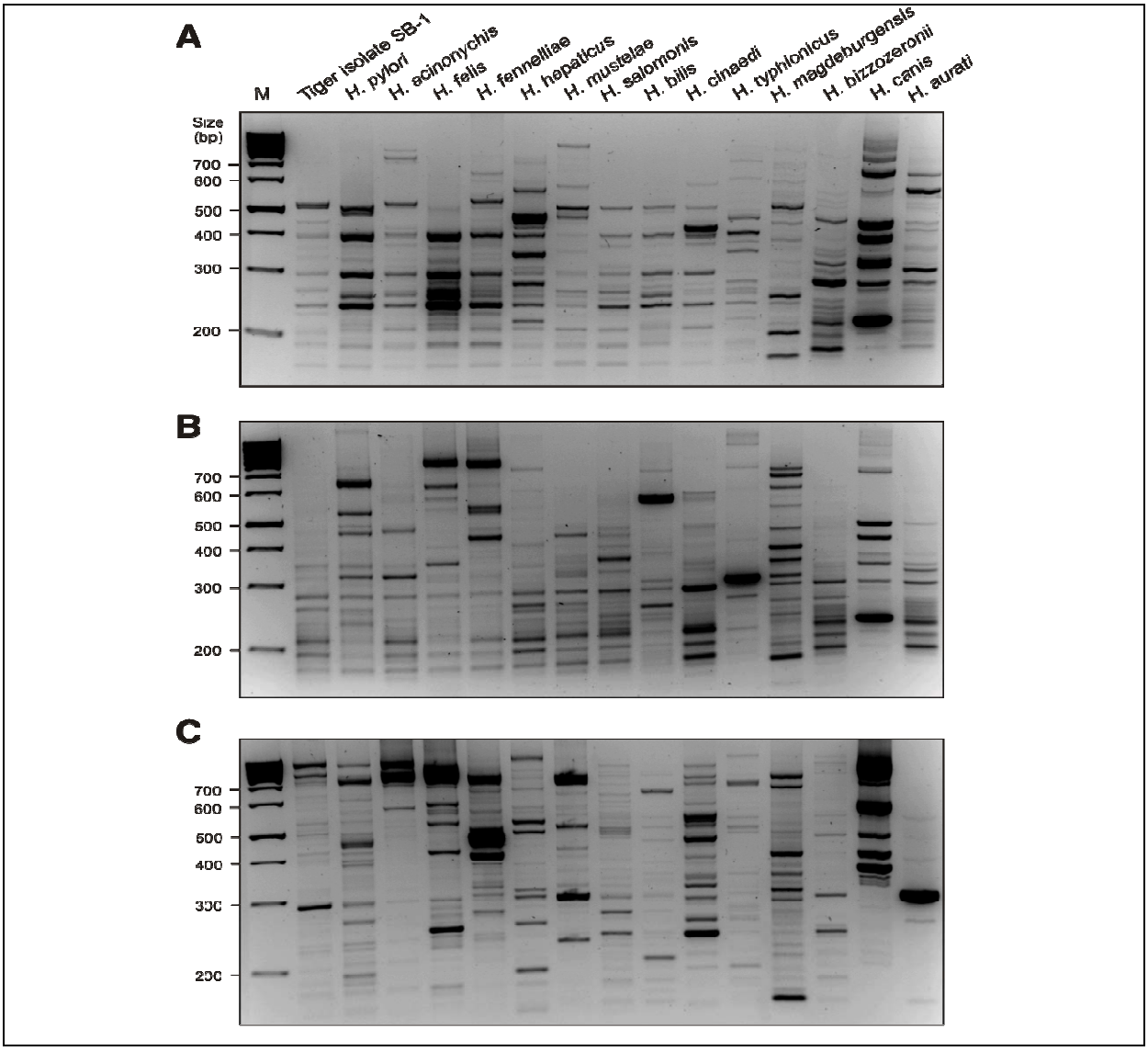


Figure 12. PCR-based randomly amplified polymorphic DNA (RAPD) fingerprinting of *Helicobacter* species. To investigate the genetic relatedness between the tiger isolates and other *Helicobacter* species, we performed RAPD analysis as described (Akopyanz *et al*., 1992). (A-C) Typical RAPD fingerprinting profiles with each of the three primers are shown for a set of single primers (D14307, D9355 or D8635).

5.11 RAPD Fingerprinting of *Helicobacter* genomic DNA

To further investigate the genetic relatedness between the tiger isolates and other *Helicobacter* species, I performed PCR-based RAPD fingerprinting analysis with all available species as described above. As shown in Fig. 12A-C, the employed *Helicobacter* species gave raise to highly diverse RAPD profiles using primers D14307, D9355 or D8635, indicating that they represent unrelated *Helicobacter* isolates. In agreement with the 16S and 23S rRNA analyses described above, it was found that the RAPD patterns of SB-1 exhibited some similarities to that of *H. acinonychis* and *H.*

pylori, but also some bands which clearly different among these three related *Helicobacters* (Fig. 12A-C).

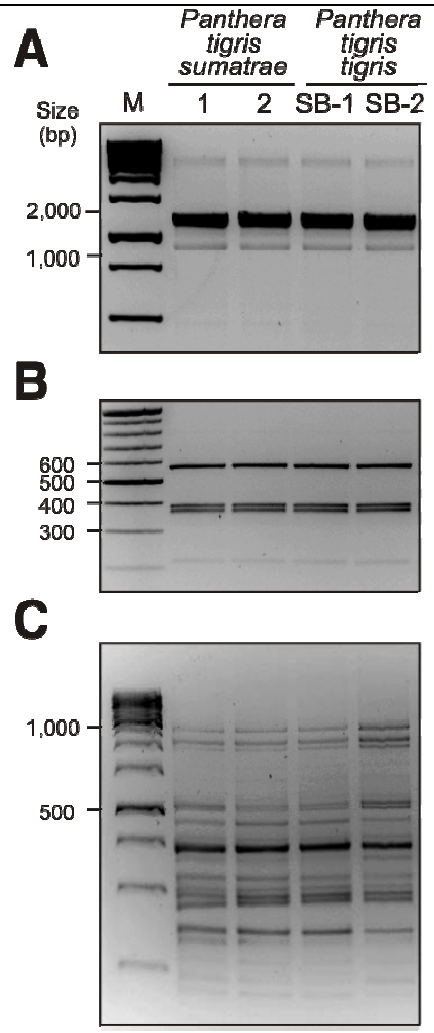


Figure 13. Analysis of 16S rRNA of different *Helicobacter* isolates from tigers (*Panthera tigris sumatrae* and *Panthera tigris tigris*) by PCR and RFLP. (A) DNA isolated from two individual clones belonging to both tiger species was investigated by conventional PCR of the 16S rRNA gene. A conserved 1.2 kb DNA fragment in the genus *Helicobacter* was amplified (Garcia *et al*., 2006). To confirm the specificity of these fragments, all PCR products were then digested with and the restriction endonuclease with *Alu*I (*B*) or *Hha*I (C), giving raise to a specific band patterns as described (Garcia *et al*., 2006), which where identical among all investigated clones.

5.12 Genetic comparison of *Helicobacter* isolates from Bengal and Sumatra tigers

Next, I compared the DNA typing patterns of two Bengal tiger isolates (SB-1 and SB-2) with that of two other *Helicobacter* isolates from Sumatra tigers maintained in captivity in a German zoo (Schröder *et al*., 1998). For this purpose, the isolated DNAs from the

Sumatra tigers were kindly provided by Prof. Dr. N. Lehn (University Regensburg). The 16S rRNA and RAPD PCRs were performed as described above. Amplification of the 1.2 kb PCR product was achieved for each of the species as expected (Fig. 13A), and restriction with *Alu*I yielded identical patterns (Fig. 13B). In agreement with this observation, the nucleotide sequence of the 16SrRNA gene was highly similar between the tiger *Helicobacter* sp. isolate SB-1 and one *Helicobacter* sp. isolate from Sumatra tigers with accession number AF057163 (Fig. 13A). In addition, the RAPD PCR patterns were also very similar between *Helicobacter* spp. from the Bengal and Sumatra tigers (Fig. 13C), indicating that closely related *Helicobacter* species colonize different tiger species.

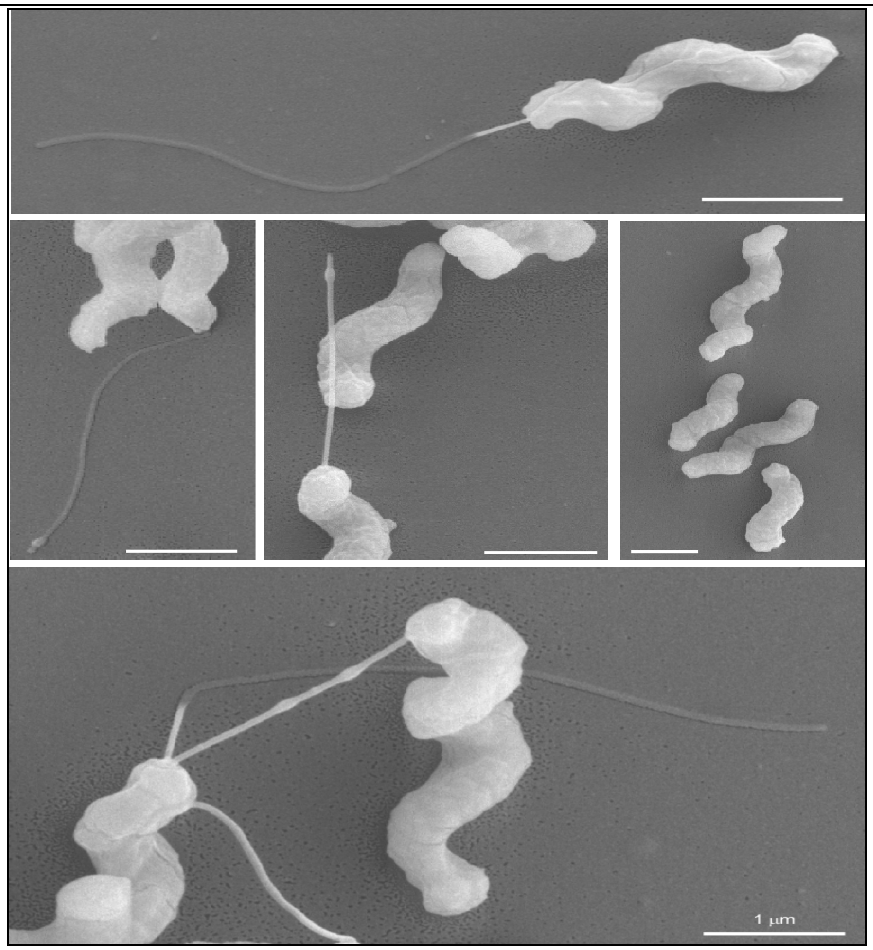


Figure 14. Morphological analyses of novel *Helicobacter* species by scanning electron microscopy (SEM). SEM revealed spiral shaped bacteria which were about 0.18-0.22 µm in diameter and varied in length from about 2.5-6 µm. The majority of bacteria contained 1-4 monopolar flagella. Representative pictures are shown from two preparations. Each bar corresponds to 1 µm. The electron microscopic pictures were taken in the lab of Prof. Dr. M. Rohde at HZI Braunschweig.

5.13 Electron microscopic characterisation

Next, the isolated tiger *Helicobacter* sp. isolates SB-1 and SB-2 were visualized. For this purpose, single bacterial colonies were grown for two days on Columbia agar plates containing 5.0 % sheep blood and prepared as described. Scanning electronic microscopic investigation revealed spiral-shaped organisms (Fig. 14). These bacteria were about 0.25-0.45 µm in diameter and varied in length from 2.5 to 6 µm. The majority of these bacteria contained 1-4 monopolar flagella with lengths of about 1.5-4.5 µm, although some bacteria were non-flagellated (Fig. 14).

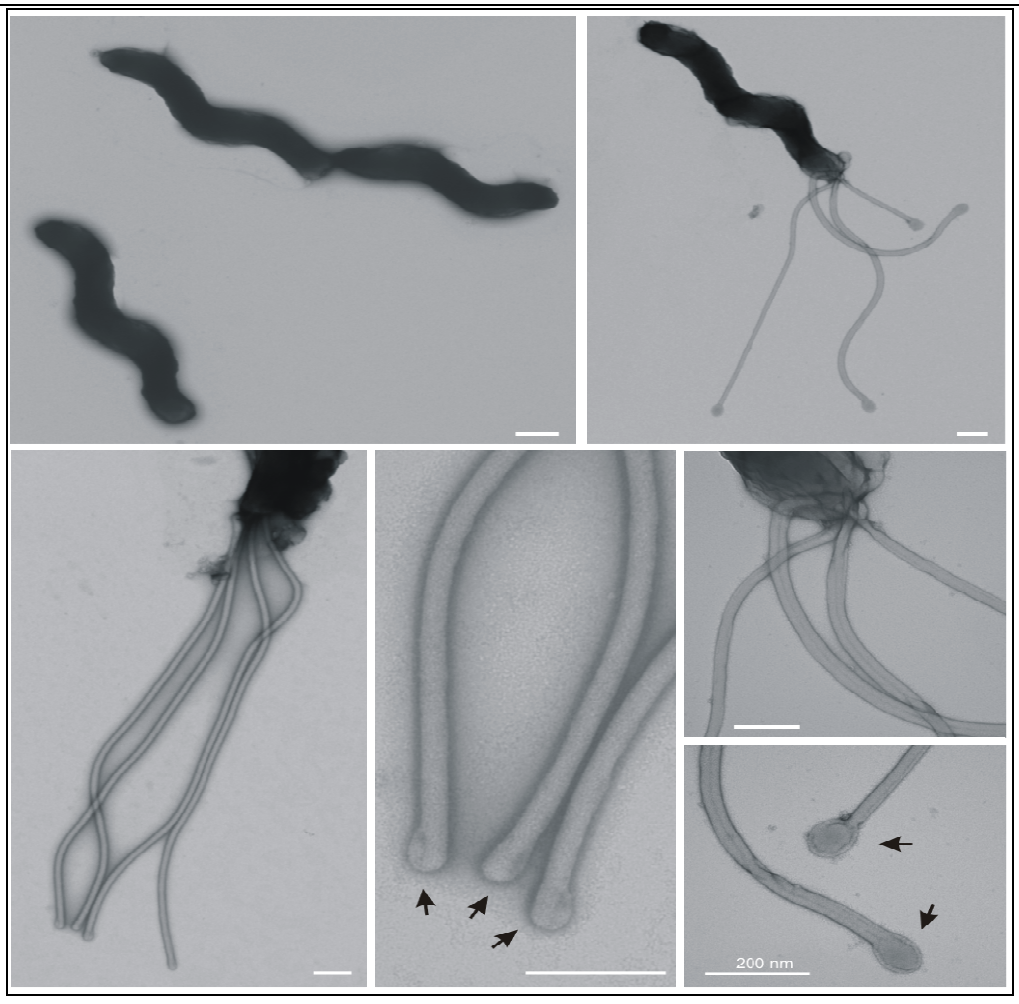


Figure 15. Morphological analyses of novel *Helicobacter* species by negative stain electron microscopy. Negative staining revealed similar results as observed for SEM as shown in Fig.6. The majority of bacteria contained 1-4 monopolar flagella. Representative pictures are shown from two preparations. Each bar corresponds to 200 nm. The electron microscopic pictures were taken in the lab of Prof. Dr. M. Rohde at HZI Braunschweig.

Preparation of the tiger *Helicobacter* isolates by another electron microscopic method (negative staining) revealed similar results and thus confirmed our findings (Fig. 15). The visualized flagella were commonly sheathed and about 43-48 nm in diameter.
The non-sheathed flagella were about 15-17 nm in diameter (see enlargement panels in Fig. 15). In addition, it was commonly detected that the flagella ends were thickened and had a bulb-like shape (see enlargement panels in Fig. 15, arrows).

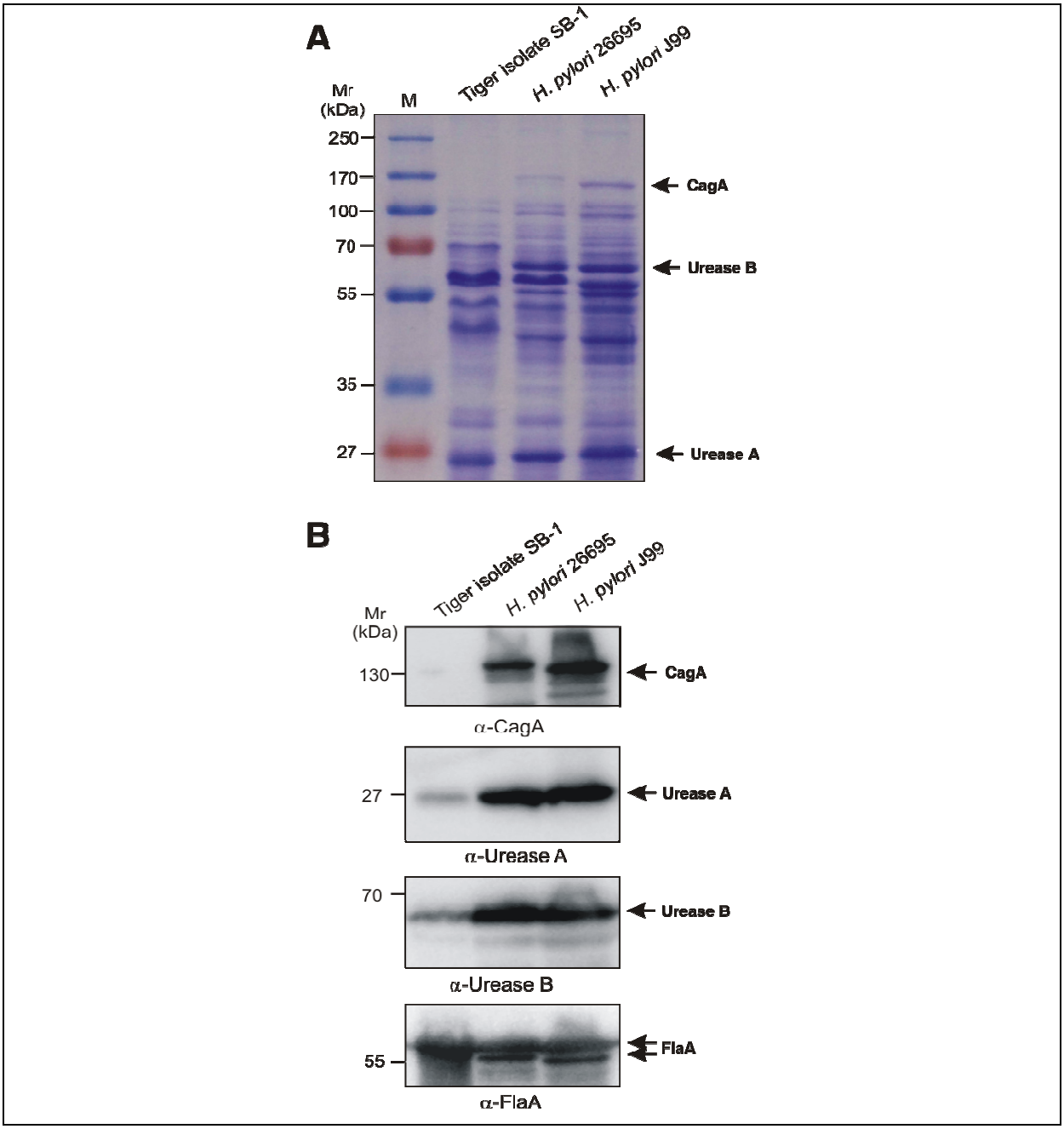


Figure 16. Total protein profiling of the *Helicobacter* isolate SB-1 from *Panthera tigris tigris* in comparison to that of *H. pylori* strains 26695 and J99. Total proteins were isolated, separated by SDS-PAGE and stained with Coomassie Blue. The protein profiles of both 26695 and J99 strains revealed the typical *H. pylori* patterns with strong bands visible for CagA and the two major urease subunits A and B as indicated. (B) Western blotting analysis using *H. pylori*-specific antibodies against CagA, UreA, UreB and FlaA. This study revealed that while FlaA is similarly expressed in SB-1, both urease subunits revealed less intense bands and no CagA expression was detectable.

5.14 Total protein profiling and Western blot analysis

The close relatedness of tiger *Helicobacter* isolates with *H. pylori* led us to screen for the presence or absence of well-known colonization and pathogenicity factors by immunoblotting. First, the total protein profiles from our tiger isolate SB-1 with that of

the fully-sequenced *H. pylori* strains 26695 and J99 were compared, also ensuring that equal amounts of protein are loaded on each gel. Coomassie-blue staining of separated total proteins revealed the presence of typical bands sizes compatible with urease subunits A and B. However, a band in the size range of CagA (*ca.* 140-150 kDa) was only observed in both *H. pylori* strains as expected, but not in SB-1 (Fig. 16A). Second, Western blot analysis confirmed the presence of expressed urease A (*ca*. 30 kDa) and urease B (*ca*. 60 kDa) subunits as well the absence of a CagA expression in SB-1 (Fig. 16B). In agreement with the observation of flagella by electron microscopy, it was also found that SB-1 expresses the flagellin component FlaA (*ca*. 55 kDa) subunit as recognised by the *H. pylori*-specific anti-FlaA antibody (Fig. 16B, bottom).

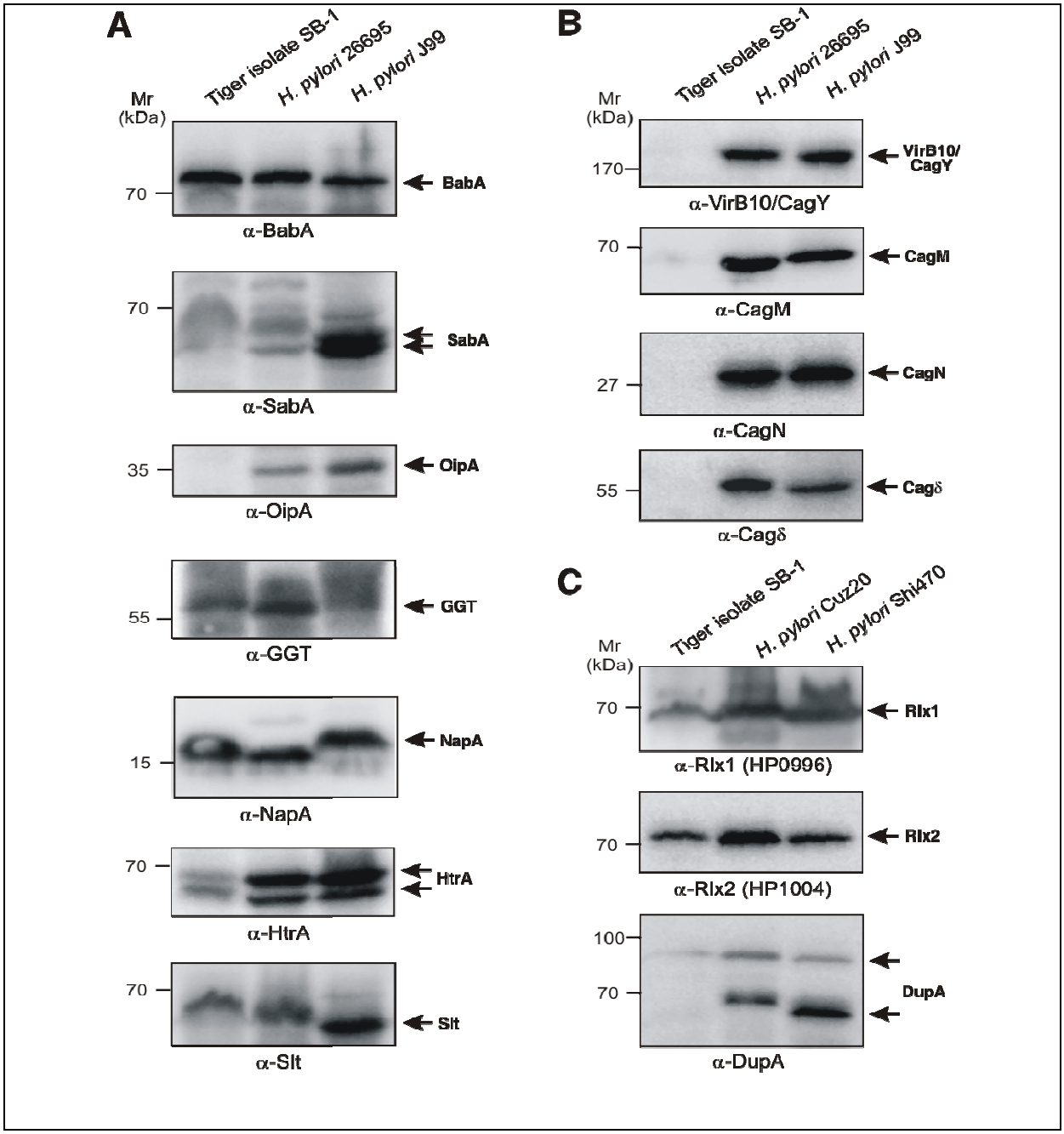


Figure 17. Western blotting analysis of the *Helicobacter* isolate SB-1 from *Panthera tigris tigris* for well-known pathogenicity-associated factors reported in *H. pylori*. Total proteins were isolated, separated by SDS-PAGE and stained with antibodies against typical *H. pylori* proteins. (A) Detection of typical *H. pylori* adhesins (BabA, SabA and OipA) and other pathogenicity-associated factors (γ-GGT, NapA, HtrA and Slt. (B) Detection of typical *H. pylori cag*PAI factors (VirB10, CagM, CagN and Cag3). (C) Western blotting analysis using *H. pylori*-specific antibodies against two potential DNA-transfer proteins Rlx1 and Rlx 2 (Backert *et al*. 1998, 2005) and the duodenal ulcer promoting gene A (DupA).

The availability of antibodies against a series of *H. pylori* proteins suggested to screen more systematically for potentially encoded homologs of certain adhesins

promoting adherence (BabA, SabA or OipA), other virulence and pathogenicity factors (NapA, HtrA, Slt, DupA), *cag*PAI factors (VirB10/CagY, Cagδ, CagM or CagN) or putative DNA transfer proteins (the VirD2 orthologs, Rlx1 and Rlx2). All antibodies were proven to specifically recognise the corresponding proteins in the *H. pylori* strains 26695 and J99, or Shi470 and Cuz20, respectively (Fig. 17A-C). It was found that while no SabA, OipA, DupA and none of the *cag*PAI proteins are expressed in SB-1, bands corresponding in size to BabA, γ-GGT, HtrA, NapA, Slt, Rlx1 and Rlx2 were detected, indicating that SB-1 encodes for genes expressing homologous *H. pylori* proteins in the tiger isolates (Fig. 17A-C). As observed in *Helicobacter* spp. from other big cats such as lions and cheetahs (Dailidiene *et al*. 2004), PCRs of DNA from the tiger strain SB-1 revealed typical products for the fragmented *vacA* gene which is not expressed in these bacteria (data not shown).

6 Discussion

Aim of this PhD thesis was to isolate and characterize at the molecular level two novel *Helicobacter* species isolated from mammals, laboratory mice and Bengal tigers. Both projects led to two independent scientific papers (Rivas *et al*., 2010; Rivas *et al*., 2011; submitted), and will therefore be discussed separately below.

6.1 Discussion (part I)

Helicobacter is a rapidly expanding bacterial genus with a wide host range but limited biological niches. The respective ecological division and taxa are often referred as gastric *Helicobacter* species (GHS) and enterohepatic *Helicobacter* species (EHS) (On, 2001). EHS are emerging as important pathogens within this genus (Fox, 2002). EHS can colonize the lower gastrointestinal tract, including the ileum, caecum, colon and biliary tree. Similarly to GHS, EHS can cause persistent infections associated with chronic inflammation and epithelial cell hyperproliferation leading to neoplastic transformation (Taylor *et al.*, 2007). EHS are also confounding factors in inflammatory bowel disease in the mouse animal model (Bohr *et al.,* 2006). The interest in keeping healthy mice in research facilities, and the studies of the zoonotic potential of these mouse populations (Azevedo *et al*., 2008) has stimulated researchers worldwide to investigate EHS in more detail. Importantly, there is very little information concerning the *Helicobacter* status in non-commercial animal facilities. Numerous recent studies using culture and PCR methods indicated that the presence of EHS can become a very common problem in commercial mouse colonies (Goto *et al*., 2000; Nielsson *et al*., 2004; Seok *et al*., 2005; Bohr *et al*., 2006). These infections often remain non-recognized but can cause severe health complications and, thus, can also change the results of animal experiments.

In recent studies using PCR assays from faeces of laboratory mice in the department of Prof. Dr. P. Malfertheiner, it was found that the most frequently detected DNA from *Helicobacter* species corresponded to that of *H. ganmani* and MIT 98-5357 (Bohr *et al*., 2006). No species-specific PCR assays or other detection methods have been established for the analysis of these bacteria in animal health screens. Therefore, these rather uncommon *Helicobacter* species often remain undetected by the routine

screening procedures, which can explain the relatively high prevalence of these rare species in *Helicobacter*-infected laboratory mice. In order to avoid the spread of *Helicobacter* infections in any animal facility of research institutions, it is therefore important to elucidate novel *Helicobacter* species, characterize them at the molecular level, and study the transmission route and possible disease outcome.

In the present thesis, I first described the direct isolation and molecular characterization of a novel urease-negative, straight spiral or curved rod-shaped Gram-negative *Helicobacter* species from laboratory mice in our animal facility on university campus. This bacterium is highly motile by means of single monopolar or bipolar sheathed flagella without helical periplasmic fibrils. Using two different electron microscopic methods (SEM and negative staining), these bacteria were measured 0.3-0.6 µm in width and had lengths ranging from about 1.0 to 6.0 µm. Analysis of the 16S rRNA revealed that the bacterium is a novel member of the genus *Helicobacter* but distinct from known species, thus, we proposed to name it *Helicobacter magdeburgensis.* Further analysis of biochemical traits and morphologic characteristics as well as genetic analysis revealed that this bacterium is closely related to a MIT 96-1001 *Helicobacter* strain, however, we could differentiate them by means of Api Campy®, RAPD fingerprinting and other methods.

In addition, the RFLP and subsequent PFGE analysis of chromosomal DNA revealed that this bacterium has an approximate genome size of 1.7 to 1.8 Mbp. Interestingly, restriction enzymes including *Apa*I, *Asc*I, *Cla*I, *Kpn*I, *Mlu*I, *Not*I, *Pac*I, *Sal*I, *Sma*I and *Xma*I failed to digest the genome, while other commonly used enzymes such as *Bgl*II, *Hind*III, *Nru*I, *Sac*I, *Spe*I, *Xba*I yielded a large number of short DNA fragments. The latter findings indicated incomplete digests, and therefore the resulting bands were found difficult to discriminate on PFGE gels. However, the digestion of chromosomal *H. magdeburgensis* DNA with two other enzymes, *Bam*HI or *Ksp*I, resulted in suitable PFGE patterns to map the entire genome. Both enzymes were therefore used to calculate the approximate genome size of *H. magdeburgensis,* being in the size range of about 1.7 or 1.8 Mbp, respectively. Both values are slightly higher than the genome of *H. hepaticus* but they are in concordance with other *Helicobacter* spp. (Saunders *et al.*, 1997; Suerbaum *et al.*, 2003). In addition, I have cloned some genomic DNA fragments for sequencing and developed a species-specific PCR assay that can be

efficiently used for rapid and specific differentiation of *H. magdeburgensis* from other common EHS. Interestingly, this 750 bp genomic DNA fragment, which was selected, is obviously absent in the MIT 96-1001, further demonstrating that *H. magdeburgensis* and MIT 96-1001 are different EHS strains.

In contrast to the plethora of *bona fide* or putative pathogenicity/virulence or immunomodulatory factors described for the genus type species, *H. pylori*, there is a paucity of information about EHS, despite the increasing evidence of their role as disease agents in immunocompetent rodent colonies as well as their association with human and non human primates disease (Bohr *et al.*, 2004; García *et al.*, 2006). Various groups worldwide have examined human IBD for the presence of EHS, from the negative studies of Bell and Grehan (2003), through those conducted by Bohr, Zhang and Laharie (2009) which have successfully demonstrated by means of molecular evidence the presence of NHPH in both, IBD and control groups of patients. *H. hepaticus*, the EHS prototype, lacks orthologs of most known *H. pylori* virulence factors, including various adhesins, the VacA cytotoxin, and almost all *cag*PAI proteins, but shares a number of orthologs present in *C. jejuni*, a human diarrhoea agent (Suerbaum *et al.*, 2003), such as periplasmic binding protein (PEB1 also called "*cell binding fraction*", CBF1), a highly conserved and a major cell adherence molecule (Pei & Blaser, 1993) as well as a CDT (Lara–Tejero *&* Galan, 2000; Johnson *&* Lior, 2003) which alternatively are not present in GHS. CDTs constitute a family of genetically related bacterial protein toxins produced by several unrelated Gram-negative mucosa-associated bacterial species able to stop the proliferation of numerous cell lines. This effect is due to their ability to trigger in target cells a signaling pathway that normally prevents the transition between the G_2 and the M phase of the cell cycle (Smith *&* Bayles, 2006). *H. hepaticus* and *C. jejuni* CDTs are obviously essential for persistent infection of the gastrointestinal tract and increase the severity of mucosal inflammation or liver disease in susceptible mouse strains, and recently, *H. hepaticus* CDT has been shown to play a crucial role in promoting the progression of infectious hepatitis to pre-malignant, dysplastic lesions via activation of a pro-inflammatory NF-□B pathway and increased proliferation of hepatocytes, providing evidence that CDT has carcinogenic potential *in vivo* (De Rycke *&* Oswald, 2001).

Taken together, with the kind help of collaborators in the Dept. of Microbiology and Dept. of Gastroenterology, Hepatology and Infectious Diseases, HZI Braunschweig and Alabama State University in Montgomery, I have identified and characterised morphologically, biochemically and genetically a novel EHS isolated from the intestine of certified SPF laboratory mice. Since unrecognized infections with diverse microorganisms may change the results of animal experiments, these studies are very important for unraveling the presence/absence these yet unknown bacteria in laboratory animals such as mice. The results of the present study are also important for more studies of the pathophysiological relevance of such infections. Future studies will therefore define whether and how *H. magdeburgensis* may contribute to certain disease in mice and other infected animals and its interaction with other microbial species in the mice intestine.

6.2 Discussion (part II)

Since the original discovery of *H. pylori* more than 25 years ago (Warren *&* Marshall, 1983), numerous other *Helicobacter* species have been identified in a wide range of mammals, possibly reflecting a long time of co-existence during evolution (Solnick *&* Schauer, 2001; Haesebrouck *et al*., 2009). The natural hosts for *H. pylori* are humans and non-human primates, and was also isolated from cats maintained as closed colony (Handt *et al*., 1994), but it can also infect other animals such as rodents (mice and gerbils) or dogs and cats in laboratory experiments (Fox *et al*., 1995). The stomach of mammalian carnivores (*e.g*. cats, dogs and cheetahs) are also often naturally infected by *Helicobacter* spp., but these commonly belong to other non-pylori species, including *H. felis, H. bizzozeronii*, and *H. salomonis,* which are genetically very different from *H. pylori* (Solnick and Schauer, 2001; Haesebrouck *et al*., 2009). Interestingly, *H. acinonychis* colonizes the stomach of large felines and is closely related to *H. pylori* with respect to ribosomal RNA sequences and multiple other genes (Daidiliene *et al*., 2004; Eppinger *et al*., 2006; Eaton *et al*., 1993). However, by comparison to *H. pylori* we know very little about the genomes of these other *Helicobacter* spp. Most of our knowledge derives from the complete genome of one strain, *H. acinonychis* Sheeba (Eppinger *et al*., 2006), and only very few other entries in Genbank databases exist. The presence of many fragmented genes in strain Sheeba, which actually represent functional genes in *H. pylori,* and other characteristics led to the proposal that *H. acinonychis* appeared as result of a host jump from humans or other primates to large felines, probably arising after a large feline became infected by eating an early human whose stomach was colonised by *H. pylori* (Eppinger *et al*., 2006). A single host jump has been proposed about 200,000 years ago (Eppinger *et al*., 2006), but other theories also exist (Daidiliene *et al*., 2004).

H. acinonychis strains have been isolated from captive American and European lions and cheetahs suffering from chronic gastritis and vomiting (Eaton *et al*., 1993; Munson *et al*., 1999, Daidiliene *et al*., 2004, Eppinger *et al*., 2006), as well as tigers (Schröder *et al*., 1998, Daidiliene *et al*., 2004). Chronic infection with *H. acinonychis* is thought to cause considerable morbidity and mortality of infected big cats (Moodley *et al.,* 2009). There are also reports of diminished appetite, chronic regurgitation and

cytological evidence of gastric inflammation in other animals (e.g. dolphins) kept in captivity consistent with the presence of gastric *Helicobacter* species (Harper *et al*., 2002). In the results part II, I have characterised at the molecular level a series of *Helicobacter* strains (SB-1 to SB-5) isolated from a captive Bengal tiger (*Panthera tigris tigris*) in Thailand suffering from gastritis and diarrhoea. The results revealed the presence of spiral-shaped bacteria, harbouring 1-4 monopolar sheathed or non-sheated flagella. The flagella had lengths of about 1.5- 4.5 µm, and their ends were commonly thickened and had a bulb-like shape of unknown nature (Fig. 15, bottom). Also a number of bacteria with the same characteristics but without flagella have been observed, and these results were corroborated by two independent electron microscopic approaches. Amplification of the 16S rRNA gene and typing with *Alu*I and *Hha*I restriction enzymes revealed distinctive patterns among all the *Helicobacter* species included in this study. Furthermore, sequencing and phylogenetic analyses based on similarity values of the 16S rRNA genes placed our tiger strains close to *H. acinonychis* AM260522, which was in concordance with the 23S rRNA phylogenetic analysis, suggesting that our tiger strains represent *H. acinonychis* rather than *H. pylori* strains. Finally, RAPD fingerprint analysis showed that these tiger strains are closely related *H. acinonychis* isolates, but clearly represent genetically distinct strains.

High bacterial motility is one of the common colonisation-associated features of *Helicobacter* species, especially important in the hostile environment close to gastric epithelial surfaces. Flagellar filaments commonly consist of two different flagellins, FlaA and FlaB, which are enveloped by a membranous flagellar sheath (Josenhans & Suerbaum, 2002). Our Western blotting and electron microscopic data indicate that the *H. acinonychis* strains SB-1 and SB-2 express a FlaA protein of 55-kDa, but the flagella structures seen were morphologically distinct. Another factor important for successive colonisation in the stomach is the gastric acid neutralisation by the urease complex, which consists of two major subunits UreA and UreB of about 30-kDa and 60-kDa, respectively, and some accessory proteins. Interestingly, urease activity is not only required to survive in an acid milieu, but also it has been postulated that urease may play a role as well in *H. pylori* metabolismus (Sachs *et al*., 2006) and disruption of transepithelial resistance (Wroblewski *et al*., 2009). Both proteins are highly conserved, and indeed both UreA and UreB proteins were expressed in our tiger isolates.

Adherence to the gastric mucosa is widely assumed to play a substantial role in initial colonization and long-term persistence of *H. pylori* in the human stomach (Odenbreit *et al*., 2005; Yamaoka, 2008). Major *H. pylori* adhesins comprise the blood antigen binding protein BabA (Ilver *et al*., 1998), outer inflammatory protein OipA (Yamaoka *et al*., 2002), sialic acid-binding adhesin SabA (Mahdavi *et al*., 2002) and some others, but the potential adhesins of *H. acinonychis* are completely unknown. The *H. pylori* adhesins were actually identified as members of the OMP family, showing specific adherence capabilities to the human gastric receptors, suggesting a specific adaptation to its human host (Odenbreit *et al*., 2005). In agreement with the host jump theory, the frequency of fragmented genes is particularly high in the sequenced *H. acinonychis* Sheeba strain as compared to *H. pylori* genomes, and this includes 12 OMPs, VacA and others (Eppinger *et al*., 2006). Thus, it is probably not surprising that I couldn't detect in Westernblots a band for expressed SabA in SB-1, while proper expression in *H. pylori* strains was documented as control. Interestingly, I observed a 72-kDa band in SB-1 reacting with a BabA antiserum, and showed the exact size of the corresponding proteins in *H pylori* strains 26695 and J99. A full-length ortholog of BabA is not present in the *H. acinonychis* Sheeba genome (accession number AM260522), but some of the OMPs exhibit homologous stretches to BabA, thus can explain our Westernblot results. Furthermore, no protein band corresponding to OipA could be found in SB-1, although one of the proteins in the Sheeba genome (OMP-7, fragment 2) shows extensive homology to *H. pylori* OipA, but again the corresponding gene is fragmented and its therefore also not clear if expressed in Sheeba. In addition, it should be noted that many of these OMP genes undergo phase variation in *H. pylori*, thus not all strains produce functional proteins. In particular, OipA is frequently switched "on" or "off" in *H. pylori*, suggesting that their expression can rapidly respond to changing conditions in the stomach or in different regions of the stomach (Yamaoka *et al*., 2002). The same can apply for *H. acinonychis* strains, which could explain the absence of Westernblot signals.

In agreement with the absence of *cag*PAI and *cagA* genes in previously published *H. acinonychis* isolates (Daidiliene *et al*., 2004, Eppinger *et al*., 2006), we were also unable to detect any protein expression for CagA and well-known *cag*PAI components such as Cagδ, CagM, CagN or VirB10 in SB-1. Furthermore, we also

failed to PCR amplify conserved fragments of *cag*A and other *cag*PAI genes (our unpublished data). However, we able to detect full-length proteins in *H. acinonychis* SB-1 Westernblots for a series of other well-known *H. pylori* pathogenicity factors including the neutrophil activating protein (NapA), γ-glutamyl transpeptidase (GGT), a secreted serine protease (HtrA) and a lytic transglycosylase (Slt), important in producing processed peptidoglycan and immune responses (Viala *et al*., 2004). The detection of GGT, NapA and Slt may help to explain the chronic gastritis as characterized by mucosal neutrophilic and mononuclear infiltrate as well as numerous lymphoid cell aggregates or follicles in the mucosa observed in some infected felines (Eaton *et al*., 1993; Schröder *et al*., 1998). The finding of a HtrA ortholog in SB-1, also raises the possibility that this protein may disturb epithelial barrier functions (Hoy *et al*., 2010) which could be involved in the gastric pathology observed in big cats.

In *H. pylori*, there has recently been considerable interest in strain-specific genes found outside of the *cag*PAI, especially genes in the so-called plasticity regions. Remarkably, almost half of the strain-specific genes in two fully sequenced *H. pylori* genomes, 26695 and J99, are located in their plasticity regions (Yamaoka, 2008). Recent work has shown that one of these plasticity regions encodes putative pathogenicity factors such as the duodenal ulcer-promoting gene A (*dupA*), which has been associated with duodenal ulceration and increased gastric inflammation (Lu *et al*., 2005), as well genes encoding putative DNA transfer enzymes such as the relaxases Rlx1 and Rlx2, also called VirD2 proteins (Backert *et al*., 1998; 2005). It has been noted that extensive size variation exists in the *dupA* genes among clinical isolates, which may interfere with their putative activity (Schmitt *et al*., 2009; Hussein *et al*., 2010). We have therefore used as controls two recently sequenced *H. pylori* strains, Shi470 and Cuz20 (accession numbers NC010698.2 and CP002076.1), which encode full-length genes for DupA's (>80-kDa) and both relaxases (*ca.* 70-kDa). Interestingly, we found by Western blotting that all three proteins are indeed expressed in both of these *H. pylori* strains, and in *H. acinonychis* SB-1 we found strong bands for both Rlx1 and Rlx2 at exactly same size as for their *H. pylori* counterparts, but no signal for DupA. The role of Rlx1 and Rlx2 is not fully clear, but they maybe involved in exchange of genetic material between bacteria of the same or other species, which requires investigation in future studies.

Taken together we have morphologically and genetically characterised a new *Helicobacter* strain isolated from a captive Bengal tiger in Thailand, which was classified as *H. acinonychis* and which shows similar genetic background as compared to previously isolated *H. acinonychis* strains from Sumatra tigers in a European zoo. Currently there is only one *H. acinonychis* genome sequence available, strain Sheeba, isolated from a lion housed at a Russian circus (Eppinger *et al*., 2007), and there is a lack of other genetic information in databases referring to different *H. acinonychis* strains isolated from big cats located in various geographic locations. Thus, this is the first report of a *H. acinonychis* isolate from an Asian big cat. In addition, we have shown that known pathogenicity factors from *H. pylori* such as flagellin A, ureaseA/B, NapA, HtrA, GGT, Slt, two relaxases and probably also a BabA-like protein are expressed in *H. acinonychis* strain SB-1. However, CagA and *cag*PAI components, as well as VacA, OipA, SabA and DupA were not detected in SB-1. An important challenge will be in future to identify the function of these and other determinants in *H. acinonychis* that contribute to colonization and disease development. Using mouse-adapted *H. acinonychis* strains as reported previously (Daidiliene *et al*., 2004) should be a valuable approach for analysing the interplay between the pathogen and its host that shapes the specificity and vigor of infections, the risks of various types of gastric diseases, and the evolutionary routes that may result.

Taken together, with the kind help of collaborators in the Dept. of Microbiology in Regensburg, HZI Braunschweig and University College Dublin, I have identified and characterised morphologically, biochemically and genetically a series of novel *H. acinonychis* isolates from a Bengal tiger. Since unrecognized infections with such diverse microorganisms is an important issue for animal health, these studies are very important for unraveling the presence/absence of certain virulence factors in uncharacterized *Helicobacter* species. The results of the present study are therefore important from scientific point of view but also for future studies on the pathophysiological relevance of such infections.

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8. Erklärung

MSc. Francisco Rivas T.
Institut für Medizinische Mikrobiologie
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Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zu dem Thema

"MORPHOLOGIC, GENETIC, AND BIOCHEMICAL CHARACTERISATION OF NOVEL *HELICOBACTER* ISOLATES FROM LABORATORY MICE AND TIGERS"

selbstständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 29.11. 2011

MSc. Francisco Rivas T.

9. CURRICULUM VITAE

Name: Francisco Rivas Traverso

Place of birth: Panama, Rep. of Panama

Nationality: panamanian

ACADEMIC DEGREES

Biology, *BSc*, 1996, University of Panama, UP, Panama

The experimental work towards Bachelor thesis:

„Isolation of *Cryptococcus neoformans* from panamanian soils"

Microbiology, *MSc*, 2000, University of Costa Rica, UCR, Costa Rica. Deutsche Akademische Austauschdients, DAAD, Scholarship

The experimental work towards Master thesis:

„Evaluation Anti-microbicidal Medicinal Plant Extracts Against *Helicobacter pylori*"

Dr rerum naturalium (Dr. rer. nat.)

Otto von Guericke Universität, Magdeburg, Germany

Secretaría Nacional de Ciencia y Tecnología, SENACYT, Scholarship

The experimental work towards Doctoral thesis:

„Morphologic, Genetic and Biochemical Characterisation of Novel *Helicobacters* Isolates from Laboratory Mice and Tigers "

OTHER STUDIES / TRAININGS

Corporación de Investigaciones Biológicas, CIB, Medellín 2000, Colombia; World Health Organisation, WHO

Otto von Guericke Universität, Magdeburg, 2003, Germany, DAAD

National Research Center for Protozoan Diseases, NRCPD, Obihiro Agricultural and Veterinary Medicine, 2004-05, Hokkaido, Japan, Japan International Cooperation Agency, JICA

LINGUISTIC COMPETENCES

	speaking	reading	comprehension	writing
english	good	good	good	good
german	(middle level courses, Institut J. W. Goethe, S.J., Costa Rica)			

PRESENT/PAST ASSOCIATIONS

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PROFESSIONAL EXPERIENCES

2000- Microbiology Department, University de Panama

2001- Microbiology Master Science Program, University of Panama

PUBLICATIONS

Rivas F, **Tegtmeyer N, Rohde M, Oyarzabal OA, Lehn N, Ferrero R, Berg DE, Fox JG, Backert** S (2011) Morphological, genetic and protein expression characterization of *Helicobacter* species isolated from tigers. submitted

Rivas F, Bohr UR, Oyarzabal OA, Rohde M, Clarici A, Wex T, Kuester D, Malfertheiner P, Fox JG, Backert S (2010) Morphologic, genetic, and biochemical characterization of *Helicobacter magdeburgensis*, a novel species isolated from the intestine of laboratory mice Helicobacter. **15**(5):403-15

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