Helicobacter

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Morphologic, Genetic, and Biochemical Characterization of *Helicobacter Magdeburgensis*, a Novel Species Isolated from the Intestine of Laboratory Mice

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Abstract

Background: The presence of enterohepatic *Helicobacter* species (EHS) is commonly noted in mouse colonies. 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. The aim of this study was to isolate and characterize a putative novel EHS that has previously been detected by PCR screening of specific-pathogen-free mice.

Materials and Methods: 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) were used to characterize isolated EHS. Genomic DNA fragments were sequenced to develop a species-specific PCR detection assay.

Results: Scanning electron microscopy revealed the presence of spiral-shaped EHS, which varied in length (2.5–6 μm) and contained single monopolar or single bipolar sheathed flagella. The bacteria were grown under anaerobic conditions, preferably on agar plates containing serum or blood. The 16SrRNA, genetic, and biochemical analyses indicated the identification of a novel EHS species, named *Helicobacter magdeburgensis*. We also examined the genome content using pulsed-field gel electrophoresis. 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.

Conclusion: We isolated and characterized a novel EHS species, *H. magdeburgensis,* morphologically, biochemically, and genetically. These results are important for future studies on the prevalence and pathophysiologic relevance of such infections. Our PCR assay can be used to detect and discriminate *H. magdeburgensis* from other *Helicobacter* species.

As *Helicobacter pylori* was the first bacterium cultivated from human gastric biopsy specimens in 1982, it has become apparent that *Helicobacter* spp. exhibit a broad host spectrum and can be isolated from the gastrointestinal tracts of humans, non-human primates, cats, dogs, cheetahs, ferrets, rodents, cows, sheep, pigs, dolphins, and birds. Members of the genus *Helicobacter* are helical curved, spiral or fusiform, Gram-negative bacteria with or without helical periplasmic fibers [1]. One of the best characterized species, *H. pylori*, was originally described as a member of the genus *Campylobacter* [2,3], but it was subsequently placed in its own genus based on 16S

rRNA sequence analysis data [4–6]. There are currently 24 internationally recognized *Helicobacter* species in the genus [7], and numerous putative new ''*Candidatus Helicobacter*'' species are described in the literature. The sequence analysis of 16S rRNA of over 225 *Helicobacter* spp. isolated from mammals and birds indicates the genus to be phylogenetically diverse, potentially containing over 30 additional taxa [8].

Helicobacter spp. have widths of about 0.3–0.6 μm and lengths ranging from 1 to 5 μm. These microorganisms are highly motile by means of single or multiple flagella [1]. Optimum temperature for growth is from 37 to 42 °C. These bacteria mostly grow under microaerobic conditions and have a respiratory type of metabolism. *Helicobacter* spp. are oxidase-producing and most strains encode for a catalase. All gastric *Helicobacter* spp. described to date produce copious amounts of urease. Although strains of several species are capable of growing on simple nutritional agar media, the majority require media supplemented with blood or serum, and some species may be exacting (e.g. *Helicobacter bizzozeronii*, *Helicobacter felis* and *Helicobacter salomonis*) [9]. Detailed biochemical, morphologic, and physiologic aspects have been previously reported for species of this genus. Most *Helicobacter* spp. can be discriminated from the neighboring genera *Campylobacter*, *Arcobacter*, *Wolinella*, *Sulfurospirillum,* and *Thiovulum* by the presence of sheathing around the flagellar apparatus. However, two *Helicobacter* species (*Helicobacter pullorum* and *Helicobacter rodentium*) possess unsheathed flagella and therefore resemble species assigned to other related genera. Genotypic data, such as 16S rRNA sequence analysis, are the major features to unequivocally differentiate the genus *Helicobacter* from all other genera.

The description of a new *Helicobacter* species or subspecies is based on features used for assigning the new taxon to the genus and on characteristics used to differentiate the new taxon from existing taxa of the genus. For critical comparisons with other species, controls consisting of type or reference strains of the appropriate taxa should be tested [1]. The use of standardized, well-described tests, and methods, such as defined phenotypic test procedures, the inoculum size, composition of the gaseous atmosphere, period of incubation, and composition of the basal growth medium has been recommended [10–16]. Putative new species of uncultured organisms for which molecular sequence data are available (such as 16S rRNA sequence) may qualify for assignment to the provisional taxonomic status *Candidatus* [17]. In accordance with the study by Murray & Stackebrandt [18], 16S rRNA sequence data are not sufficient to assign *Candidatus* status. Therefore, morphotype, Gram-reaction, and other preliminary metabolic data should also be collected. With culturable *Helicobacter* spp., it is preferred that five isolates from different sources be studied, and that multiple sequences of the putative new ''*Candidatus Helicobacter*'' species cluster together in phylogenetic analyses [1]. This convention has been used for ''*Candidatus Helicobacter suis*'' [19] and ''*Candidatus Helicobacter* bovis'' [20], and may be appropriate for other uncultured *Helicobacter* spp., such as other gastric spiral organisms described by Solnick et al. [21].

Our groups are interested in the investigation and characterization of novel enterohepatic *Helicobacter* spp. (EHS), which are an emerging group of microaerobic, motile pathogens carrying flagella with variable styles in number and locations [22,23]. EHS are known to persistently colonize multiple animal species. They can be isolated from the lower intestine, hepatobiliary system, and diarrheic feces and are potentially associated with chronic inflammation and epithelial cell hyperproliferation leading to neoplasic disease [24,25]. *Helicobacter hepaticus*, considered the prototype of all known EHS [22], induces chronic active hepatitis and hepatocellular carcinoma in A/JCr and B6C3F1 mice strains as well as typhlocolitis in A/JCr inbred mice. This species is also used experimentally to induce cholesterol gallstones, inflammatory bowel disease (IBD), and in certain strains of mice induces colon cancer [26,27]. Because lesions caused by EHS in mice often mimic those seen in humans with cholecystitis, their possible role in hepatobiliary disease in humans has been proposed [28]. In addition, the possible zoonotic origin of important clinical manifestations in humans and the health status of mice housed in research facilities have recently attracted the attention of scientists [29]. In contrast to *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 and Lior [30], is encoded in the genomes of several *Helicobacters* species [31].

To evaluate the prevalence of EHS infections in mouse strains harbored in our specific-pathogen-free (SPF) facilities, we tested 40 mouse lines that were permanently living in nine colony rooms using a group-specific PCR, which detects all *Helicobacter* species currently known [32]. When *Helicobacter*-negative and infected mice shared the same cage, transmission of the infection occurred within two weeks at very high frequency (100%). Furthermore, we found that mice from commercial breeding facilities may carry undetected *Helicobacter* infections [32]. We also showed that infection with EHS may occur and spread frequently in mice under SPF conditions, and despite extensive safety

precautions. Our 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. Here we describe a novel EHS isolated from SPF mice. We propose to name this EHS *Helicobacter magdeburgensis*.

Materials and Methods

Laboratory Mice

Mice from various mouse lines (BALB/c, C3H, and C57BL/6) that were contaminated with putative novel *Helicobacter* species were separated in individually ventilated cages. Fecal samples of each isolated mouse were then tested for presence of *Helicobacter* DNA as described previously [32]. Subsequently, the infection was transferred from individual infected animals to several PCR-proven *Helicobacter*-free C57BL/6 mice by harboring the mice in the same cage for 2 weeks. After transfer of the infection was detected by PCR [32], *Helicobacter*-infected mice were then used for bacterial isolation and culturing.

Bacterial Isolation

Helicobacter-infected mice were euthanized and organs were immediately dissected in a laminar flow hood. Ileum and the entire large intestine were cut into small pieces with sterile scissors, incubated with BHI (brain heart infusion) medium (5 mL per gram material), and shaken for 20 min at 37 °C in 50 mL Falcon tubes at 1000× ***g***. The mixture was then centrifuged for 10 min at 2000× ***g*** to remove larger particles like cells and intestinal debris. The supernatant was removed and passed through sterile filter paper (Whatman) to further remove cell debris. Bacteria were then cultured in different amounts (100, 50, 25 or 5 μL) on different agar plates (*Helicobacter pylori* selective agar plates (HP), GC agar plates with 10% horse serum, *Campylobacter* selective plates, Müller-Hinton agar plates, and Columbia agar plates containing 5% sheep blood). These plates were incubated for 2, 3, 4, and 7 days. The Campygen™, Anaerogen™ from Oxoid, Anaerocult® from Merck gas generating systems, and one anaerobic chamber (5% N_2, 4.5% CO_2 and 3% H_2) were used for incubation at 37–42 °C. Single bacterial colonies were picked and grown for further analysis.

Bacterial Control Strains and Growth Conditions

Helicobacter pylori, *Helicobacter* MIT 96-1001, *Helicobacter typhlonicus*, *Helicobacter hepaticus*, *Helicobacter bilis* ATCC 51630, *Helicobacter mustelae,* and *Campylobacter jejuni* strains were included as controls and grown under standard conditions. Briefly, *H. pylori* were cultivated on HP agar plates under microaerophillic conditions, and all other strains were cultivated on Columbia 5% sheep blood agar plates in an anaerobic chamber at 37 °C for 48 hours [9,10].

DNA Isolation and Purification

Bacteria were harvested with a sterile cotton swab and suspended in 100 μL of lysis buffer (50.0 mM Tris-HCl [pH 7.6], 1 mM EDTA, 0.5% Tween-20, 20 mg of proteinase K per mL) and incubated at 58 °C for 2 hours. The proteinase K was inactivated by conventional phenol/chloroform extraction. Purified DNA was then precipitated with 2.5 volume of 96% ethanol and washed with 70% ethanol.

Amplification and Sequencing of a 1.6-kb PCR Product of the 16S rRNA Gene

For amplification of the complete 16S rRNA gene, primers C70 (5′-AGA GTT TGA TYM TGG C-3′, forward) and B37 (5′-TAC GGY TAC CTT GTT ACG A-3′, reverse) were used [28]. Amplicons were then purified and sequenced directly by using the amplification primers C70 and B37, as well as internal primers C97-20: 5′- GGC TAT GAC GGG TAT CCG GC-3′ (forward), H5A: 5′-CGC GTG GAG GAT GAA GG-3′ (forward), C98: 5′-GAT TTT ACC CCT ACA CCA-3′ (reverse), H2: 5′-TCG CAA TGA GTA TTC CTC TT-3′(reverse), and H3A-20: 5′-GCC GTG CAG CAC CTG TTT TC-3′ (reverse) [24]. The gene has the NCBI GenBank accession number EF990624.

16S rRNA Sequence Analysis

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 [5]. The database used contains approximately 400 *Helicobacter*, *Wolinella*, *Arcobacter*, and *Campylobacter* sequences and more than 1000 sequences of other bacteria. 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 [33]. A phylogenetic tree from the distance matrix was created with growtree using the UPGMA method [34].

Restriction Fragment Length Polymorphism (RFLP) of the 16S rRNA Gene

For restriction fragment analysis of the 16S rRNA gene, we amplified by PCR a specific and conserved 1.2-kb subfragment using primers C97 (5′- GCT ATG ACG GGT ATC C - 3′) and CO5 (5′- ACT TCA CCC CAG TCG CTG - 3′) by the method of Fox et al. [28]. RFLP patterns of amplified respective PCR products were obtained with each of the following enzymes: *Alu*I, *Hha*I, *Apa*LI [25,35] in the appropriate buffer as recommended by the manufacturer (New England Biolabs, Acton, MA, USA). The resulting DNA cleavage products were compared with the RFLP patterns of *H. magdeburgensis* and known *Helicobacter* spp. after separation on agarose gels.

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 [36] and sequenced using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). The consensus sequence was deposited in GenBank (accession number HM222564).

Randomly Amplified Polymorphic DNA (RAPD) Fingerprinting PCR

The RAPD fingerprinting method established for *H. pylori* strains [37] was used to compare the diversity of the DNA sequences among the *Helicobacter* strains tested. 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 genomic DNA from each strain as template, 20 pmol of each primer (5′- GAG CGG CCA AAG GGA GCA GAC-3′ D8635, 5′-CCG GAT CCG TGA TGC GGT GCG-3′ D9355, 5′-GGT TGG GTG AGA ATT GCA CG-3′ D14307), 1U Taq DNA-polymerase (Qiagen, Hilden, Germany) and 250 μM from each dNTP, 1× buffer, and water for a total volume of 50 μL. A Perkin-Elmer thermal cycler model 9700 was used for amplification reactions. The cycling program was four cycles of 94 °C, 5 min; 40 °C, 5 min; 72 °C, 5 min; low stringency amplification, and a final incubation at 72 °C for 10 min.

Agarose Gel Electrophoresis

Five microliters aliquots of each PCR reaction or 20 μL restriction digests were electrophoretically analyzed in 0.8–2.0 g/mL agarose gels containing 0.5 μg/mL ethidium bromide in 1× Tris acetate running buffer. The electrophoresis was for 2 hours at 100V. The 1-kb or 100-bp DNA ladder (Fermentas GeneRuler) was used as the size marker (M) in all gels.

Scanning Electron Microscopy (SEM)

Bacterial cells were harvested and fixed in a sterile solution containing 5% formaldehyde, 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M 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 m M Tris, 1 m M 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. 10.0–11.0 nm thick gold film by sputter coating (SCD040; 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.

Electron Microscopic Analysis of 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.

Gram-staining and Biochemical Characterization Using the API Campy kit

Gram-staining of the isolated bacteria (Crystal violet, Gram's iodine solution, acetone/ethanol (50 : 50 v:v), 0.1% basic fuchsin solution) was applied. For the biochemical characterization, the API Campy kit was used according to the recommendations of the manufacturer (bioMerieux, Marcy I'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. It 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 (bioMerieux).

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

After 24–48 hours of growth, 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 0.25 M EDTA (pH 9.0), 0.5% lauroyl sarcosyl, and 0.5 mg of proteinase K per ml, as described previously [38]. 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.

Restriction Digests and Pulsed-field Gel Electrophoresis (PFGE)

The LMP agarose plugs containing genomic DNA were preincubated with 100 μL of the appropriate 1× restriction enzyme buffer before digestion was carried out with 50 U of enzyme in fresh 1× 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, *Sal*I, *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, MA, USA) was used as the DNA marker for 12 hour gels, and a digestion of *Salmonella choleraesuis* ss. *Choleraesuis* serotype Braenderup H9812 (ATCC BAA-664) genomic DNAs were used as the size markers for 18-hour gels. The markers were included in both sides of each PFGE gel with another one in the middle. Gels were stained with ethidium bromide, visualized with a UV transilluminator (Gel-Doc System, Bio-Rad), and pictures were recorded using GeneSnap (Syngene, Frederick, MD, USA). BioNumerics version 4.50 (Applied Maths, Austin, TX, USA) was used to perform a 10% background substraction of densitometric curves to identify bands and to determine band sizes and to perform genome calculations.

Cloning of Chromosomal DNA and Species-specific PCR Assay

To design species-specific PCR primers, we cloned fragments of chromosomal DNA. For this purpose, purified genomic DNA was prepared as described earlier and was digested at 37 °C for 3 hours using restriction endonuclease *Sau*3AI in 1× buffer provided by the manufacturer (New England BioLabs) and the resulting fragments were ligated into pBluescript II SK (+/−) vector. Ligated DNA was then transformed into *E. coli* Top 10 cells. Twenty five clones were sequenced as described earlier. Based on the sequence derived from one clone, specific primers with the following sequences 538F (5′-ATG CCG CCC TTG CAT CTG TC-3′) and 538R (5′- GGC GTA AAA ACT GAT GAA GCG AT-3′) were synthesized (Eurofins MWG GmbH, Ebersberg, Germany). The amplification conditions were 1U Taq DNA12 polymerase (Qiagen, Hilden), 1× 12.5 mM $MgCl_2$, 1× PCR buffer, 20 ng template DNA, 200 μM dNTP, 20 pmol of each primer in a total volume of 50 μL at 95 °C, 5 min; 94 °C, 30 s; 68 °C, 30 s; 72 °C, 1 min (30–35 cycles); and finally 72 °C for 10 min. This PCR protocol specifically amplified a 750-bp PCR product of the cloned 800-bp *Sau*3AI fragment from the *H. magdeburgensis* genome.

Results

Isolation of *Helicobacter* from Mouse Intestines

Recently, the presence of EHS-DNA in 35 of 40 mouse strains harbored at our animal facility 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 [32]. Here we 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 harboring 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. We included *Helicobacter pylori* (HP) selective agar plates, GC agar plates with 10% horse serum, *Campylobacter* selective plates, Müller-Hinton agar plates, and Colombia agar plates containing 5% sheep blood. 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 (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.

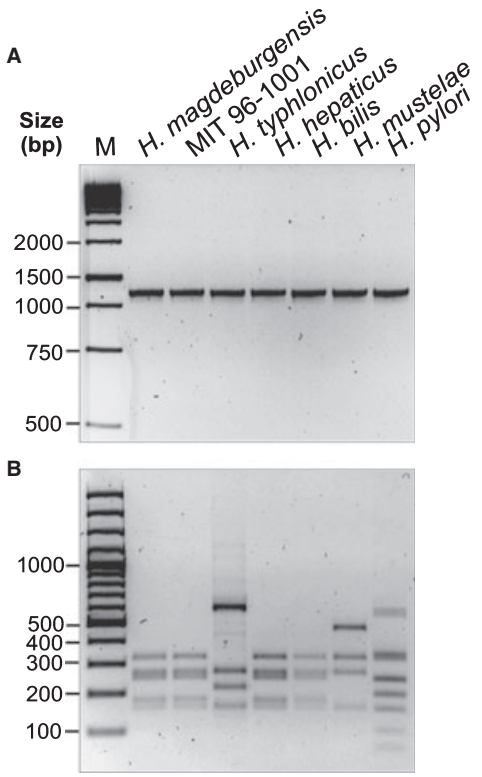

Figure 1 Investigation of 16S rRNA genes of different *Helicobacter* species by PCR and RFLP analyses. (*A*) DNA isolated from bacteria belonging to the genus *Helicobacter* (*H. magdeburgensis*, *H. typhlonicus*, *H. hepaticus*, *H. bilis*, *H. mustelae*, *H. pylori*, and the MIT strain 96-1001) was applied for conventional PCR of the 16S rRNA gene. A conserved 1.2-kb DNA fragment in the genus *Helicobacter* was amplified [28]. (*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 [25].

16S rRNA and 23S rRNA Phylogenetic Analysis

We suspected that the isolated bacteria belonged to the genus *Helicobacter*. To test this hypothesis, we isolated DNA for conventional PCR of the 16S rRNA gene. For this purpose, we amplified a 1.2-kb DNA subfragment of the 16S rRNA gene, which is highly conserved within the genus *Helicobacter* [28]. We 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. 1A). To confirm the specificity of these fragments, all PCR products were then digested with the restriction endonuclease *Alu*I, which yields specific band patterns [25]. 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. 1B). Similar results were obtained using other recommended restriction enzymes such as *Hha*I and *Apa*LI (data not shown). 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. 2A–C).

Next, the complete 16S rRNA gene sequence of the isolates HM006, HM007, and HM009 was determined by sequencing of a 1617-bp PCR product as described in Materials and Methods. 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).

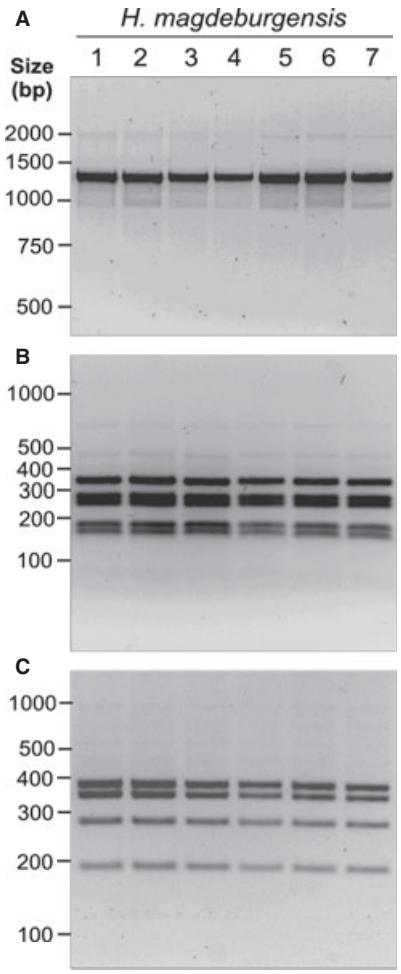

Figure 2 Analysis of 16S rRNA of different *Helicobacter magdeburgensis* isolates by PCR and RFLPs. (*A*) DNA isolated from seven individual clones belonging to *H. magdeburgensis* was investigated by conventional PCR of the 16S rRNA gene. A conserved 1.2-kb DNA fragment in the genus *Helicobacter* was amplified [28]. 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 [25] and was identical among all investigated clones.

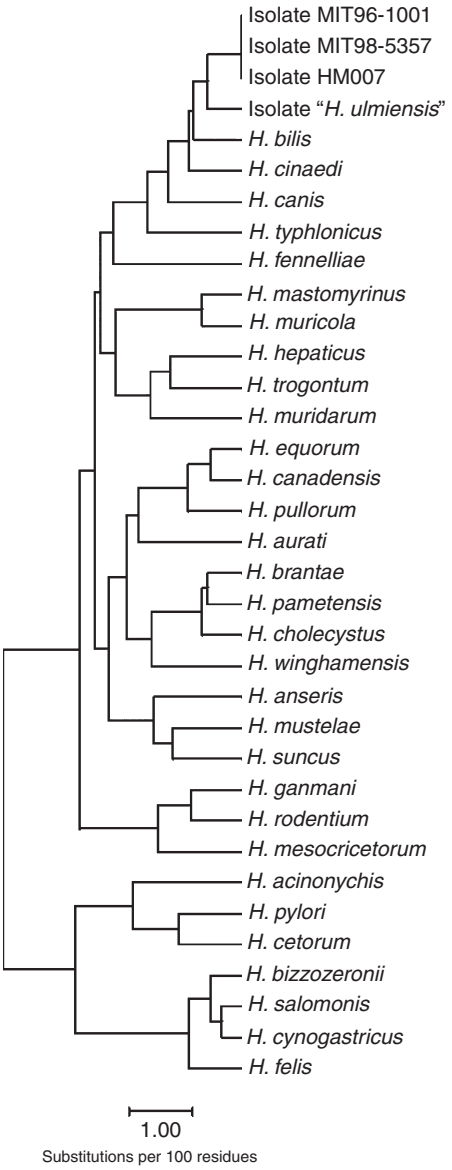

Figure 3 Phylogenetic tree of 16S rRNA sequences of different *Helicobacter* species including *H. magdeburgensis*.

Phylogenetically, the novel *Helicobacter* species isolated from HM006, HM007, and HM009 (hereafter named *H. 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. 3).

Interestingly, the 16S rRNA gene of *H. magdeburgensis* contained an intervening sequence (IVS) of 179 bp. Intervening sequences 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 intervening sequences in the 23S rRNA genes, which alter or may even produce a loss of phylogenetic information in these genes [36].

Morphologic Description of the Isolated *Helicobacter* ssp.

Next, we visualized the isolated *H. magdeburgensis* from HM006, HM007, and HM009. For this purpose, single bacterial colonies were grown for 2 days on Columbia agar plates containing 5% sheep blood and prepared as described. Scanning electronic microscopic investigation revealed in all cases spiral-shaped bacteria (Fig. 4A–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 nonsheathed 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. 4D).

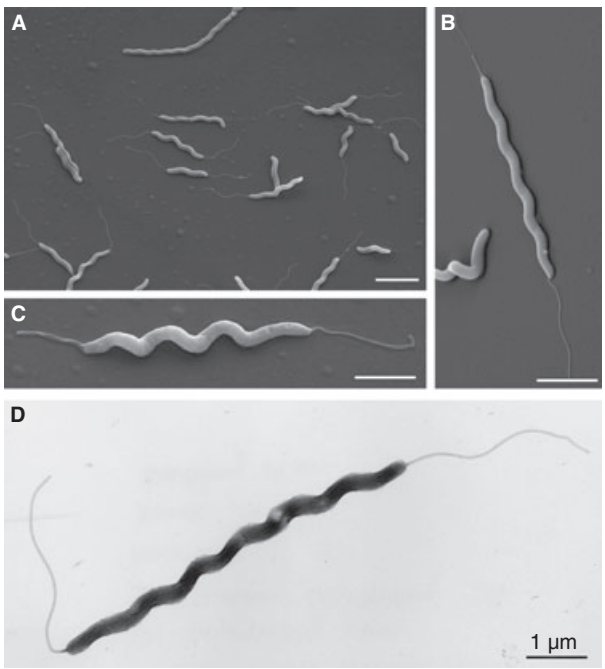

Figure 4 Morphologic analyses of novel *Helicobacter* species by electron microscopy. (*A–C*) Scanning electron microscopy revealed spiral-shaped bacteria that were about 0.18–0.22 μm in diameter and varied in length from about 2.5–6 μm. The majority of bacteria contained single monopolar or single bipolar 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 1 μm.

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, we performed PCR-based randomly amplified polymorphic DNA (RAPD) fingerprinting analysis as described elsewhere [37]. 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 [39]. Typical RAPD fingerprinting profiles with each of the three primers are shown in Fig. 5A–C. All control strains tested gave different RAPD profiles, indicating that they represent unrelated *Helicobacter* isolates. In agreement with the 16S rRNA analysis described earlier, we found that the RAPD pattern of our strain with two primers was very similar to that of MIT 96-1001 (Fig. 5B,C). However, using the primer D14307, we 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. 5A, arrows). In addition, a 1.1-kb band present in MIT 96-1001 is absent in our strain using primer D9355 (Fig. 5B, arrow) implying that *H. magdeburgensis* and the MIT 96-1001 represent different strains.

Biochemical Characterization

To further characterize our *H. magdeburgensis* isolate, we determined the biochemical activity of specific enzymes using the conventional API Campy kit and compared the data of the representative HM007 isolate with that obtained for the MIT strain 96-1001 (Table 1). *Helicobacter 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 *H. pylori* 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 1). These results further demonstrate that *H. magdeburgensis* and MIT 96-1001 are closely related but not identical.

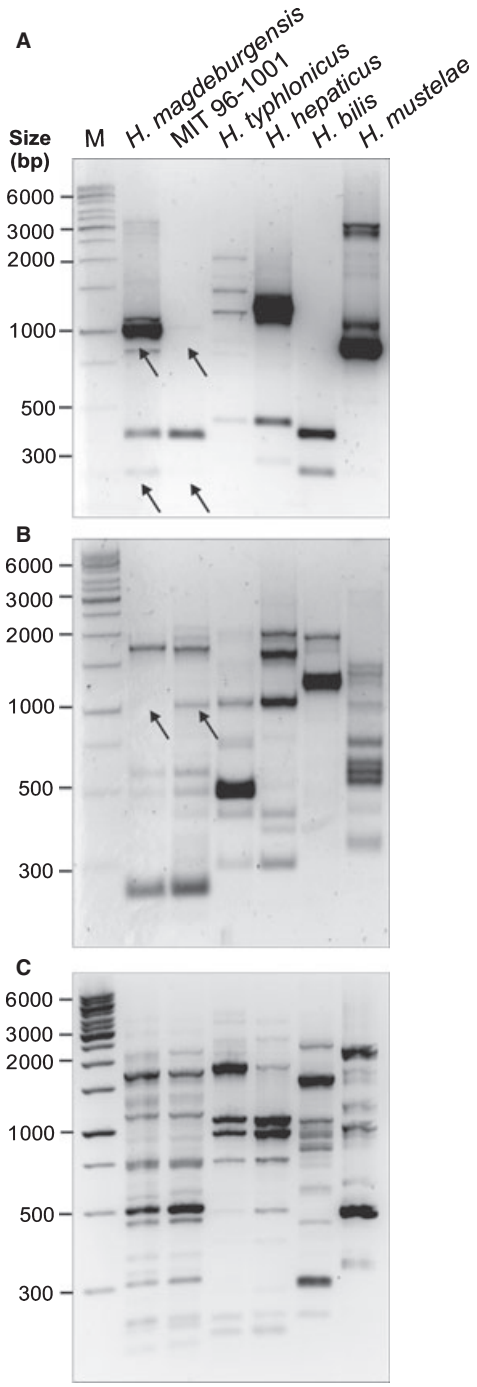

Figure 5 PCR-based randomly amplified polymorphic DNA (RAPD) fingerprinting of *Helicobacter* species. To investigate the genetic relatedness between *H. magdeburgensis* and the closest known relative (MIT 96-1001) and other strains, we performed RAPD analysis as described [37]. (*A–C*) This method uses a set of single primers (D14307, D9355 or D8635), which arbitrarily anneal and amplify genomic DNA resulting in strain-specific fingerprinting patterns [39]. 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.

Table 1 Enzymatic analysis of *Helicobacter* spp. by api® Campy test

Tests	Reactions	Results[a]	
		H. magdeburgensis	MIT 96-1001
URE	UREase	–	–
NIT	Reduction of NITrates	+	–
EST	ESTerase	–	–
HIP	HIPpurate	–	–
GGT	Gamma Glutamyl Transferase	–	–
TTC	Reduction of Triphenyl Tetrazolium Chloride	+	±
PyrA	Pyrrolidonyl Arylamidase	–	–
ArgA	L-Arginine Arylamidase	–	–
AspA	L-Aspartate Arylamidase	–	–
PAL	ALkaline Phosphatase	+	+
H_2S	Production of H_2S	–	–
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)	–	–

URE, urea; NIT, potassium nitrate; EST, 5-bromo-4-chloro-3-indoxyl acetate; HIP, sodium hippurate; GGT, γ–L-glutamic acid-β–naphthylamide; TTC, triphenyltetrazolium chloride; PyrA, pyroglutamic acid β–naphtilamide; ArgA, L-arginine-4-methoxy-β-naphthylamide; AspA, aspartic acid-β-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, ±

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

In the next set of experiments, we analyzed *H. magdeburgensis* HM007-1 in terms of restriction enzyme digests and estimation of its genome size. For this purpose, a series of commonly used restriction enzymes were 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, *Sal*I, *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. 6). However, the digestion with *Bam*III resulted in 16 DNA fragments with sizes ranging from 12.86 to

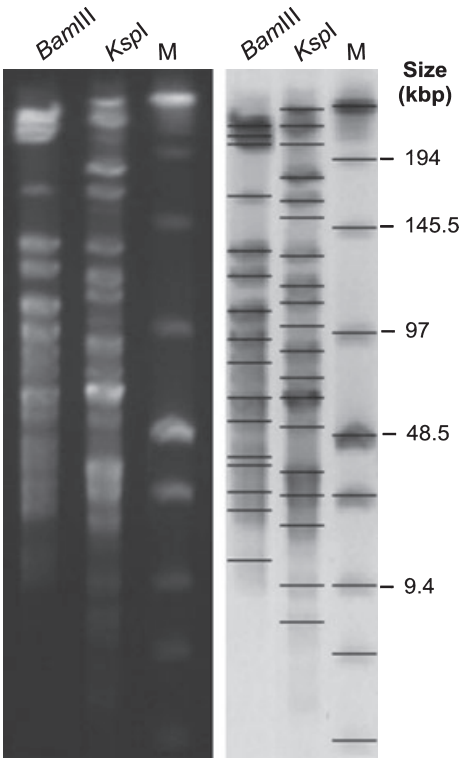

Figure 6 Pulsed-field gel electrophoresis (PFGE) analysis of *Helicobacter magdeburgensis*. Chromosomal DNA was digested with the restriction endonucleases *Bam*III and *Ksp*I, respectively. Low Range PFGE Marker was used as the DNA size marker (M). BioNumerics software was used to identify bands and to determine band sizes. The values from the genome calculations are summarized in Table 2.

231.98 kbp, and the digestion with *Ksp*I resulted in 17 fragments with sizes between 7.99 and 243.23 kbp (Table 2). 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 2). Digests with *Bam*III revealed a total genome size of about 1695 kb and the restriction with *Ksp*I yielded a size of about 1793 kb, respectively.

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

Finally, to discriminate our new isolate from other *Helicobacter species,* we developed a *H. magdeburgensis*-specific PCR assay. For this purpose, we digested isolated *H. magdeburgensis* 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. The

Table 2 Determination of *Helicobacter magdeburgensis* genome size by Restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) analysis[a]

	Restriction Enzyme	
	*Bam*III	*Ksp*I
Detected band sizes (in kbp)	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

[a]A representative PFGE gel is shown in Fig. 6.

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, we 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. 7).

Discussion

Helicobacter is a rapidly expanding bacterial genus with a wide host range but limited biologic niches. The respective ecologic division and taxa are often referred as gastric *Helicobacter* species (GHS) and enterohepatic *Helicobacter* species (EHS) [3,40]. EHS are emerging as important pathogens within this genus [7]. EHS can colonize the lower gastrointestinal tract, including the

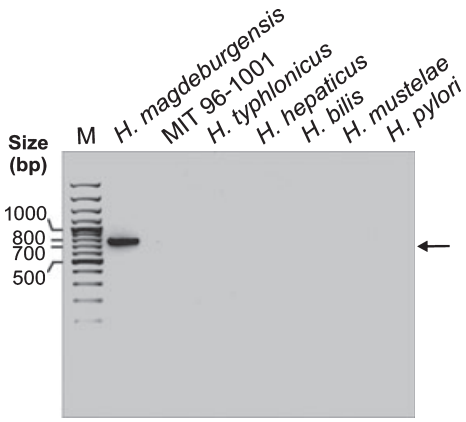

Figure 7 Development of a *H. magdeburgensis*-specific PCR detection assay. We designed specific PCR primers as described in the Materials and Methods section. Using these primers, we developed a PCR assay giving rise to a single *H. magdeburgensis*-specific 750-bp DNA fragment, which is clearly absent in all other *Helicobacter* isolates tested, even after 35 PCR cycles, including the MIT strain 96-1001, *H. typhlonicus*, *H. hepaticus*, *H. bilis*, *H. mustelae*, and *H. pylori*.

ileum, cecum, colon, and biliary tree. Similarly to GHS, EHS can cause persistent infections associated with chronic inflammation and epithelial cell hyperproliferation leading to neoplastic transformation [29]. EHS are also confounding factors in inflammatory bowel disease in the mouse animal model [32]. The interest in keeping healthy mice in research facilities, and the studies of the zoonotic potential of these mouse populations [41] has stimulated researchers to investigate EHS in more detail. Importantly, there is very little information concerning the *Helicobacter* status in noncommercial 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 [32,42–44]. These infections often remain nonrecognized but can cause severe health complications and, thus, can also change the results of animal experiments.

In recent studies using PCR assays from feces of our laboratory mice, we identified that the most frequently detected DNA from *Helicobacter* species corresponds to that of *H. ganmani* and MIT 98-5357 [32]. 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 remain undetected by the routine screening procedures, which can explain the relatively high prevalence of these rare species in *Helicobacter*-infected mice. To avoid the spread of *Helicobacter* infections in any animal facility of research institutions, it is important to elucidate novel *Helicobacter* species, characterize them at the molecular level, and study the transmission route and possible disease outcome.

In the present report, we describe 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. This bacterium is highly motile by means of single monopolar or bipolar sheathed flagella without helical periplasmic fibers. 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 propose 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 [45,46]; however, we could differentiate these two strains by means of ApiCampy, RAPD fingerprinting, and other methods. Interestingly, *Helicobacter magdeburgensis* grows under anaerobic conditions, but its definition as a strict anaerobic bacterium will require further studies.

Our RFLP and subsequent PFGE analysis of chromosomal DNA revealed that this bacterium has an approximate genome size of 1.7 to 1.8 Mbp. 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; 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*III 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. These values are slightly higher than the genome of *H. hepaticus* but are in agreement with other *Helicobacter* spp. [47,48]. Finally, we cloned some genomic DNA fragments for sequencing and to develop 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 is obviously absent in the MIT 96-1001, further demonstrating that *H. magdeburgensis* and MIT 96-1001 are different EHS strains.

Taken together, we have identified and characterized morphologically, biochemically, and genetically, a novel EHS isolated from the intestine of certified specific-pathogen-free laboratory mice. As unrecognized infections with diverse microorganisms may change the results of animal experiments, our studies are very important for unraveling the presence/absence of these unknown bacteria in laboratory animals such as mice. The results of this study are also important for future studies of the pathophysiologic relevance of such infections. The isolate most closely related to *H. magdeburgensis*, strain MIT 96-1001, has been shown to exhibit pathogenic properties in both the liver and lower bowel of infected A/J and scid mice and to express a CDT ortholog [45,46]. Future studies will therefore define whether and how *H. magdeburgensis* may contribute to disease conditions in mice and other infected animals, and the interaction of *H. magdeburgensis* with other microbial species in the mouse intestine.

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