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# '*Candidatus* Helicobacter suis', a gastric helicobacter from pigs, and its phylogenetic relatedness to other gastrospirilla

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**'*Gastrospirillum suis*' is an uncultured, tightly spiral micro-organism that has been associated with ulcer disease in the stomachs of pigs. It was the purpose of this study to determine the phylogenetic position of '*G. suis*'. Stomachs of five slaughterhouse pigs, originating from different Belgian and Dutch farms, were selected on the basis of the presence of '*G. suis*'-like bacteria, as demonstrated by biochemical, immunohistochemical and electron microscopical data. Bacterial 16S rDNA was amplified by PCR using broad-range primers and five helicobacter-like sequences were determined either by direct or indirect sequence analysis. An inter-sequence homology of 99·7% was observed, suggesting that the sequences originated from strains belonging to a single species. Phylogenetic analysis of the consensus sequence placed the organism within the genus *Helicobacter*, where it formed a distinct sub-group together with other gastrospirillum-like bacteria (*Helicobacter felis*, *Helicobacter bizzozeronii*, *Helicobacter salomonis* and '*Helicobacter heilmannii*' types 1 and 2). Diagnostic PCR primers and a probe were developed that differentiated the porcine sequences from all known helicobacters. These results indicate that the porcine sequences represent a single taxon within the genus *Helicobacter*. The low similarity level towards *H. salomonis* (96·6%), its closest validly named neighbour, strongly suggests that this taxon is a novel *Helicobacter* species. *In situ* hybridization experiments linked the reference sequence to the '*G. suis*'-like bacteria. On the basis of these results, we propose the name '*Candidatus* Helicobacter suis' for this gastric helicobacter from pigs.**

**Keywords:** *Helicobacter*, '*Gastrospirillum suis*', pig, stomach, PCR

## INTRODUCTION

At the end of the 19th century, spiral organisms were described for the first time in the stomachs of animals by Rappin (1881) and Bizzozero (1893). It was not until 1984, after the isolation of *Helicobacter pylori* from the human stomach (Marshall & Warren, 1984) and its association with chronic gastritis, peptic ulcer disease and gastric cancer (Cover & Blaser, 1995; Blaser *et al*., 1991; Parsonnet *et al*., 1991), that scientific interest in these organisms emerged. Over the years, other *Helicobacter* species have been isolated from many different animals. To date, the genus *Helicobacter* consists of 18 validly named species (On, 1996; Franklin *et al*., 1996; Mendes *et al*., 1996b; Jalava *et al*., 1997; Shen *et al*., 1997) and constitutes, together with the genera *Wolinella*, *Campylobacter* and *Arcobacter*, the ε-subdivision of the *Proteobacteria*, also known as rRNA superfamily VI (Vandamme *et al*., 1991).

Within the genus *Helicobacter*, a phylogenetic subgroup of morphologically similar bacteria can be distinguished. These bacteria, characterized by their

The GenBank accession number for the 16S rDNA nucleotide reference sequence of '*Candidatus* Helicobacter suis' V2BXA is AF127028.

long and tightly coiled (gastrospirillum-like) appearance, have been observed in gastric biopsies of humans, cats, lemurs, dogs, pigs and exotic carnivores (Dent *et al*., 1987; Lee *et al*., 1988; O'Rourke *et al*., 1992; Hänninen *et al*., 1996; Jalava *et al*., 1997; Queiroz *et al*., 1990; Eaton *et al*., 1993; Jakob *et al*., 1997). Three species with this morphology (*Helicobacter felis*, *Helicobacter bizzozeronii*, *Helicobacter salomonis*) have been cultured and characterized from gastric samples of cats and dogs (Paster *et al*., 1991; Hänninen *et al*., 1996; Jalava *et al*., 1997).

In pigs, gastrospirillum-like bacteria were observed in the antral pits and at the mucosal surface of the stomach (Queiroz *et al*., 1990) and have provisionally been named '*Gastrospirillum suis*' (Mendes *et al*., 1990). Histopathological studies associated this bacterium with pyloric lymphonodular gastritis (Mendes *et al*., 1991) and gastric ulcer disease of the pars oesophagea in pigs (Barbosa *et al*., 1995; Queiroz *et al*., 1996). Although cultivation of '*G. suis*' *in vitro* has been unsuccessful (Queiroz *et al*., 1990; unpublished data), cultivation *in vivo* in mice and rats has been reported (Moura *et al*., 1993; Mendes *et al*., 1996a). Several helicobacter-like bacteria have been cultured from the stomach of swine, but none had the characteristic gastrospirillum morphology (Seymour *et al*., 1994; Dewhirst *et al*., 1994; On *et al*., 1995).

As '*G. suis*' remains unculturable, an official species designation is impossible according to the guidelines of the International Code of Nomenclature of Bacteria (Lapage *et al*., 1992), which states the necessity of a broad range of phenotypic and phylogenetic data. Murray & Schleifer (1994) anticipated this problem and proposed a provisional status to record the properties of putative taxa of prokaryotes. This proposal was implemented in 1995 by the International Committee on Systematic Bacteriology, by the introduction of the provisional status *Candidatus* for the description of uncultivable organisms based upon genomic data and to a certain extent on structural, metabolic, reproductive and environmental characteristics (Murray & Stackebrandt, 1995).

It was the object of this study to determine the taxonomic position of the porcine gastrospirillum-like bacteria according to the guidelines of Murray & Schleifer (1994). In the present study, we propose a new candidate species, '*Candidatus* Helicobacter suis', a gastric *Helicobacter* species from pigs.

## METHODS

**Samples.** Stomachs from five healthy slaughterhouse pigs were selected, all originating from different farms in Belgium and the Netherlands. The stomachs were opened longitudinally along the greater curvatura and rinsed gently with tap water. A small mucosal fragment was taken from each stomach near the torus pyloricus and placed into an urease test tube (CUTest; Temmler Pharma) for 2 h at 37 °C. Mucosal biopsies from the antral part of the stomach were taken along the curvatura major ($n=2$) and the curvatura minor ($n=2$) for immunohistochemical evaluation and placed into 4% buffered formalin. For electron microscopy, samples were taken from the same places and fixed in 0·1 M cacodylate buffer (pH 7·0) containing 5% glutaraldehyde and 0·15% (w/v) ruthenium red. A mucosal fragment of each stomach was also taken for PCR, placed into sterile PBS and frozen in liquid nitrogen. Special care was taken during sampling to avoid cross-contamination.

**Reference strains.** Fifteen *Helicobacter* strains were used to test the specificity of the '*Candidatus* Helicobacter suis'-specific PCR assay (Table 1). Strains were grown on 5% Mueller–Hinton blood agar and incubated at 37 °C in a microaerobic atmosphere containing approximately 5% $O_2$, 3·5% $CO_2$, 7·5% $H_2$ and 84% $N_2$. Bacteriological purity was checked by plating and Gram-staining.

**Immunohistochemistry.** An immunohistochemical staining procedure was performed on the formalin-fixed, paraffin-embedded pyloric samples of each animal, to look for the presence of gastrospirillum-like organisms. Sections (4 μm thick) of formalin-fixed, paraffin-embedded tissues were placed on 3-aminopropyl triethoxysilane (APES)-coated slides (Sigma) and dried overnight at 60 °C. After dewaxing with xylene and rehydration in series with ethanol and distilled water, sections were placed in citrate buffer (0·1 M with 2% urea) and boiled (one period of 3 min, two periods of 5 min) in an 800 W microwave oven (Whirlpool M611) to elicit antigen retrieval. Slides were then incubated with 12% $H_2O_2$ in methanol (30 min) in order to block endogenous peroxidase activity and were subsequently pre-incubated with 30% normal goat serum in PBS for 30 min to reduce non-specific antibody binding. The sections were incubated overnight at 21 °C in a moist chamber with a mouse polyclonal antibody directed against *H. pylori* (DAKO), diluted 1:20 in PBS. The sections were washed and incubated with biotinylated swine anti-rabbit immunoglobulin (21 °C, 30 min) and, after rinsing, were covered with peroxidase-conjugated streptavidin–biotin complex (ABC). Peroxidase activity was developed using $H_2O_2$ with diaminobenzidine (DAB) as a chromogen (Fast DAB Tablet Set; Sigma). Finally, the sections were counter-stained with Mayer's haematoxylin and mounted. The *H. pylori*-specific polyclonal antibodies were replaced by foetal calf serum in Tris/HCl buffer (pH 7·6) for negative controls. A section of a mouse stomach experimentally infected with *H. pylori* LMG 7539$^T$ was taken as a positive control.

**Transmission electron microscopy.** Two different antral biopsies were selected for electron microscopic evaluation on the basis of the high numbers of gastrospirillum-like organisms in the corresponding immunostained slides. After dehydration in a graded series of acetone washes, the samples were embedded in Spurr low-viscosity resin. Ultrathin sections were post-stained with uranyl acetate and lead citrate and examined with an electron microscope (Phillips 201 TEM) at an accelerating voltage of 60 kV.

**DNA extraction.** DNA was isolated from the scrapings of the gastric biopsies and from the reference strains by lysis with guanidinium isothiocyanate and bound to silica particles (Boom *et al*., 1990).

**Primers and PCR amplification of 16S rDNA.** Broad-range PCR primers *H33f*, *H61f* and *H1368r* were selected from rRNA superfamily VI-specific regions of the 16S rRNA gene by aligning multiple 16S rDNA sequences from *Helicobacter*, *Campylobacter*, *Arcobacter* and *Wolinella* species. The use of broad-range primer *1492RPL* was suggested by

***Table 1.*** *Helicobacter* strains used for the evaluation of the '*Candidatus* Helicobacter suis'-specific PCR

| Taxon | Source | Strain |
|---|---|---|
| *H. acinonychis* | Cheetah gastric mucosa | LMG 12684$^T$ |
| *H. cinaedi* | Human faeces | LMG 7543$^T$ |
| *Helicobacter* sp. CLO-3 | Human rectal swab | LMG 7792 |
| *H. fennelliae* | Human faeces | LMG 11759 |
| *H. pametensis* | Tern faeces | LMG 12678$^T$ |
| *Helicobacter* sp. Bird B | Bird faeces | LMG 12679 |
| *Helicobacter* sp. Bird C | Bird faeces | LMG 13642 |
| *H. hepaticus* | Mouse liver | LMG 16316$^T$ |
| *H. pullorum* | Broiler chicken mucosa | LMG 16318 |
| *H. mustelae* | Ferret gastric mucosa | LMG 18044$^T$ |
| *H. canis* | Dog faeces | LMG 18086$^T$ |
| *H. muridarum* | Mouse intestinal mucosa | LMG 14378$^T$ |
| *H. bizzozeronii* | Dog gastric mucosa | 12A |
| *H. salomonis* | Dog gastric mucosa | CCUG 37845$^T$ |
| *H. felis* | Cat gastric mucosa | CCUG 28539$^T$ |

***Table 2.*** Oligonucleotide primers and probe used for PCR amplification, sequencing of genes encoding 16S rRNA and Southern blot hybridization

Primer locations are given relative to the sequence of the *E. coli* 16S rRNA gene.

| Primer | Sequence (5′–3′) | Location |
|---|---|---|
| *H33f* | ACGCTGGCGGCGTGCCTAATACATGCAAGTCG | 33–64 |
| *H1368r* | GGTGAGTACAAGACCCGGGAACGTATTCACCG | 1368–1388 |
| *H61f* | TGCAAGTCGAACGATGAAGC | 61–76 |
| *H274f* | AGGCTATGACGGGTATCCGGCCTGAGA | 274–299 |
| *1492RPL* | GCCGCCCGGGTTACCTTGTTACGACTT | 1491–1517 |
| *H390f* | GCAGCAACGCCGCGTGGAGGATGA | 390–413 |
| *H1053r* | ACGAGCTGACGACAGCCGTG | 1053–1072 |
| *V832f* | TTGGGAGGCTTTGTCTTTCCA | 832–852 |
| *V1000f* | AGGAATTCCCTAGAAATAGGG | 1000–1020 |
| *V1261r* | GATTAGCTCTGCCTCGCGGCT | 1261–1281 |

Weisburg *et al*. (1991). A genus *Helicobacter*-specific primer *H274f* was adapted from primer *274r* described by Dewhirst *et al*. (1994) (Table 2). Primer combinations *H33f–H1368r*, *H274f–1492RPL* and *H61f–1492RPL* were used to amplify ~1·3, ~1·2 and ~1·4 kbp fragments, respectively. PCR was performed in a volume of 50 µl containing 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 3·5 mM $MgCl_2$, 200 µM of each dNTP, 1·5 U Ampli*Taq* Gold (Perkin-Elmer) and 25 pmol of both forward and reverse primers (Eurogentec). Reaction mixtures were covered with mineral oil and PCR was performed in a Biomed-60 thermocycler under the following conditions: 9 min pre-incubation at 94 °C to activate Ampli*Taq* Gold, followed by 50 cycles of 30 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C. Final extension was performed for 5 min at 72 °C.

**Analysis of PCR products.** PCR products were separated on 1% agarose gels and stained with ethidium bromide. DNA extractions of *Helicobacter acinonychis* LMG 12684$^T$ (Trüper & De'Clari, 1997) and *Helicobacter mustelae* LMG 8776 were used as positive controls. In order to determine whether PCR products were derived from helicobacter-like organisms, the desired DNA bands were cut from the gels, diluted 1:1 in distilled water and sequenced using 5′-indocarbocyanin (Cy5)-labelled *H61f* and *1492RPL* primers. Partial sequences were screened for homologous sequences using the NCBI GENINFO BLAST network service (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997).

**DNA cloning and sequencing.** PCR products comprising the 16S rDNA sequences from two different stomach samples (V2B, V4A) were cloned into plasmid vector pGEM-T (Promega Biotech) according to the manufacturer's instructions and transformed into *Escherichia coli* JM109 using standard procedures. Plasmids were purified using the Easy Prep Plasmid Prep kit (Pharmacia Biotech). Sequences were determined by the T7 sequencing system (Pharmacia

***Table 3.*** Strains used for phylogenetic analysis

| Taxon | Source | Accession no. | Strain/clone |
|---|---|---|---|
| '*H. heilmannii*' type 1 | Human gastric mucosa | L10079 | Clone G1A1 |
| '*H. heilmannii*' type 2 | Human gastric mucosa | L10080 | Clone G2A9 |
| *H. acinonychis* | Cheetah gastric mucosa | M88148 | Eaton 90-119-3 |
| *H. bilis* | Mouse liver | U18766 | MIT 93-1909 |
| *H. bizzozeronii* | Dog gastric mucosa | Y09404 | CCUG 35045 |
| *H. canis* | Dog faeces | L13464 | NCTC 12739 |
| *H. cholecystus* | Mouse liver | U46129 | Hkb-1 |
| *H. cinaedi* | Human faeces | M88150 | CCUG 18818 |
| *H. felis* | Cat gastric mucosa | M57398 | CS1 |
| *H. fennelliae* | Human faeces | M88154 | CCUG 18820 |
| *H. hepaticus* | Mouse liver | U07574 | FRED1 |
| *H. muridarum* | Mouse intestinal mucosa | M80205 | ST1 |
| *H. mustelae* | Ferret gastric mucosa | M35048 | ATCC 43772 |
| *H. nemestrinae* | Macaque gastric mucosa | X67854 | ATCC 49396$^T$ |
| *H. pametensis* | Swine faeces | M88155 | M17 Seymour |
| *H. pullorum* | Broiler chicken caecum | L36141 | NCTC 12824 |
| *H. pylori* | Human gastric mucosa | M88157 | ATCC 43504 |
| *H. salomonis* | Dog gastric mucosa | Y09405 | CCUG 37845$^T$ |
| *H. trogontum* | Rat colon mucosa | U65103 | LRB 8581 |
| *H. rodentium* | Mouse intestinal mucosa | U96297 | MIT 96-1312 |
| *Arcobacter butzleri* | Human | L14626 | CCUG 10373 |
| *Campylobacter jejuni* | Human faeces | L14630 | CCUG 24567 |
| *Wolinella succinogenes* | Cattle abomasal mucosa | M88159 | ATCC 29543 |

Biotech). Two primers flanking the multiple cloning sites (*T7* and *SP6*) as well as internal primers *H390f* and *H1053r* (Table 2) were used. Sequence analysis was performed with the PCGENE software (IntelliGenetics).

PCR amplicons of three other gastric samples (V5, V14, V19) were sequenced without prior cloning (referred to below as direct sequence analysis).

**Phylogenetic analysis.** Phylogenetic analysis was performed using the GENCOMPAR 2.0 software package (Applied Maths). All five '*Candidatus* Helicobacter suis' sequences and the sequences of strains belonging to the same phylogenetic lineage (Table 3) were aligned. A phylogenetic tree and corresponding similarity matrix were constructed using the neighbour-joining method. Unknown bases and gaps were not considered in the numerical analysis.

**Diagnostic PCR assay and Southern blot hybridization.** '*Candidatus* Helicobacter suis'-specific primers (*V832f* and *V1261r*) were selected from variable rDNA regions of the sequences determined by direct and indirect sequence analysis, comprising a ~0·4 kbp 16S rDNA fragment. Within this fragment, a '*Candidatus* Helicobacter suis'-specific probe, *V1000f* (Table 2), was selected for hybridization purposes. PCR was performed in a volume of 50 µl containing 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 2·5 mM $MgCl_2$, 200 µM of each dNTP, 1·5 U Ampli*Taq* Gold and 25 pmol of both forward and reverse primers. PCR amplification was performed under the following conditions: 9 min pre-incubation at 94 °C to activate Ampli*Taq* Gold, followed by 40 cycles of 30 s at 94 °C, 45 s at 60 °C and 90 s at 72 °C. Final extension was performed for 5 min at 72 °C. Plasmid DNA from the cloned 16S rDNA fragments (V2B, V4A) was used as a positive control. DNA extracted from the stomach of a gnotobiotic piglet was used as a negative control.

To test the specificity of the primers, PCR was also performed on DNA extracts of 15 different *Helicobacter* species (Table 1), including all of the closest phylogenetic neighbours of '*Candidatus* Helicobacter suis' and a random selection of other helicobacters.

PCR products were separated on 2% agarose gels, stained with ethidium bromide and transferred to Hybond-N+ (Amersham) by electro-blotting. Southern blot hybridization was performed with the [$\gamma$-$^{32}$P]ATP-labelled probe *V1000f* according to standard procedures (Amersham Pharmacia Biotech). In order to ensure the specificity of the probe hybridization, blots were washed twice with 0·1× SSC, 0·1% SDS at 55 °C.

***In situ* hybridization.** To link the derived sequence to the corresponding organism, an *in situ* hybridization procedure was performed on the formalin-fixed, paraffin-embedded pyloric samples from each animal. A ~0·4 kb digoxigenin-labelled probe was synthesized using the PCR Dig Probe Synthesis kit (Boehringer Mannheim) in combination with the '*Candidatus* Helicobacter suis'-specific primers *V832f* and *V1261r* (Table 2). PCR conditions were identical to those described in the diagnostic PCR assay. The resulting PCR product was purified using the High Pure PCR Product Purification kit (Boehringer Mannheim) following the manufacturer's instructions.

Sections (4 µm) of the paraffin-embedded tissues were mounted on APES-coated slides (Sigma) and fixed by heating for 1 h at 60 °C. The sections were deparaffinized in xylene (2×5 min), rehydrated through a graded ethanol

series and washed twice in PBS for 5 min each. Sections were then treated with proteinase K (DAKO) for 15 min at 37 °C in a humidified chamber. The enzyme was inactivated by treatment with 0·2 % glycine in PBS for 3 min. Sections were washed twice in PBS for 5 min each, dehydrated in graded ethanol series and air-dried. Tissues were circumscribed with a DAKO Pen (DAKO) to avoid liquid spillage during further processing. For the hybridization step, sections were covered with 5–15 µl hybridization solution consisting of 5 ng labelled probe $\mu l^{-1}$ in 50 % deionized formamide, 2 × SSC, 10 % dextran sulfate, 0·25 µg yeast tRNA $\mu l^{-1}$, 0·5 µg heat-denatured salmon sperm DNA $\mu l^{-1}$ and 1 × Denhardt's solution. Sections were covered with a coverslip. To denature the probe, sections were heated for 10 min at 95 °C and chilled on ice for 10 min. Slides were then incubated overnight at 37 °C in a humidified chamber. To remove the unbound probe, the coverslips were removed and the sections were washed in 2 × SSC and 1 × SSC at room temperature for 10 min each followed by washes in 0·3 × SSC at 40 °C for 10 min and at room temperature for 10 min.

All steps involving the immunological detection of the hybridized probe were performed at room temperature. The sections were treated first for 30 min in buffer 1 (100 mM Tris/HCl, 150 mM NaCl, pH 7·5) containing 2 % normal goat serum and 0·3 % Triton X-100, followed by a 3 h incubation with diluted (1 : 30 in buffer 1) anti-digoxigenin antibodies conjugated to horseradish peroxidase (DAKO). Unbound antibodies were removed by washing gently on a shaker, first with buffer 1 and then with buffer 2 (100 mM Tris/HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9·5) for 15 min each. To optimize the level of detection, the Tyramid Signal Amplification system (NEN Life Science Products) was applied on each section, following the manufacturer's instructions. The hybridized probe was then visualized using $H_2O_2$ with DAB as the chromogen. Finally, the sections were counter-stained with Mayer's haematoxylin and mounted.

**Nucleotide sequence accession numbers.** Accession numbers of the 16S rDNA gene sequences used for the phylogenetic analysis are listed in Table 3.

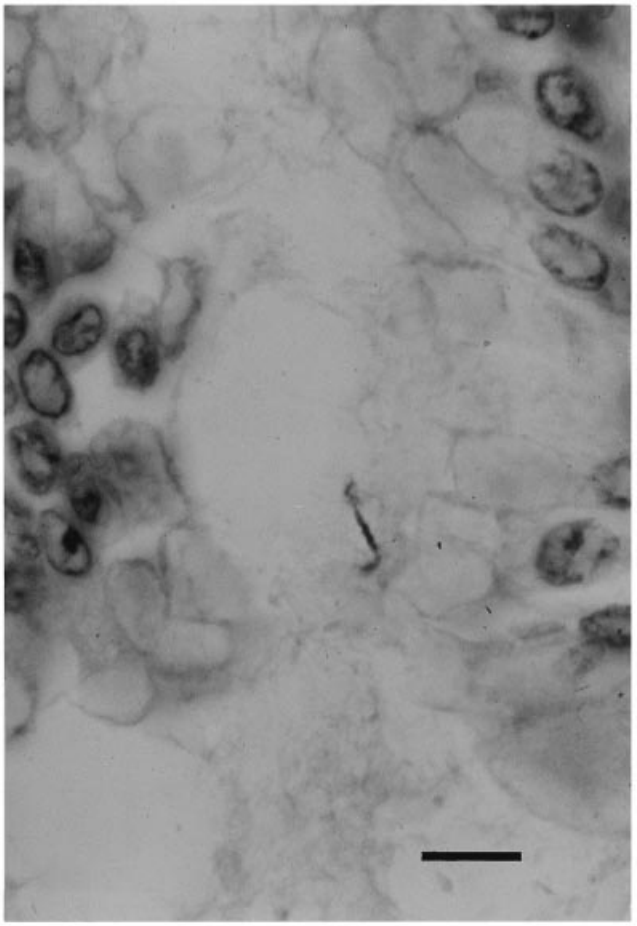

***Fig. 1.*** Immunohistochemical staining of a gastrospirillum-like organism in the gastric crypts of a pig showing a spiral morphology and bipolar multiple flagella. Bar, 10 µm.

## RESULTS

### Urease activity and immunohistochemical evaluation

Tightly coiled, immunostained spiral organisms, morphologically similar to '*G. suis*' (Queiroz *et al*., 1990), were observed in all stomach samples (5/5), which was consistent with the presence of urease activity (5/5). The gastrospirillum-like organisms were seen lying separately or in small clusters with a patchy distribution over the sample and were found mostly in the superficial part of the gastric crypts. Some bacteria revealed bipolar immunostained flagella (Fig. 1). Immunostained coccoid-like organisms were also observed in the pyloric crypts. Only *H. pylori*-like bacteria were stained in the positive control, while no labelling was observed in the negative controls.

### Transmission electron microscopy

Longitudinal and transversal sections of spiral organisms could be seen within the gastric crypts of the antral region. All bacteria had the same characteristic, tightly coiled appearance, typical of helicobacters with the gastrospirillum morphology (Fig. 2). The length of cells varied from 2·5 to 3·5 µm and they were approximately 0·6 µm wide. Multiple complete spiral turns with a wavelength of ~ 600 nm were seen in all longitudinal sections. Polarly flagellated cells were observed, the number of flagella ranging from one to five. The presence of a flagellar sheath was unclear. The bacteria were not seen within cells, nor was there any obvious association with the surrounding epithelial cells.

### Amplification, cloning and sequencing of helicobacter-like 16S rDNA fragments

Several combinations of PCR primers yielded sequences of the expected size. The length of these amplified fragments varied between 1·2 (*H274f–1492RPL*) and 1·4 kbp (*H61f–1492RPL*). The latter primer combination was used to examine all samples. The 16S rDNA sequences of two different clones were

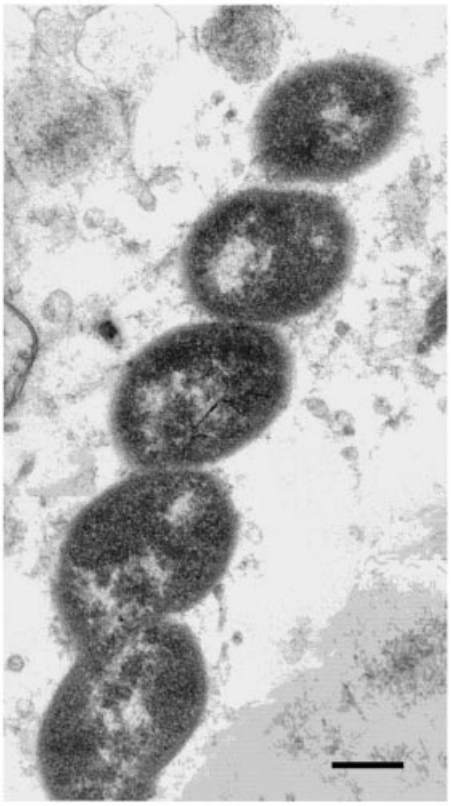

***Fig. 2.*** Transmission electron micrograph showing a cross-section of a '*G. suis*'-like bacterium in the crypts of the antral part of the stomach of a pig. Bar, 300 nm.

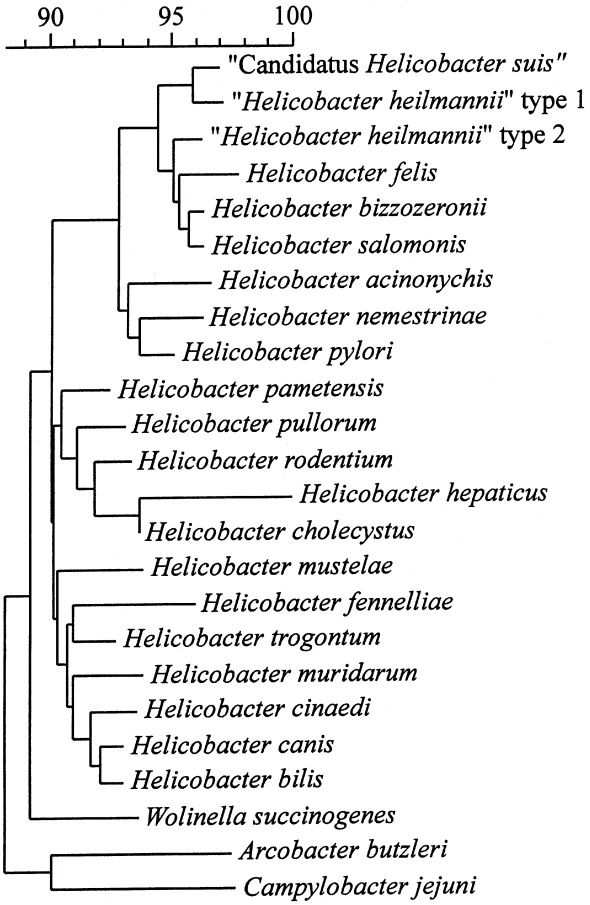


***Fig. 3.*** Phylogenetic tree for 23 strains of *Helicobacter*, *Campylobacter*, *Arcobacter* and *Wolinella* species on the basis of 16S rDNA sequence similarity. Divergence of nucleotide sequences, as determined by measuring the length of horizontal lines connecting any two species, is shown as a percentage.

determined (V2BXA, V4AXA). Additional sequences of three other samples (V5, V14, V19) were determined by direct sequence analysis.

### Sequences and phylogenetic analysis

The five sequences that were determined varied in length from 1345 to 1421 bp. Pairwise comparisons between 1345 bp consensus fragments of these sequences revealed a minimum similarity of 97·7%. One reference sequence of 1421 bp, obtained from PCR product V2BXA, was used for phylogenetic analysis. A similarity matrix was calculated based on comparisons of 16S rDNA sequences of all *Helicobacter* species, '*Helicobacter heilmannii*' types 1 and 2, *Campylobacter jejuni*, *Arcobacter butzleri* and *Wolinella succinogenes* (Table 3). The sequence of the porcine gastrospirillum-like organism formed a distinct sub-group in this analysis within the *Helicobacter* lineage, together with other gastrospirilla: *H. felis*, *H. bizzozeronii*, *H. salomonis* and '*H. heilmannii*' types 1 and 2. The sequence was highly similar to that of '*H. heilmannii*' type 1 (99·5% similarity). The similarity to other gastrospirillum-like bacteria, *H. felis*, *H. bizzozeronii*, *H. salomonis* and '*H. heilmannii*' type 2, was clearly lower: 96·4, 96·5, 96·6 and 96·8%, respectively. The reference sequence was clearly distinct from sequences belonging to other genera from superfamily VI, as shown by 86·2, 84·7 and 89·6% similarity with *C. jejuni*, *A. butzleri* and *W. succinogenes*, respectively. A phylogenetic tree based on this analysis is shown in Fig. 3.

### '*Candidatus* Helicobacter suis'-specific PCR and Southern blot hybridization

Amplification of helicobacter DNA using the primers *V832f* and *V1261r* produced a 433-bp fragment from all five stomach samples. All PCR products hybridized with the *V1000f* probe after Southern blot hybridization. No amplification product was obtained using DNA preparations from any of the *Helicobacter* strains, including *H. felis*, *H. bizzozeronii* and *H. salomonis* (Table 1), nor from the negative control. PCR with the cloned reference material (2BXA) yielded a ~ 0·4 kbp product as expected.

### *In situ* hybridization

*In situ* hybridization of '*G. suis*'-like bacteria with the '*Candidatus* Helicobacter suis'-specific probe was seen in sections from all (5/5) stomachs. Bacteria were observed as dark-brown spiral organisms in the superficial mucus layer and the gastric crypts. In some cases, helical organisms, located deeply in the crypts, were labelled weakly or were negative. A faint background staining, seen as finely stained strings, was sometimes observed in the surrounding cells. This background staining was also observed in the *H. pylori*-infected mouse stomach that was used as a negative control. The *H. pylori* cells in this control did not hybridize with the '*Candidatus* Helicobacter suis'-specific probe, however.

## DISCUSSION

The presence of helicobacter-like organisms in the stomachs of pigs was reported for the first time in 1990 (Queiroz *et al.*, 1990). Since then, the morphology, prevalence and potential pathogenic role of these organisms have been described in several other studies (Mendes *et al.*, 1990, 1991; Barbosa *et al.*, 1995; Queiroz *et al.*, 1996). The name '*G. suis*' was proposed (Mendes *et al.*, 1990), as these bacteria were morphologically similar to '*Gastrospirillum hominis*', a unculturable bacterium seen in human gastric biopsy specimens (McNulty *et al.*, 1989).

In the present study, 16S rDNA sequences were obtained from five different porcine stomach samples containing gastrospirillum-like bacteria. A maximum divergence of less than 0·3 % was found between these sequences, suggesting that they originate from strains belonging to a single species. Phylogenetic analysis placed the organism characterized by the reference sequence 2BXA within the genus *Helicobacter*, where it formed a distinct sub-group together with other gastrospirillum-like bacteria (*H. felis*, *H. bizzozeronii*, *H. salomonis* and '*H. heilmannii*' types 1 and 2) (Fig. 3). A very high level of 16S rDNA sequence similarity (99·5 %) was found towards '*H. heilmannii*' type 1. This is in agreement with previously reported data on the phylogenetic position of '*G. suis*', described in a poster presented at the 7th workshop of the European *Helicobacter pylori* Study Group (Mendes *et al.*, 1994) (the 16S rDNA sequence determined in the latter study has not been deposited in an international database).

A clearly lower level of 16S rRNA similarity was observed towards the other species characterized by the gastrospirillum morphology, and alignment of the V2BXA 16S rDNA sequence allowed the design of primers and a probe that were specific for the sequences derived from the porcine samples. DNA from none of the other helicobacters examined yielded a product. *In situ* hybridization with a specific probe associated the reference sequence to the spiral organisms seen in the gastric crypts of pigs, confirming that our 16S rRNA sequences corresponded to the '*G. suis*' cells observed in the gastric specimens. These results indicate that the porcine sequences represent a taxon that can be differentiated from all known helicobacter species. The low level of similarity towards *H. salomonis* (96·6 %), its closest validly named neighbour, strongly suggests that this taxon is a novel helicobacter species. Conforming with the guidelines of Murray & Schleifer (1994) and Murray & Stackebrandt (1995), we propose to assign the porcine taxon to the category *Candidatus* pending further characterization of this bacterium after *in vitro* cultivation of strains. The availability of the nearly complete 16S rDNA sequence and the specific PCR assay and probe provide the scientific community with an instrument to analyse the prevalence and role of this bacterium.

Analysis of 16S rDNA sequences of human gastrospirilla ('*G. hominis*') revealed not only that these bacteria also belong to the genus *Helicobacter* (and as a consequence the name '*H. heilmannii*' has been used to denote the same organism), but that there are at least two different types, commonly referred to as types 1 and 2 (O'Rourke *et al.*, 1992; Solnick *et al.*, 1993). This observation was based on a 3·5 % sequence difference, suggesting that the two sequences represented two different species. '*H. heilmannii*' type 2 belongs phylogenetically to the *H. felis–H. bizzozeronii–H. salomonis* sub-cluster and its relatedness towards these three species needs further study. As '*H. heilmannii*' type 1 shares 99·5 % of its 16S rRNA sequence with '*G. suis*' (Queiroz *et al.*, 1996; this study), it is tempting to speculate that both taxa represent the same species and that '*H. heilmannii*' type 1 infection is a zoonosis from pigs. However, strains of different species may share such high levels of 16S rDNA sequence similarity (Stackebrandt & Goebel, 1994), as is illustrated by the high similarity between, for instance, *H. felis*, *H. bizzozeronii* and *H. salomonis* (Jalava *et al.*, 1997). As the relationship between '*H. heilmannii*' type 1 and '*G. suis*' can be determined fully only after cultivation of strains from both taxa, we propose the name '*Candidatus* Helicobacter suis' for the porcine gastrospirillum.

The description is as follows: '*Candidatus* Helicobacter suis' [(*ε-Proteobacteria*), genus *Helicobacter*; porcine isolates have been propagated in mice, but not *in vitro*; Gram-negative; Helical; NAS (GenBank accession no. AF127028), oligonucleotide sequence complementary to unique region of 16S rRNA gene 5′-AGCCGCGAGGCAGAGCTAATC-3′; morphology, 2·5–3·5 × 0·6 μm, multiple complete spiral turns with a wavelength of ~ 600 nm, multiple bipolar flagella; symbiotic (*Sus*, stomach); strong urease activity, cross-reaction with polyclonal *H. pylori*-derived antibodies].

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