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Novel urease-negative *Helicobacter* sp. '*H. enhydrae* sp. nov.' isolated from inflamed gastric tissue of southern sea otters

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ABSTRACT: A total of 31 sea otters *Enhydra lutris nereis* found dead or moribund (and then euthanized) were necropsied in California, USA. Stomach biopsies were collected and transected with equal portions frozen or placed in formalin and analyzed histologically and screened for *Helicobacter* spp. in gastric tissue. *Helicobacter* spp. were isolated from 9 sea otters (29%); 58% (18 of 31) animals were positive for helicobacter by PCR. The *Helicobacter* sp. was catalase- and oxidase-positive and urease-negative. By electron microscopy, the *Helicobacter* sp. had lateral and polar sheathed flagella and had a slightly curved rod morphology. 16S and 23S rRNA sequence analyses of all '*H. enhydrae*' isolates had similar sequences, which clustered as a novel *Helicobacter* sp. closely related to *H. mustelae* (96–97%). The genome sequence of isolate MIT 01-6242 was assembled into a single ~1.6 Mb long contig with a 40.8% G+C content. The annotated genome contained 1699 protein-coding sequences and 43 RNAs, including 65 genes homologous to known *Helicobacter* spp. and *Campylobacter* spp. virulence factors. Histological changes in the gastric tissues extended from mild cystic degeneration of gastric glands to severe mucosal erosions and ulcers. Silver stains of infected tissues demonstrated slightly curved bacterial rods at the periphery of the gastric ulcers and on the epithelial surface of glands. The underlying mucosa and submucosa were infiltrated by low numbers of neutrophils, macrophages, and lymphocytes, with occasional lymphoid aggregates and well-defined lymphoid follicles. This is the second novel *Helicobacter* sp., which we have named '*H. enhydrae*', isolated from inflamed stomachs of mustelids, the first being *H. mustelae* from a ferret.

KEY WORDS: *Helicobacter* · Gastritis · Otter

INTRODUCTION

Sea otter populations have suffered dramatically due to extirpation associated with hunting in the eighteenth and nineteenth centuries (1741 to 1911) until they were protected under the International Seal Treaty (Larson et al. 2012). The southern sea otter *Enhydra lutris nereis,* listed as Threatened under the US Endangered Species Act, has declined in numbers in the 1970s through the mid-late 1990s, and their population continues to be suboptimal despite having been legally protected for >100 yr. Slow population recovery of the southern sea otter is undoubtedly multifaceted; both traumatic and infec-

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tious causes can impact individual survival and population growth (Kreuder et al. 2003). In a recent systematic review of published cases of marine mammal diseases from 1972 to 2012, bacterial cases represented approximately 20% of the total number of calculated cases reported (Simeone et al. 2015). Of the bacterial-associated diseases in sea otters, fatal infection by *Streptococcus phocae* and other beta-hemolytic streptococci has been associated with skin trauma (Bartlett et al. 2016); *S. infantarius* has been linked to septicemia and fatal vegetative endocarditis (Carrasco et al. 2014, Counihan et al. 2015); novel *Bartonella* spp. have been identified by PCR-based assays, along with *Streptococcus* spp. in vegetative valvular endocarditis cases. Antibodies to *Toxoplasma gondii, Leptospira interrogans,* and *Brucella* spp. have also been recorded in sea otters inhabiting southern California (Hanni et al. 2003). The prevalence of *T. gondii* infection has been associated with water-runoff contaminated with *T. gondii* oocysts (Miller et al. 2002), which are trapped by kelp; the kelp biofilm is then ingested by snails, which in turn are ingested by the otters (Mazzillo et al. 2013). *Sarcocystis neurona* infections identified in sea otters are also the result of fecal-associated exposure (Miller et al. 2010). Other enteric pathogens, including *Campylobacter* spp., *Clostridium perfringens,* and *Vibrio parahaemolyticus,* are cultured from feces of southern sea otters living in coastal urban areas with higher freshwater runoff exposure (Miller et al. 2006).

Although gastric ulcers have been noted in both northern and southern sea otters, an etiological agent has not been previously identified (Lipscomb et al. 1993, Kreuder et al. 2003). Given that another member of the *Mustelidae* family, the domestic ferret *Mustela putorius furo*, is known to be colonized with a gastric helicobacter, *Helicobacter mustelae,* which is associated with gastritis and gastric ulcers, we initiated a survey of southern sea otters to ascertain whether these animals were also colonized with a gastric *Helicobacter* sp. (Fox et al. 1990, 1991, 1992, 1993).

MATERIALS AND METHODS

Sample collection

A total of 31 southern sea otters *Enhydra lutris nereis* found dead or moribund and euthanized were necropsied in California. In accordance with Section 109(h) of US Marine Mammal Protection Act (MMPA) and the US Fish and Wildlife Service's (Service) regulations implementing the MMPA at 50 CFR 18.22(a), and in accordance with the Service's regulations implementing the US Endangered Species Act at 50 CFR 17.21(c)(3), the samples that were used to complete this work were collected from fresh, necropsied sea otter carcasses taken from the wild by an official or employee of the California Department of Fish and Wildlife (CFDW) in the course of his or her duties as an official or employee of CDFW. Stomach biopsies from the pylorus and the gastric body were collected, placed in freeze media containing 20% glycerol in *Brucella* broth (BD) and frozen for *Helicobacter* culture and PCR; samples were also placed in formalin for histological evaluation. The biopsy samples were shipped to Massachusetts Institute of Technology, Cambridge, MA. The initial study was conducted in 2001 with 11 animals; additional samples were collected from 20 animals in 2015. Samples were processed for helicobacter isolation and PCR shortly after receiving the biopsies.

Helicobacter PCR

The High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals) was used for extraction of DNA from both the gastric samples from 31 sea otters and the bacterial isolates following the manufacturer's instructions. *Helicobacter* genus-specific primers C97 (5′-GCT ATG ACG GGT ATCC-3′) and C05 (5′-ACT TCA CCC CAG TCG CTG-3′) were used to amplify a 1.2 kb PCR product from the16S rRNA gene (Fox et al. 1998). PCR amplifications were performed using the Expand High Fidelity PCR System (Roche Molecular Biochemicals). The following conditions were used for amplification: 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1.5 min, followed by an elongation step of 7 min at 72°C. The 1200 bp PCR products were sequenced using BigDye Terminator Cycle Sequencing method (Thermo Fisher Scientific).

Helicobacter culture

Biopsy samples from 31 sea otter stomachs were homogenized, and gastric body and pyloric aliquots were applied separately to the surface of CVA (cefoperazone, vancomycin, and amphotericin B) agar

plates. Additional aliquots of each sample were passed through a 0.65 µm syringe filter onto a trypticase soy agar plate with 5% sheep blood (Remel Laboratories). All plates were incubated at 37°C under microaerobic conditions in a vented jar containing N_2, H_2, and CO_2 (80, 10, and 10%, respectively) and were inspected for bacterial growth every 2 to 3 d for 3 wk.

Detailed biochemical characterization analysis was performed on 5 individual isolates using the RapID™ NH System (Remel Laboratories) and API Campy kit (bioMérieux). Urease, catalase, and oxidase productions, sensitivity to nalidixic acid and cephalothin, as well as the growth in the presence of 1% glycine were assessed, as previously described (Shen et al. 2005). A disc assay was used to screen for indoxyl acetate hydrolysis (Kaur et al. 2011). Suspected bacterial growth was identified as *Helicobacter* on the basis of gross colony morphology, compatible bacterial morphology on phase microscopy and Gram stains, biochemical testing, helicobacter-specific PCR, and 16S rRNA gene sequencing. The full 16S rRNA sequence of 5 strains was amplified with primer 9F (5′-GAG TTT GAT YCT GGC TCA G-3′) and 1541R (5′-AAG GAG GTG WTC CAR CC-3′). Sequence alignments and phylogenetic analysis of 16S rRNA and 23S rRNA were performed using the Lasergene software package (Lasergene 12 DNASTAR).

Histological evaluation

Full-thickness postmortem gastric biopsies from 26 sea otters were formalin-fixed (10%), paraffin-embedded, and sliced into 5 µm sections stained by hematoxylin and eosin (H&E) and Warthin-Starry stains for histological assessment by a board-certified pathologist (V. Bakthavatchalu).

Electron microscopy

Sea otter isolate *Helicobacter* sp. MIT 01-6242 was examined by transmission electron microscopy (B. Paster). Cells grown on blood agar plates were centrifuged and gently suspended in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of ~10^8 cells ml^{-1}. Samples were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 s. Specimens were examined and measured via a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

Whole genome sequencing of strain MIT 01-6242

Genomic DNA was sequenced using the Single Molecule Real-Time (SMRT) sequencing method with a PacBio RS II machine (Pacific BioSciences). The sequencing reads were assembled using the RS_HGAP_Assembly.3 workflow from the SMRT Portal 2.3. The assembled genome was annotated with the Rapid Annotation using Subsystem Technology (RAST) using the RASTtk workflow (http://rast.nmpdr.org) (Overbeek et al. 2014). Annotated protein sequences were further analyzed for conserved domains using Batch CD-Search (www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) (Marchler-Bauer et al. 2015). Multi-genome comparisons and identification of homologous virulence factor were performed with the Pathosystems Resource Integration Center (PATRIC) (Wattam et al. 2014).

RESULTS

Helicobacter prevalence in sea otters

For samples collected in 2001, 82% of pyloric and 45% of gastric body samples were positive for *Helicobacter* sp. by PCR; *Helicobacter* sp. was isolated from 1 pyloric sample. For samples collected during 2015, 45% of pyloric samples and 10% of gastric body samples were positive for *Helicobacter* spp. by PCR. A novel *Helicobacter* sp. was isolated from 40% of the 2015 pyloric samples (Table 1). For sea otters with gastric ulcers, 4 of 5 (80%) of the stomachs were positive by PCR in 2001, while in 2015, 5 of 10 (50%) of gastric samples with ulcers were positive by PCR for *Helicobacter* spp.

Characteristics of helicobacter isolates from sea otters

Helicobacter-like organisms were isolated from the pylorus of 9 sea otters (Table 1). Gram-negative bacteria were visible on CVA and blood agar plates as single colonies following 3 to 5 d of incubation under microaerobic conditions. The biochemical characteristics of 5 isolates were compared with those of other closely related *Helicobacter* species (Table 2). All isolates were oxidase- and catalase-positive and urease-negative. The isolates did not reduce nitrate to nitrite and did not hydrolyze alkaline phosphate or indoxyl acetate. The bacteria did not have γ-glutamyl transpeptidase activity, and all isolates were sensitive to

Table 1. Prevalence of *Helicobacter* spp. detection (percentage) in gastric mucosal biopsies from necropsied southern sea otters *Enhydra lutris nereis* as well as ulcer rates in the necropsied animals

Expt	Antrum (pylorus)	Body	Animals	Ulcer	Ulcer rates
2001					
PCR	9/11 (82)	5/11 (45)	9/11 (82)	4/5 (80)	5/11 (45)
Culture	1/9 (11)	0/9 (0)	1/9 (11)	0/3 (0)	3/9 (33)
2015					
PCR	9/20 (45)	2/20 (10)	9/20 (45)	5/10 (50)	10/20 (50)
Culture	8/20 (40)	0/20 (0)	8/20 (40)	4/10 (40)	10/20 (50)

nalidixic acid and resistant to cephalothin. The organism grew in 1% glycine and at 37 and 42°C, but not at 25°C.

Electron microscopy

By electron microscopy, the novel sea otter isolate *Helicobacter* sp. is a slightly curved rod (1 to 3 µm long by 0.5 µm wide) (Fig. 1). The organisms had lateral and polar sheathed flagellae. Coccoid bacterial forms with similar flagellae were also noted.

Phylogenetic analysis

The 16S rRNA gene sequences from all 9 sea otter isolates were sequenced and shared over 99% sequence similarity with each other. The sea otter isolates clustered as a novel *Helicobacter* sp. most closely related to *H. mustelae* (96–97%) (Fig. 2A). The 23S rRNA gene sequences of 2 of the isolates,

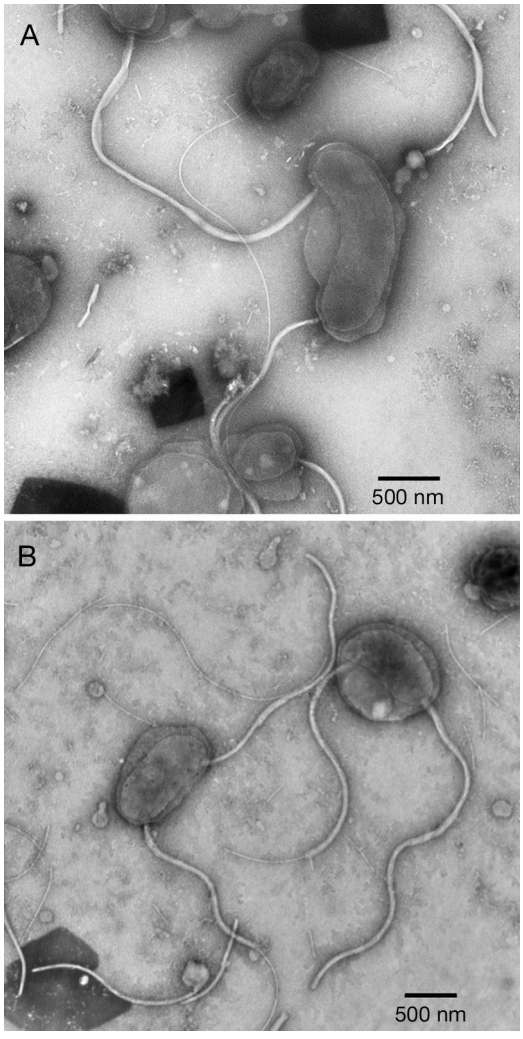

Fig. 1. Transmission electron micrograph of negatively stained *Helicobacter enhydrae* MIT 01-6242, demonstrating (A) a slightly curved bacterial rod with sheathed lateral flagellae and (B) a more coccoid bacterial form

Table 2. Biochemical properties of *Helicobacter enhydrae* isolates from southern sea otters *Enhydra lutris nereis* in relation to other *Helicobacter* spp. NO_3: nitrate reduction; IAH: indoxyl acetate hydrolysis; GGT: gamma-glutamyl transpeptidase; PO_4: alkaline phosphatase hydrolysis; NA: nalidixic acid 30 µg; CE: cephalothin 30 µg; S: susceptible; R: resistant; I: intermediate; ND: no data

Taxon	Catalase	Oxidase	NO_3	Urease	IAH	GGT	PO_4	25°C	37°C	42°C	1% glycine	NA	CE
Sea otter isolate *H. enhydrae* sp. nov.	(+) 5/5	(+) 5/5	(–) 5/5	(–) 5/5	(–) 5/5	(–) 5/5	(–) 5/5	(–) 5/5	(+) 5/5	(+) 5/5	(+) 5/5	(S) 5/5	(S) 5/5
Previously characterized													
H. mustelae	+	+	+	+	ND	+	+	–	+	+	–	S	R
H. cetorum	+	+	–	+	–	+	–	–	+	+	ND	R/S	S
H. acinonychis	+	+	–	+	–	+	+	–	+	–	–	R	S
H. pylori	+	+	–	+	–	+	+	–	+	–	–	R	S
H. macacae	+	–	–	–	–	–	–	–	+	+	+	R	R
H. bilis	+	+	+	+	–	+	–	–	+	+	+	R	R
H. marmotae	+	+	–	+	–	–	+	–	+	–	+	R	R
H. canis	–	+	–	–	+	+	+	–	+	+	–	S	I
H. hepaticus	+	+	+	+	+	–	–	–	+	–	+	R	R

MIT 01-6242 and MIT 15-1068, were analyzed and compared with the 23S rRNA gene sequences of other *Helicobacter* spp.; the 2 sequences were also most closely related to *H. mustelae,* with 97% identity (Fig. 2B).

Whole genome sequencing

The complete genome sequence of *Helicobacter* sp. MIT 01-6242 was obtained using PacBio's SMRT sequencing method. In total, 70 216 reads with a mean read length of 9773 base pairs and N50 read length of 13 584 base pairs were obtained at ~350-fold coverage. The reads were assembled into a single 1.6 Mb long contig with a G+C content of 40.8%, which was similar to representative genomes from the gastric, enterohepatic, and marine *Helicobacter* species *H. mustelae* 12198, *H. pylori* 26695, *H. hepaticus* ATCC 51449, and *H. cetorum* MIT 99-5656. Likewise, the RASTtk annotated genome of *Helicobacter* sp. MIT 01-6242 contained comparable numbers of protein coding sequences and RNA genes as the representative *Helicobacter* genomes. Using PATRIC's proteome comparison service to perform a multi-genome bi-directional BLASTP (parameters: 30% minimum coverage, 10% minimum identity, 1e–5 minimum E-value), >50% of the annotated protein sequences from *Helicobacter* sp. MIT 01-6242 were homologous to those from the other *Helicobacter* spp. (Table 3).

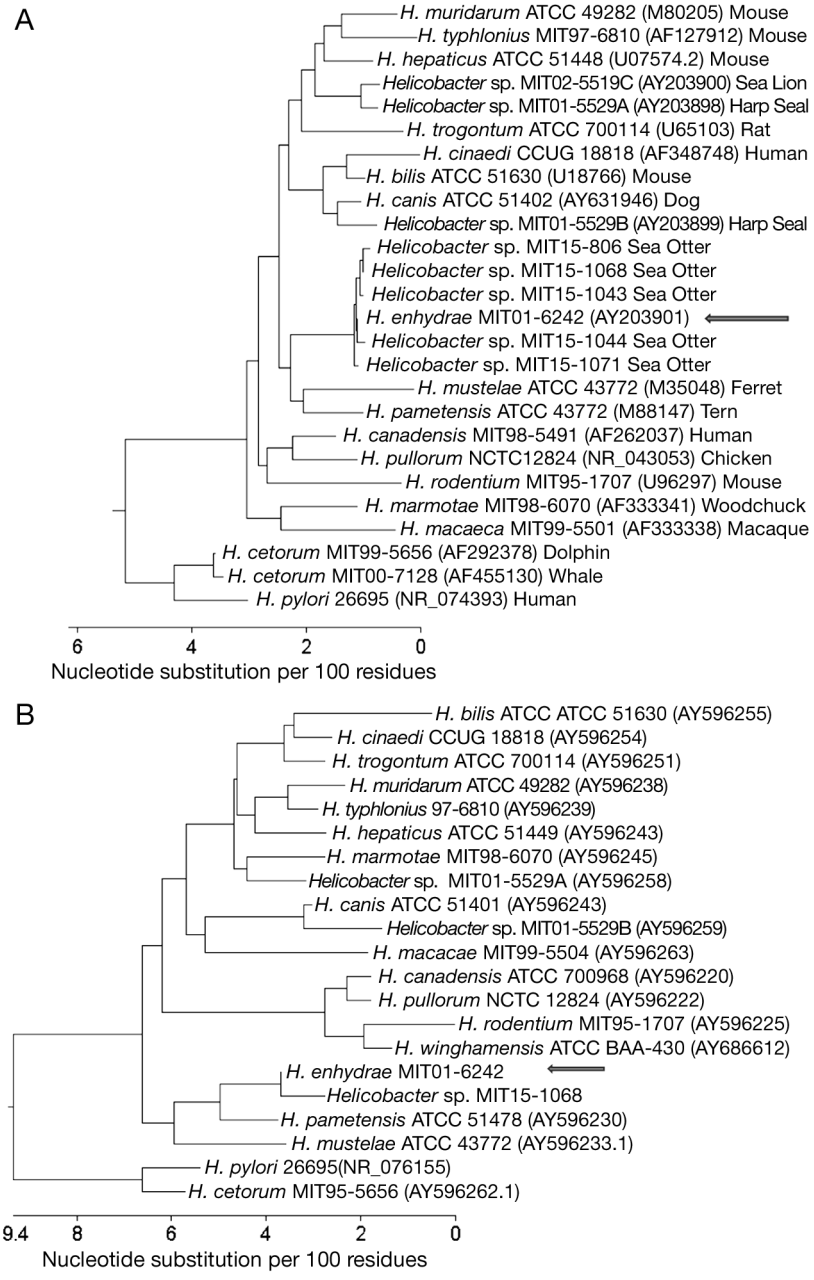

Fig. 2. (A) Phylogenetic placement of *Helicobacter enhydrae* in relation to other (A) *Helicobacter* 16S or (B) *Helicobacter* 23S rRNA gene sequences, based on neighbor-joining analysis. Arrows point to *H. enhydrae* MIT01-6242

Virulence factors from the RASTtk annotated genomes of *H. mustelae* 12198 (NCBI GenBank: FN555004.1), *H. pylori* 26695 (NCBI GenBank: AE000511.1), *H. cetorum* MIT 99-5656 (NCBI GenBank: CP003481.1), *H. hepaticus* ATCC 51449 (NCBI GenBank: AE017125.1), *H. canis* NCTC 12740 (NCBI Reference Sequence: NZ_KI669458.1), *H. canadensis* MIT 98-5491 (NCBI GenBank: ABQS00000000.1), and *Campylobacter jejuni* subspecies jejuni NCTC 11168 (NCBI Reference Sequence: NC_002163.1) were identified from the Victors database through PATRIC and then cross-referenced against *Helicobacter* sp. MIT 01-6242 using the proteome comparison results. In total, 65 genes homologous to known virulence factors from *Helicobacter* and *Campylobacter* spp. were detected in *Helicobacter* sp. MIT 01-6242 (see full list in the Supplement at www.int-res.com/articles/suppl/d123p001_supp.xls). Notable virulence genes included 2 copies of flagellin (*flaA*) necessary for infection, colonization, and pathogenicity by *H. mustelae*, high temperature requirement A (*htrA*) that cleaves

Table 3. Profile characteristics and comparison of *Helicobacter enhydrae* and other related *Helicobacter* species genomes. Genomes downloaded from NCBI and annotated with RASTtk: *H. hepaticus* ATCC 51449 (GenBank: AE017125.1), *H. mustelae* 12198 (GenBank: FN555004.1), *H. cetorum* MIT 99-5656 (GenBank: CP003481.1), and *H. pylori* 26695 (GenBank: AE000511.1). Virulence factor genes: *cadF*: fibronectin/fibrinogen-binding protein; *cagA*: cytotoxin-associated gene A; *cdtB*: cytolethal distending toxin subunit B; *dnaJ*: chaperone protein DnaJ; *flaA*: flagellin; *ggt*: gamma-glutamyltranspeptidase; *hsr*: *Helicobacter* surface rings; *htrA* protease; nap: neutrophil-activating protein; *ure*: urease; *vacA*: vacuolating cytotoxin

Characteristics	*H. enhydrae* MIT 01-6242	*H. hepaticus* ATCC 51449	*H. mustelae* 12198	*H. cetorum* MIT 99-5656	*H. pylori* 26695
Genomic size (bp)	1 594 790	1 799 146	1 578 097	1 833 666	1 667 867
G+C content	40.8%	35.6%	38.9%	42.5%	35.9%
No. of protein genes	1699	1853	1667	1822	1688
No. of RNA genes	43	38	43	41	40
Genes homologous to *H. enhydrae*	–	1079 (64.7%)	1048 (62.8%)	975 (58.6%)	965 (57.9%)
Select virulence factors	*cadF, dnaJ flaA, htrA, nap*	*cdtB, htrA, nap, ure*	*flaA, ggt, hsr, htrA, nap, ure*	*flaA, ggt, htrA, nap, ure*	*cagA, flaA, ggt, htrA, nap, ure, vacA*

E-cadherin, the pro-inflammatory cytokine stimulator neutrophil-activating protein (*nap*), and the adherence/colonization factors fibronectin/fibrinogen-binding protein (*cadF*) and chaperone protein DnaJ (*dnaJ*) (Table 3). Homologous sequences to urease (*ure*), cytotoxin-associated gene A (*cagA*), *virB/D* type IV secretion system components (T4SS), vacuolating cytotoxin (*vacA*), cytolethal distending toxin (*cdtA/B/C*), and gamma-glutamyltranspeptidase (*ggt*) were not found in *Helicobacter* sp. MIT 01-6242. Although the gene encoding *Helicobacter* surface rings (*hsr*), a unique morphological feature of *H. mustelae* 12198 required for pathogenic gastric infection, was not present in *Helicobacter* sp. MIT 01-6242, a protein sequence with a homologous C-terminal autotransporter domain to *hsr* was annotated as a major ring-forming surface antigen precursor (Patterson et al. 2003).

Additionally, multi-genome proteome comparison revealed numerous locations in the genome of *Helicobacter* sp. MIT 01-6242 that contained clusters of protein sequences, almost exclusively annotated as hypothetical proteins, lacking corresponding homologs in the representative *Helicobacter* genomes (Fig. 3). The 2 largest regions, 46 genes from positions 1 157 093 to 1 215 003 bp and 82 genes from 1 446 155 to 1 478 648 bp, both consisted of >60% hypothetical proteins but also contained several genes associated with viral/phage replication and structure, such as integrase and capsid proteins. Batch CD-Search was used as an attempt to identify conserved domains in the hypothetical proteins within these clusters (see Table S1). Four hypothetic proteins within the ~1.15 to ~1.2 Mb cluster contained domains found in bacterial polymorphic toxin systems, such as secreted RNase toxins, which may have virulence functionality (Table S1) (Jamet & Nassif 2015). Lastly, throughout the genome in smaller cluster regions, a total of 30 hypothetical pro-

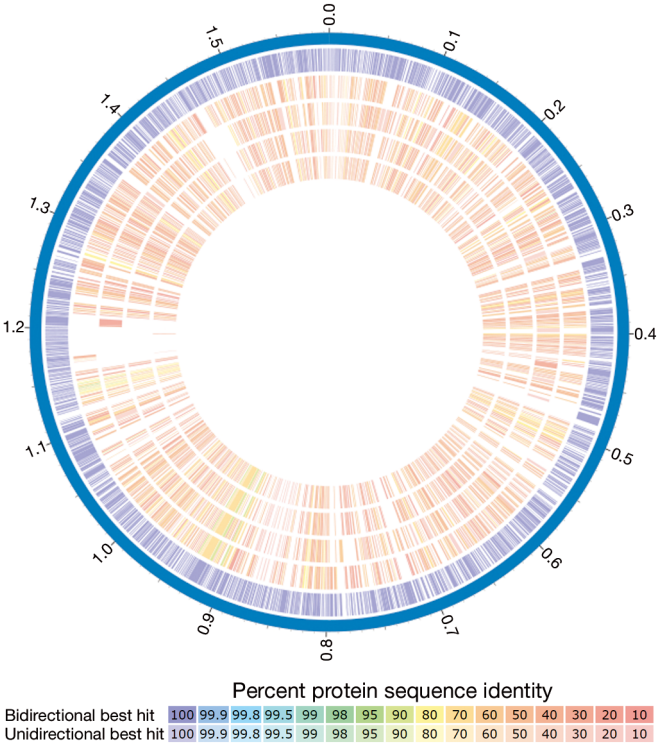

Fig. 3. PATRIC proteome comparison. The RASTtk annotated protein sequences from *Helicobacter enhydrae* were compared to those of *H. hepaticus* ATCC 51449, *H. mustelae* 12198, *H. cetorum* MIT 99-5656, and *H. pylori* 26695 using bi-directional BLASTP. Homologs are presented as bidirectional or unidirectional best hits in accordance with percent protein sequence identity in reference to *H. enhydrae*. Rings from outer to inner represent *H. enhydrae*, *H. hepaticus* ATCC 51449, *H. mustelae* 12198, *H. cetorum* MIT 99-5656, and *H. pylori* 26695

teins were identified and assigned autotransporter domains secreted by a Type V system and associated with virulence (Table S1) (Tseng et al. 2009). This finding was corroborated by identification of the gene components needed for a complete Sec translocase, and thus, a putatively functional Type V secretion system (T5SS) exists in the genome of *Helicobacter* sp. MIT 01-6242.

Histopathology findings

Histological changes in gastric tissues from PCR and/or culture-positive sea otters ranged from mild cystic degeneration of gastric glands to severe erosions and ulcers. In severely affected animals, the erosions and ulcers were characterized by partial or complete loss of gastric mucosa (Fig. 4). The affected areas were disrupted by minimal to moderate amounts of cellular and karyorrhectic debris, showed hemorrhage, and were covered by abundant bacilli. The underlying mucosa and submucosa were infiltrated by low numbers of neutrophils, macrophages, and sparse lymphocytes. The submucosa was mildly expanded by inflammatory cells, fibrin, and edema. Adjacent venules contained fibrin thrombi, and lymphatics were ectatic. Occasionally, the lumen of gastric glands adjacent to affected areas was dilated, with variable surface erosion or attenuated epithelium, and contained scant cellular debris. On some sections, the gastric mucosa and submucosa were disrupted by lymphoid aggregates and well-defined lymphoid follicles composed of mature lymphocytes admixed with rare immunoblasts (Fig. 5). On Warthin-Starry stained slides, numerous 1 to 2 µm by 0.5 to 1.0 µm bacilli lined the mucosa and extended deep into gastric pits and gastric glands (Fig. 6). Bacteria comparable with *H. enhydrae* were also noted at the margins of ulcers in 7 of the affected stomachs (Fig. 7).

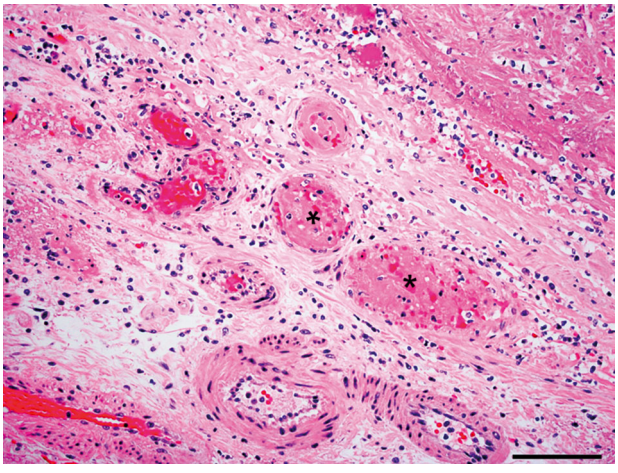

Fig. 4. The gastric submucosal interstitium of a southern sea otter was mildly expanded by neutrophils, macrophages, edema and scant fibrin. The lumen of adjacent venules was obstructed by fibrin thrombi (asterisks). H&E. Scale bar = 100 µm

DISCUSSION

We identified by culture, ultrastructure, biochemical characterization, 16S rRNA and 23S rRNA sequence analysis, and whole genome sequencing a novel helicobacter, for which we propose the name *Helicobacter enhydrae*. Like *H. mustelae,* which colonizes ferrets with gastritis and ulcers, the gastric bacteria were identified by PCR and culture in the inflamed gastric tissue of stranded southern sea

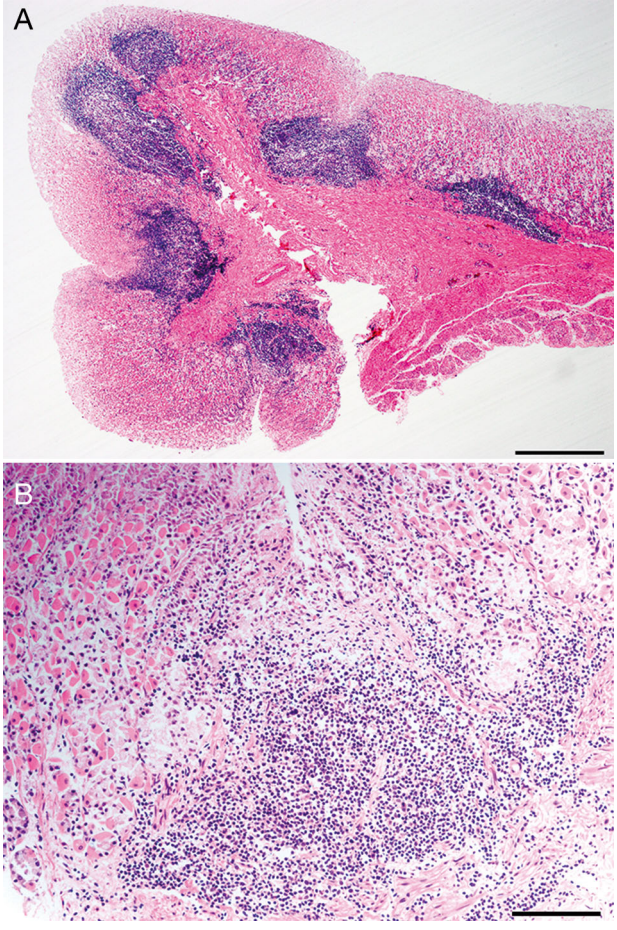

Fig. 5. (A) Cross-section of the gastric body: of a southern sea otter. Multiple lymphoid follicles are present at the junction of the mucosa and submucosa. (B) Higher magnification view of a single lymphoid follicle, with a predominance of mature lymphocytes. H&E. Scale bars = (A) 500 µm, (B) 100 µm

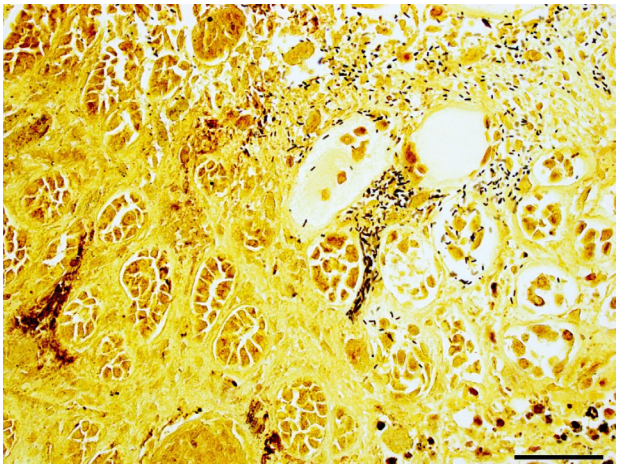

Fig. 6. Mucosa of the gastric pylorus of a southern sea otter. Numerous short, stout and mildly recurved bacilli are scattered within and adjacent to pyloric glands exhibiting epithelial necrosis and surface erosion. Warthin-Starry stain. Scale bar = 50 µm

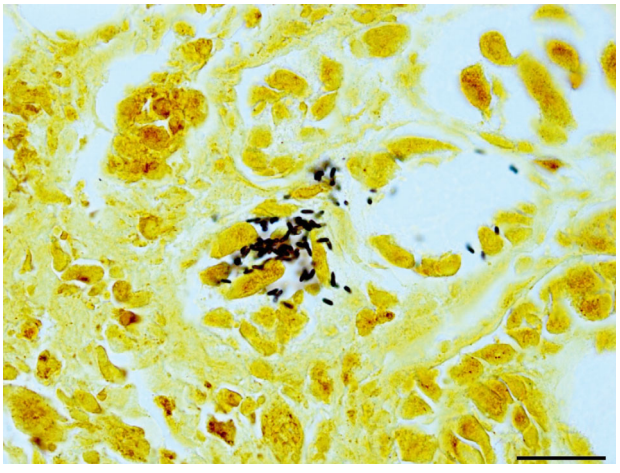

Fig. 7. Mucosa of the gastric pylorus of a southern sea otter. Higher magnification view of bacilli clustered within and adjacent to the gastric glands. Warthin-Starry stain. Scale bar = 20 µm

otters. Interestingly, the ultrastructure of the novel *Helicobacter* sp. is similar to *H. mustelae* with lateral and polar sheathed flagella (O'Rourke et al. 1992). The slightly curved silver-stained bacterial rods noted at the periphery of gastric ulcers and within crypts and on surface gastric epithelium in *H. enhydrae*-infected southern sea otters has the same histologic morphology and anatomic distribution as *H. mustelae* in infected ferrets (Fox et al. 1990, O'Rourke et al. 1992).

Although closely related taxonomically, one distinct difference is that *H. mustelae* is urease-positive, whereas *H. enhydrae* is urease-negative. *H. pylori*, the human gastric pathogen that causes gastritis, peptic ulcers, and occasionally gastric cancer, is also urease-positive. The large gastric *Helicobacter* sp., *H. suis,* which occasionally colonizes humans and also infects the stomachs of pigs and nonhuman primates, and the gastric spirals that colonize dogs and cats, *H. felis,* are also urease-positive (Haesebrouck et al. 2009). The ability of these *Helicobacter* spp. to colonize the stomach is largely attributed to the urease enzyme, which enzymatically converts urea to ammonia, which in turn buffers the organisms from the acidic pH of the stomach. It is of interest that *H. enhydrae* can apparently colonize the sea otter stomach without the buffering capacity of urease. *H. cinaedi,* a urease-negative enteric *Helicobacter* sp. that normally colonizes the lower intestine of humans and other mammals including cats, dogs, and rodents, has been identified on occasion in the stomachs of humans (Peña et al. 2002, Han et al. 2010). Experimentally, *H. cinaedi* also persistently colonizes the gastrointestinal tract of mice, including the stomach (Shen et al. 2009). This may be partially attributed to the higher gastric pH noted in the mouse and the coprophagic nature of the mouse, which allows steady exposure to *H. cinaedi* that persistently colonizes the lower bowel (Shen et al. 2009). Given sea otters do not routinely practice coprophagy, this feature is unlikely to be operative in the southern sea otter. However, the ferret and southern sea otter have short gastrointestinal (GI) transit times, and the ferret is also noted to have episodes of hypochlorhydria, which in combination facilitates the ease of culturing *H. mustelae* from ferret's feces (Fox et al. 1992, 1993), raising the possibility that sea otters have the same anatomic and physiologic features observed in the ferret, thus allowing gastric colonization of urease-negative *Helicobacter* spp. *H. mustelae* and *H. enhydrae* cluster phylogenetically more closely to enterohepatic *Helicobacter* spp., which may suggest that both of the mustelid *Helicobacter* spp. could be classified as gastrointestinal *Helicobacter* spp., rather than strictly gastric species. *H. mustelae* is easily cultured from the feces of ferrets, which may infer intestinal colonization or simply transit of the organism from its niche in the stomach (Fox et al. 1988). Whether *H. enhydrae* colonizes the lower gastrointestinal tract, a more suitable environment inhabited by several urease-negative enterohepatic *Helicobacter* spp. (EHS), requires further studies. These data in part may explain why urease-negative *H. enhydrae* can colonize the stomachs of sea otters. However, in contrast, it should be noted that isogenic mutants of *H. mustelae* and *H. pylori* lacking urease activity do not colonize

the stomach of ferrets and gnotobiotic swine, respectively (Eaton & Krakowka 1994, Andrutis et al. 1995).

It is tempting but premature to ascribe the gastric ulcers observed in the sea otters examined in this study to the gastric colonization of the novel bacteria *H. enhydrae.* However, it is interesting that Warthin-Starry-positive bacteria with morphology consistent with *H. enhydrae* were observed in the periphery of the gastric ulcers and inflamed gastric tissue histologically. Further studies will be needed to better characterize this relationship. Whether stress, inadequate food supply, exposure to chemical contaminants, or a myriad of other factors contribute to gastric ulcer formation is unknown. Whether *H. enhydrae* identification by PCR and culture in this study reflects the true prevalence is unknown, and the identified prevalence could be lower (or higher) than the actual prevalence of the organism in southern sea otter stomachs because sampling was restricted to small areas and specific anatomic locations and was performed postmortem on stranded animals. Because the bacteria were identified in only 40 to 80% of gastric samples with grossly apparent ulcers, more extensive evaluation of otters with and without gastric ulcers or mural inflammation is required. In ferrets, *H. mustelae* colonizes a high percentage of ferrets, and like *H. pylori* in humans, only a small percentage of *H. mustelae*-infected ferrets develop gastric ulcers (Fox et al. 1990).

There are reports describing *H. acinonychis,* a helicobacter colonizing the cheetah stomach. In captivity, cheetahs infected with this bacterium have severe gastritis and gastric ulcers; in the wild, though colonized with the same gastric helicobacter, stomachs histologically are, in large part, normal (Eaton et al. 1993a,b). Authors have attributed the severe gastric disease noted in captive cheetahs to stress of captivity and other undefined variables that trigger gastritis and ulcers (Terio et al. 2012). Authors have also argued that generations of inbreeding of the cheetah has created a genetic bottleneck, resulting in the lack of genetic diversity in this species, resulting in a population that, when subjected to captivity, expresses increased susceptibility to gastric *H. acinonychis* (O'Brien et al. 1987, Munson et al. 2005). A similar circumstance could be playing a role in the sea otter, which suffered at least one historic population bottleneck due to hunting of these mammals for fur in the 18th and 19th century (Larson et al. 2012). Indeed, of the sea otter populations along the Pacific coast, California sea otters have the lowest genetic diversity. Perhaps this low diversity accounts for increased susceptibility to certain infectious diseases, including gastric ulcers associated with '*H. enhydrae*' infection.

The sea otter is not unique among aquatic living mammals in being colonized with gastric helicobacters. We first isolated a gastric *H. cetorum* from gastric ulcers and inflamed stomachs of dolphins in 2003 (Harper et al. 2000, 2002a), followed by the identification of novel gastric helicobacters in harp seals, sea lions, and beluga whales (Harper et al. 2002b, 2003). These novel helicobacters are also urease-positive, except for a novel *Helicobacter* sp. isolated from the stomach of harp seals, which was urease-negative (Harper et al. 2003). It is likely that these novel *Helicobacter* spp. in sea mammals persist in the stomachs of these animals in a manner similar to that of *H. pylori* and *H. mustelae.* The finding of *H. enhydrae* in sea otters in 2001 and again in 2015 supports this hypothesis.

In summary, we have identified a novel helicobacter, *H. enhydrae,* in the stomach of the southern sea otter. Whether the novel *Helicobacter* sp. is involved in gastric ulcer disease in these sea mammals will require further study. In addition, it will be interesting to ascertain whether *H. enhydrae* also colonizes the stomachs of northern sea otters.

Description of *Helicobacter enhydrae* sp. nov.

Helicobacter enhydrae sp. *nov.* (en.hy'drae. N.L. gen. n. *enhydrae* of the sea otter *Enhydra*). The organism is motile; cells are slightly curved (2–3 µm) with lateral and polar sheathed flagella. The bacteria are Gram negative and non-sporulating. The organism grows slowly at 37°C and 42°C, but not at 25°C, under microaerobic conditions. It appears on solid agar as single colonies. The bacterium is oxidase and catalase positive, but urease, alkaline phosphatase, indoxyl acetate hydrolysis and nitrate reduction are negative. It grows on 1% glycine and is sensitive to nalidixic acid and resistant to cephalothin. The type strain MIT 01-6242 has a DNA G+C content of 40.8%, and its genome is ~1.6 Mb in length. The genome of *Helicobacter* sp. MIT 01-6242 has been submitted under GenBank accession number CP016503.

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