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Campylobacter mustelae, a New Species Resulting from the Elevation of *Campylobacter pylori* subsp. *mustelae* to Species Status

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The name *Campylobacter pylori* subsp. *mustelae* was recently proposed for strains belonging to the genus *Campylobacter* that were isolated from the gastric mucosa of ferrets because of the high levels of deoxyribonucleic acid relatedness (85 to 100%) of these strains to the type strain of *Campylobacter pylori*. Subsequent deoxyribonucleic acid relatedness studies done independently in three laboratories by three different methods indicate that the original results were in error. Strains isolated from ferrets in the United States, England, and Australia are members of a single species that is substantially different from *C. pylori* (10 to 20% relatedness at 50°C and 3 to 10% relatedness at 65°C as determined by the hydroxyapatite method; less than 10% relatedness at 65°C as determined by the nylon membrane blot method; and 30 to 49% relatedness at 66 to 68°C as determined by the spectrophotometric method). The strains isolated from ferrets also differ from human *C. pylori* strains in their ability to reduce nitrates, their negative leucine arylamidase reaction, their susceptibility to nalidixic acid, and their resistance to cephalothin. The strains from ferrets possess both polar and lateral flagella, whereas only polar flagella have been demonstrated in human *C. pylori* strains. Thus, the strains isolated from ferrets are members of a species that is distinct from *C. pylori*, and we propose elevating *C. pylori* subsp. *mustelae* to species status as *Campylobacter mustelae* sp. nov.

Campylobacter-like organisms have been isolated from normal and inflamed gastric mucosa of ferrets in the United States, England, Canada, and Australia (3, 4, 14, 16; J. A. Hollingsworth et al., Conference on *C. pylori* Infections, 1987; Goodwin, unpublished data). The isolates from ferrets most closely resemble *Campylobacter pylori*, but differ from this species in having lateral flagella as well as polar, sheathed flagella, in their ability to reduce nitrates, in their negative leucine arylamidase reaction, in their susceptibility to nalidixic acid, and in their resistance to cephalothin (3, 4).

In 1988 deoxyribonucleic acid (DNA) hybridization studies were published (4) indicating that isolates from ferrets in the United States were very highly related to one another and to the type strain of *C. pylori*. Despite the fact that the isolates from ferrets had a different host and different phenotypic characteristics, their genomic relatedness to *C. pylori* allowed these organisms to be classified as a subspecies, *Campylobacter pylori* subsp. *mustelae* (4). After this study was accepted for publication, the authors learned from C. S. Goodwin that the level of DNA relatedness of an isolate from an Australian ferret to *C. pylori* was quite low, indicating that the two organisms are separate species.

This led to an exchange of strains and to the reexamination of the relatedness of *C. pylori* to the strains from ferrets described in this paper. Our results clearly demonstrate that the DNA relatedness results presented by Fox et al. (4) were in error (the responsibility for which is solely that of P.E. and D.J.B.) and that the isolates from ferrets represent a new species, for which the name *Campylobacter mustelae* is proposed.

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MATERIALS AND METHODS

Bacterial strains. *C. pylori* NCTC 11637 is the original culture of the type strain (= ATCC 43504) and was isolated from a human at Royal Perth Hospital in 1982. *C. mustelae* NCTC 12032 was isolated from a ferret in England and was obtained from the National Collection of Type Cultures, London, England. *C. mustelae* 15896 was isolated from a ferret in Western Australia. These strains were cultured on brain heart infusion agar containing 7% saponin-lysed horse blood and were incubated at 37°C for 2 to 3 days in vented jars containing a microaerophilic atmosphere (6% O_2, 10% CO_2, 84% N_2). Isolates were stored at −70°C in peptone water containing 10% glycerol. All other strains were isolated in the United States and were cultivated as previously described (4).

DNA base composition. DNA base composition was determined by two modifications of the thermal denaturation method. The method used in the United States has been described previously (4). In Australia, DNAs were extracted by the Marmur method (10), with the addition of protease treatments after cell lysis by sodium dodecyl sulfate and again after ribonuclease treatment (1). The guanine-plus-cytosine (G+C) values were determined by the T_i method of Sly et al. (15), using a model 2600 microprocessor-controlled spectrophotometer fitted with a thermal programmer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

DNA hybridization. DNA hybridization was done by three methods. The hydroxyapatite method (method 1) and the use of thermal elution profiles to determine divergence within related DNA sequences were done in the United States as described previously (4).

The nylon filter blot method (method 2) was used in Perth,

TABLE 1. DNA relatedness of *C. mustelae* and *C. pylori*

Test strain	Reference strain												
	C. pylori ATCC 43504^T: method 1[a]			*C. pylori* NCTC 11637^T		*C. mustelae* ATCC 43772^T				*C. mustelae* NCTC 12032			
						Method 1[a]			Method 2: % binding at 65°C[b]	Method 1[a]			Method 3: % relatedness at 68°C[c]
	% Binding at 50°C	D (%)	% Binding at 65°C	Method 2: % binding at 65°C[b]	Method 3: % relatedness at 66°C[c]	% Binding at 50°C	D (%)	% Binding at 65°C		% Binding at 50°C	D (%)	% Binding at 65°C	
C. pylori ATCC 43504^T	100	0.0	100			13		6		16	25.0	5	
C. pylori NCTC 11637^T				100	100				2				49
C. mustelae ATCC 43772^T	10	25.0	3	7	30	100	0.0	100	100	90	0.0	92	
C. mustelae NCTC 12032	12	25.5	4	1	44	98	0.0	100	75	100	0.0	100	100
C. mustelae 15896				8		94	0.5	83	96				
C. mustelae R85-13-11	12	25.0	3			94	0.0	92		98	1.0	98	
C. mustelae R85-13-12						100	0.0	100					

[a] Hydroxyapatite method. D, Divergence within related sequences, calculated to the nearest 0.5%.
[b] Filter blot method.
[c] Spectrophotometric method.

Australia. DNA was extracted and purified as described by Majewski and Goodwin (9), except that cells were harvested from plates rather than broth cultures, and the DNA solutions were treated with ribonuclease A (50 μg/ml) at 37°C for 30 min and reprecipitated. DNA pellets were dissolved in TE buffer [10 mM tris (hydroxymethyl)aminomethane chloride, 1 mM ethylenediaminetetraacetate, pH 8.0] to an absorbance at 260 nm of approximately 0.7. The solutions were then sonicated for 5 min at 40 kHz to obtain DNA fragments that were 0.5 to 1.0 kilobases long. All DNA concentrations were then adjusted to an absorbance at 260 nm of 0.5. Samples were denatured by boiling and blotted in triplicate onto a positively charged nylon membrane (Biotrace; Gelman Sciences, Inc., Ann Arbor, Mich.) with the aid of a vacuum manifold. Each blot contained 0.5 μg of DNA. The membranes were baked at 80°C for 1 to 2 h to immobilize the DNA. Portions (50 ng) of DNA from *C. pylori* NCTC 11637^T (T = type strain) and *C. mustelae* ATCC 43772^T and NCTC 12032 were labeled with [α-^{32}P]cytidine triphosphate by random priming, using a commercial kit (Multiprime; Amersham International plc, Buckinghamshire, United Kingdom) according to the directions of the manufacturer. Nylon membranes were sealed in plastic bags; prehybridization and hybridization were done at 65°C in a shaking water bath. Membranes were prehybridized for 1 h in 3 to 5 ml of hybridization fluid containing 1.5× SSPE (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M ethylenediaminetetraacetate, pH 7.7), 1% sodium dodecyl sulfate, 0.5% nonfat milk powder, 10% dextran sulfate, and 0.5 mg of denatured salmon sperm DNA per ml. For hybridization the prehybridization fluid was removed and replaced with 3 to 5 ml of the same fluid containing 10 ng of labeled DNA per ml, and the preparations were incubated overnight. After incubation the membranes were removed from the bags and washed at 65°C once for 15 min in 2× SSC (3 M NaCl plus 0.3 M sodium citrate), twice for 15 min in 2× SSC containing 0.1% sodium dodecyl sulfate, and twice for 15 min in 0.1× SSC containing 0.1% sodium dodecyl sulfate. The membranes were then air dried and autoradiographed. Radioactivity was assayed by dividing the membranes into 1-cm^2 pieces, each containing one DNA blot. These were placed into glass vials and dissolved by adding 1 ml of concentrated formic acid. A 9-ml portion of scintillation fluid (Instagel; Packard Instrument Co., Inc., Downers Grove, Ill.) was added to each vial, which was then counted in a liquid scintillation counter (model 1219; LKB Instruments, Inc., Bromma, Sweden). A homologous control reaction (labeled and unlabeled DNAs from the same strain) was used to represent 100% relatedness.

The spectrophotometric renaturation rate method (method 3, used in Queensland, Australia) for DNA hybridization was performed as described by De Ley et al. (2) and modified by Huss et al. (7). The optimal temperatures for renaturation were calculated by using the method of Gillis et al. (5) to be 68°C for *C. mustelae* NCTC 12032 and 66°C for *C. pylori* NCTC 11637^T.

RESULTS

DNA base composition. G+C contents were determined spectrophotometrically for *C. mustelae* strains both in Australia and in the United States. The G+C content of strain ATCC 43772^T was calculated to be 39 mol% in the United States and 37 mol% in Australia. Strain R85-13-11 had a G+C content of 42 mol% (United States). The G+C content of strain NCTC 12032 was calculated to be 40 and 41 mol% in the United States and Australia, respectively, and the G+C content of strain 15896 was calculated to be 40 and 36 mol% in the United States and Australia, respectively.

DNA hybridization. Hydroxyapatite, filter blot, and spectrophotometric DNA hybridization methods were used to determine the levels of DNA relatedness of the isolates from ferrets to one another and to *C. pylori* (Table 1). In 50°C hydroxyapatite reactions the isolates from ferrets were 90 to 100% interrelated, with 0 to 1% divergence within the related sequences. The isolates from ferrets were 10 to 16% related to the type strain of *C. pylori*, with 25% divergence within the related sequences. In 65°C hydroxyapatite and filter blot reactions the isolates from ferrets were 75 to 100% interrelated and showed 2 to 8% relatedness to *C. pylori*. As determined by the spectrophotometric method at 66 or 68°C, *C. pylori* was 30 to 49% related to the isolates from ferrets.

DISCUSSION

Isolates obtained from normal and inflamed gastric mucosa of ferrets in the United States were recently classified as a subspecies of *C. pylori* on the basis of high levels of DNA relatedness to the type strain of *C. pylori* (4). In our study these experiments were repeated and extended by using three hybridization methods, new preparations of DNA, and additional isolates from ferrets. Our results con-

firm that the isolates from ferrets from the United States are a single species and establish that isolates from ferrets in England and Australia belong to this species.

Our results contradict the previous observation that the isolates from ferrets belong in the species *C. pylori* because they were clearly distinguishable from *C. pylori*. The isolates from ferrets were 16% or less related to *C. pylori* as determined by the hydroxyapatite and filter blot methods. *C. pylori* and the isolates from ferrets appeared to be substantially more closely related when the spectrophotometric method was used, but were still recognizable as different species. Spectrophotometric method data have been reported to correlate well with membrane filter method data at levels of relatedness above 80%, but are unreliable at levels below 30% (7).

We (D.J.B. and P.E.) do not know the source of the error in the original experiments (4), but it was most likely due to a contaminated DNA preparation from *C. pylori* type strain ATCC 43504. The taxonomic changes required by the present, corrected data are correction of the emended description of *C. pylori*, elevation of *C. pylori* subsp. *mustelae* to species rank, and abolition of *C. pylori* subsp. *pylori*. The first two of these changes are made below, and the third will occur without formal action.

Emended description of *Campylobacter pylori* (Marshall, Royce, Annear, Goodwin, Pearman, Warren, and Armstrong 1984) Marshall and Goodwin 1987; Fox, Taylor, Edmonds, and Brenner 1988. Fastidious, gram-negative, oxidase-positive, microaerophilic, nonencapsulated, curved rods. Cells are 2.5 to 5 μm long and 0.5 to 1.0 μm wide with a spiral periodicity (11). Darting motility by means of multiple sheathed unipolar flagella with terminal bulbs. Slow growth in brain heart infusion broth and other liquid media unless shaken. Grows within 2 to 5 days on brain heart infusion agar, blood agar, and chocolate agar. Colonies are nonpigmented, translucent, and 1 to 2 mm in diameter (11–13). Optimal growth at 37°C; growth at 30°C, but not at 25°C; variable growth at 42°C. Growth in air enriched with 10% CO_2, but no growth anaerobically. No growth in the presence of 3% NaCl or 1% glycine. Grows in the presence of 0.5% glycine and 0.04% triphenyltetrazolium chloride. Catalase positive. H_2S production is negative on triple sugar iron agar and variable on lead acetate paper. Urea is rapidly hydrolyzed. Does not reduce nitrates or hydrolyze hippurate. Exhibits leucine arylamidase, alkaline phosphatase, and gamma-glutamyltranspeptidase activities. Susceptible to ampicillin, amoxicillin, cephalothin, erythromycin, gentamicin, kanamycin, penicillin, rifampin, and tetracycline (6, 8, 11). Resistant to sulfonamides, trimethoprim, and vancomycin (6, 7). Variable resistance to nalidixic acid and polymyxin (6, 7).

Isolated from the gastric mucosa of humans. Found in cases of gastritis and gastric and duodenal ulcers. Causative agent of type B gastritis, but a role in gastric and duodenal ulcers has not yet been established.

The G+C content is 36 to 38 mol%. The type strain is Royal Perth Hospital isolate 13487 (= NCTC 11637 = ATCC 43504), which has a G+C content of 37 mol%.

Description of *Campylobacter mustelae* sp. nov. *Campylobacter mustelae* (mus. tel' ae. L. gen. n. *mustelae*, of a ferret). The description is the same as that given previously for *C. pylori* subsp. *mustelae* (4). The characteristics are similar to those of *C. pylori* except as noted below. Multiple lateral flagella, as well as polar sheathed flagella with terminal bulbs. Growth at 42°C. No growth in air enriched with 10% CO_2, but grows anaerobically with CO_2. Reduces nitrates. Does not exhibit leucine arylamidase activity. Susceptible to nalidixic acid (30 μg) and resistant to cephalothin (30 μg) (4).

Isolated from the normal or inflamed gastric mucosa of ferrets.

The G+C content is 36 to 41 mol%.

The type strain is strain R85-13-6 (= ATCC 43772); it has a G+C content of 37 to 39 mol%. The characteristics of the type strain are the same as those of the species (4).

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