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Campylobacter pylori, the Spiral Bacterium Associated with Human Gastritis, Is Not a True *Campylobacter* sp.

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Comparison of partial 16S rRNA sequences from representative *Campylobacter* species indicates that the *Campylobacter* species form a previously undescribed basic eubacterial group, which is related to the other major groups only by very deep branching. This analysis was extended to include the spiral bacterium associated with human gastritis, *Campylobacter pylori* (formerly *Campylobacter pyloridis*). The distance between *C. pylori* and the other *Campylobacter* species is sufficient to exclude the pyloric organism from the *Campylobacter* genus. The results indicate that *C. pylori* is more closely related to *Wolinella succinogenes* than it is to the other *Campylobacter* species inspected. Another close relative of the campylobacters was found to be *Thiovulum*, a sulfide-dependent marine bacterium.

Campylobacter species are important human and animal pathogens (24, 26). *Campylobacter fetus*, the type species of the genus, has long been recognized as an etiologic agent of infertility and septic abortion in sheep and cattle (24). In humans, *C. fetus* is an opportunistic pathogen and has been isolated from people with a variety of systemic infections, including septicemia (17, 24). The thermotolerant species *Campylobacter jejuni* and *Campylobacter coli* also cause disease in animals, and over the past few years they have become recognized as important bacterial enteric human pathogens (4, 14, 24). *Campylobacter* spp. consistently rank among the top three bacterial enteric human pathogens in prevalence. There are also increasing reports of the isolation of *C. jejuni* from humans with a variety of systemic infections (18). Other campylobacters may also have pathogenic roles in humans, and recently, a number of *Campylobacter*-like organisms have been isolated from people with a variety of clinical conditions (5, 19). One of these has garnered considerable attention, as it has been found to be associated with human gastric ulcers and may play a pathogenic role in gastritis (R. A. Burnett, J. A. H. Forrest, R. W. A. Girdwood, and C. R. Fricker, Letter, Lancet i:1349, 1984; M. L. Langenberg, G. N. J. Tytgat, M. E. I. Schipper, P. J. G. M. Rietra, and H. C. Zanen, Letter, Lancet i:1348, 1984). This organism has been designated *Campylobacter pylori* (formerly *Campylobacter pyloridis*) (19).

Classification of species in the genus *Camplyobacter* has always presented problems. The campylobacters are generally inert in standard biochemical tests, so that their classification is dependent upon a few morphological and physiological features (10, 15, 24). For example, the division of *C. fetus* into two subspecies is based on a single growth property, the ability to grow in the presence of 1% glycine (23). Of the thermotolerant species, *C. jejuni* and *C. coli* are distinguished from one another largely on the basis of the hippurate hydrolysis test, which has been reported to be at best 81% reliable (10, 23), while the proposed species *Campylobacter laridis* (2) is distinguished from *C. coli* primarily on the basis of resistance to nalidixic acid (15, 23). The inclusion of *C. pylori* in the genus is contentious, as this bacterium possesses markedly different fatty acid and protein composition from those of other *Campylobacter* species, as well as several morphological features in conflict with the current definition of the genus *Campylobacter* (7; A. D. Pearson, L. Bamforth, L. Booth, G. Holdstock, A. Ireland, C. Walker, P. Hawtin, and H. Millward-Smith, Letter, Lancet i:1349–1350, 1984). It is difficult to determine whether the various differences are to be reflected in a relaxation of the criteria used to define *Campylobacter* spp., or whether these morphological differences exclude the pyloric organism from classification as a *Campylobacter* sp.

The phylogenetic comparison of rRNA sequences has become a powerful method for the systematic classification of microbial organisms (6, 20). The recent introduction of a rapid technique for generating partial 16S rRNA sequences has facilitated the use of phylogenetic analysis for the rapid identification of newly isolated species (17). By using reverse transcriptase, crude RNA as a template, and three oligonucleotide primers complementary to highly conserved sequences in eubacterial 16S rRNA, it is possible to go from a cell pellet to 800 to 1,000 nucleotides of sequence data in 3 days (17). For a poorly defined genus like *Campylobacter*, such an approach can provide an unambiguous answer to the question of classification, particularly when putative new species like *C. pylori* are involved. We report here the results obtained by comparing partial 16S rRNA sequences from five representative *Campylobacter* species and *C. pylori*. The relationship of the genus *Campylobacter* to the 10 major eubacterial groupings has also been determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Campylobacter* strains used in this study were *C. jejuni* VC79 (NCTC 11392; National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom), *C. fetus*

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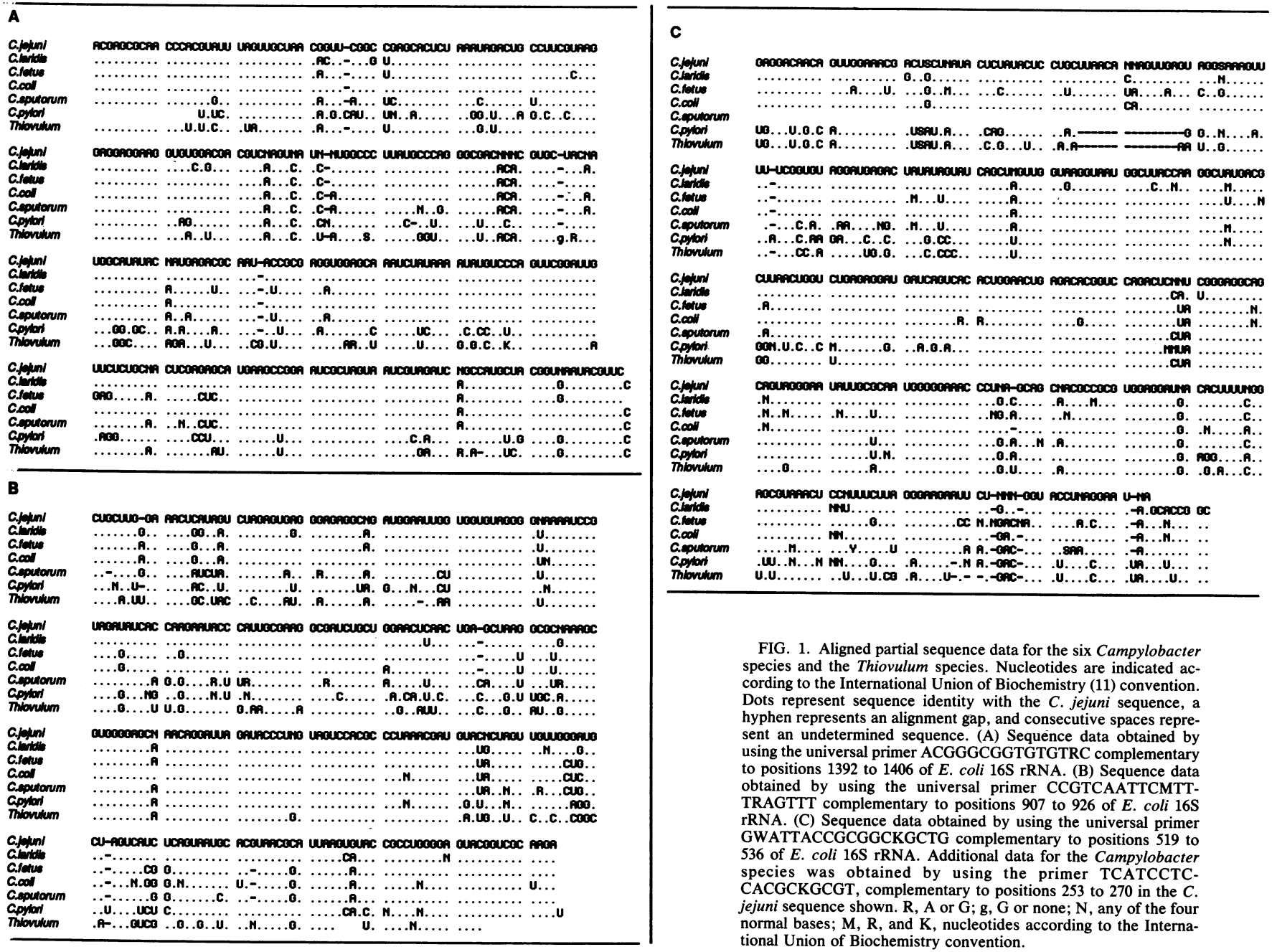

FIG. 1. Aligned partial sequence data for the six *Campylobacter* species and the *Thiovulum* species. Nucleotides are indicated according to the International Union of Biochemistry (11) convention. Dots represent sequence identity with the *C. jejuni* sequence, a hyphen represents an alignment gap, and consecutive spaces represent an undetermined sequence. (A) Sequence data obtained by using the universal primer ACGGGCGGTGTGTRC complementary to positions 1392 to 1406 of *E. coli* 16S rRNA. (B) Sequence data obtained by using the universal primer CCGTCAATTCMTTTRAGTTT complementary to positions 907 to 926 of *E. coli* 16S rRNA. (C) Sequence data obtained by using the universal primer GWATTACCGCGGCKGCTG complementary to positions 519 to 536 of *E. coli* 16S rRNA. Additional data for the *Campylobacter* species was obtained by using the primer TCATCCTCCACGCKGCGT, complementary to positions 253 to 270 in the *C. jejuni* sequence shown. R, A or G; g, G or none; N, any of the four normal bases; M, R, and K, nucleotides according to the International Union of Biochemistry convention.

subsp. *fetus* VC78 (CIP 5396; Collection de l'Institut Pasteur, Paris, France [type species of the genus]), *C. coli* VC80 (NCTC 11366), *C. laridis* VC187 (NCTC 11352), *C. sputorum* subsp. *sputorum* VC188 (H. Lior strain C6926; National Enteric Reference Centre, Ottawa, Canada), and *C. pylori* 5443 (A. Lee, School of Microbiology, University of New South Wales, Sydney, Australia). Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol-Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). For RNA isolation, *Campylobacter* species were grown at 37°C in anaerobic jars for 48 h on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) in 150-mm petri dishes. An atmosphere containing 5% oxygen and 10% CO_2 was produced with a gas generation kit for campylobacters (Oxoid Ltd., Basingstoke, United Kingdom). *C. pylori* was grown for 48 h on twice-lysed human blood-blood agar base no. 2 (Oxoid) in anaerobic jars without a catalyst under an atmosphere produced with an anaerobic gas generation kit (Oxoid). Bacteria were harvested from plates and stored at −70°C until used for the isolation of RNA.

Isolation of RNA. Total RNA was isolated from 0.1 to 0.4 g (wet weight) of bacteria by a hot sodium dodecyl sulfate-phenol extraction method (22). Large RNA was selectively precipitated from solution at 4°C by the addition of 2 M NaCl (22). The RNA was dissolved in distilled water and precipitated by addition of 3 volumes of ethanol and incubation for 30 min at −70°C. After centrifugation at 17,000 × *g* for 10 min at 4°C, the RNA pellet was dried in vacuo. All RNA samples were dissolved in an appropriate volume of distilled water to give a solution with a final concentration of 2 mg/ml. RNA was stored at −20°C.

Partial sequencing of 16S rRNA. Partial sequencing of the 16S rRNA was done by using a dideoxynucleotide method (17), with reverse transcriptase, large rRNA as a template, and three oligonucleotide primers complementary to highly conserved sequences in eubacterial 16S rRNA. Typically, 250 to 300 nucleotides of sequence were obtained from each primer. To provide additional sequence information near the 5′ end of the 16S rRNA, a fourth, mixed oligonucleotide primer having the sequence dTCATCCTCCACGC(T,G) GCGT was used.

Comparison of RNA sequences. Sequence homologies were calculated by using methods described elsewhere (20). Regions of the *Campylobacter* sequences which could not be aligned unambiguously with other procaryotic sequences were omitted from homology calculations. Alignment gaps were assigned half the value of a nucleotide in the same position.

RESULTS AND DISCUSSION

The original classification of the genus *Campylobacter* with *Spirillum* into the family *Spirillaceae* was based upon the presence of a general spiral morphology, a single unsheathed polar flagellum, the inability to accumulate polyhydroxybutyric acid, and a 29 to 38 mol% G+C composition of DNA (15, 16). More recently, because of the uncertain status of the family *Spirillaceae*, the genus *Campylobacter* has been grouped with a variety of other gram-negative spiral organisms of uncertain taxonomic status (16). DNA hybridization analysis has shown that the classification to species level resulting from the restricted list of phenotypic properties has been surprisingly accurate (1, 2, 9, 23). Indeed, there is very little cross-hybridization between species, with the exception of a moderate homology between the DNAs of *C. jejuni*, *C. coli*, and *C. laridis*.

TABLE 1. Homology of *Campylobacter* 16S rRNA sequences compared with other procaryotic species

Species (reference)	% Homology (745 positions) and corrected % difference[a] for:																
	C. jejuni	*C. laridis*	*C. coli*	*C. fetus*	*C. sputorum*	*C. pylori*	*W. succinogenes*	*Thiovulum* sp.	*A. tumefaciens*	*P. testosteroni*	*E. coli*	*Anacystis nidulans*	*Bacillus subtilis*	*Flavobacterium heparinum*	*Myxococcus xanthus*	*Methanococcus vannielii*
C. jejuni		98.1	97.9	95.7	92.9	85.0	86.6	85.3	75.9	76.7	76.0	77.4	75.3	73.1	76.9	62.2
C. laridis	2.0		96.8	94.8	92.4	84.6	87.1	85.2	76.7	76.5	76.4	76.7	76.6	73.3	77.2	62.3
C. coli	2.1	3.3		96.8	96.8	85.0	86.9	85.1	76.1	76.5	76.2	77.3	75.5	73.1	77.1	62.0
C. fetus	4.5	5.4	3.3		95.2	85.5	87.4	84.3	75.9	77.3	77.3	77.0	75.9	73.6	77.5	62.6
C. sputorum	7.4	8.1	6.3	5.0		84.8	87.1	84.3	75.9	77.1	76.9	77.2	75.6	74.1	77.3	61.4
C. pylori	16.7	17.2	16.7	16.1	17.0		90.2	82.6	76.7	76.0	74.6	76.6	78.4	71.6	76.7	61.6
W. succinogenes[b]	14.7	14.2	14.4	13.8	14.1	10.5		84.4	79.2	78.6	77.8	79.1	79.0	73.6	79.5	63.8
Thiovulum sp.	16.3	16.5	16.6	17.6	17.6	19.7	17.5		77.8	76.2	77.0	74.7	74.0	71.4	75.6	60.8
A. tumefaciens (29)	29.1	27.8	28.8	29.1	29.1	27.9	24.3	26.3		78.9	81.7	80.2	81.4	76.1	82.2	65.1
P. testosteroni (29)	27.9	28.1	28.2	27.0	27.4	28.9	25.2	28.6	24.8		81.0	77.8	78.7	75.8	80.9	65.9
E. coli (3)	28.9	28.3	28.6	27.0	27.7	31.1	26.4	27.5	21.0	21.9		77.4	78.7	75.9	81.2	64.3
Anacystis nidulans (25)	26.9	27.9	27.0	27.5	27.2	28.0	24.5	30.9	23.0	26.4	27.0		80.3	73.9	80.9	67.9
Bacillus subtilis (8)	29.9	28.0	29.6	29.1	29.6	25.5	24.7	31.9	21.3	25.0	25.0	22.8		76.2	82.2	67.6
Flavobacterium heparinum (27)	33.3	33.1	33.4	32.5	31.8	35.6	32.5	36.0	28.8	29.2	29.0	32.1	28.6		75.5	64.7
Myxococcus xanthus (21)	27.7	27.3	27.4	26.7	27.1	27.9	23.9	29.4	20.3	22.1	21.7	22.1	20.3	29.6		65.8
Methanococcus vannielii (12)	52.6	52.3	53.0	51.7	54.3	53.8	49.5	55.5	46.9	45.5	48.5	41.8	42.5	47.8	45.7	

[a] Percent difference is corrected for superimposed mutations as described in reference 12. The percent difference values are given in the lower part of the table.
[b] Lau et al., submitted for publication.

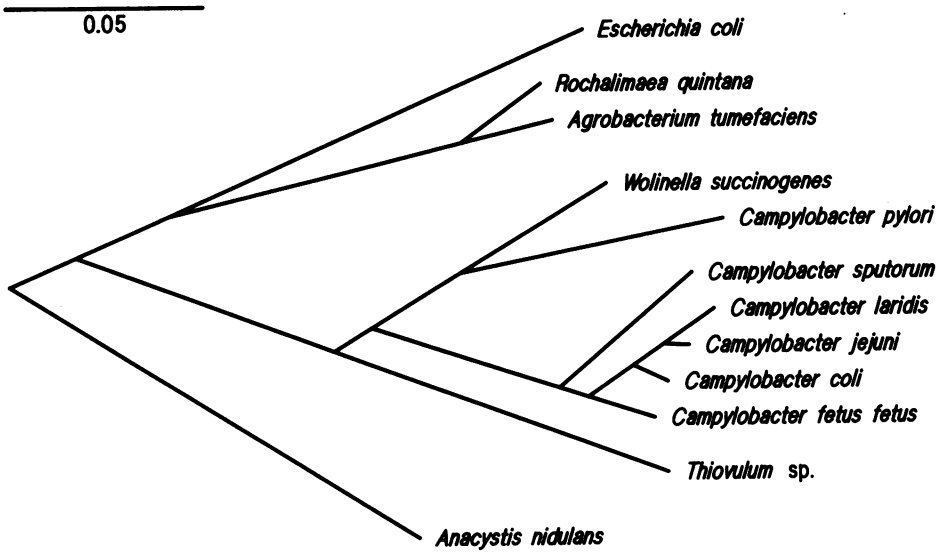

FIG. 2. Phylogenetic relationship of *Campylobacter* species to representatives of the purple photosynthetic bacterial and cyanobacterial phyla and to *C. pylori*, *W. succinogenes*, and *Thiovulum* species. The homology data shown in Table 1 are abstracted from the larger set (all pairwise comparisons) used to construct the tree by using an iterative method (20). The scale bar represents an evolutionary distance of 0.05 fixed nucleotide changes per sequence position (20). Note that the horizontal component of the tree segments represent evolutionary distance, and vertical positions are arbitrary. The root of this tree is based on analogous trees derived from complete 16S rRNA sequences (29).

The results obtained from reverse transcriptase sequencing of 16S rRNA isolated from five *Campylobacter* species, *C. pylori*, and a *Thiovulum* species are shown in Fig. 1. *C. sputorum* subsp. *sputorum* was chosen as a representative of a catalase-negative *Campylobacter* species, and the remainder of the species were catalase-positive, pathogenic campylobacters. With the exception of *C. pylori*, the representative *Campylobacter* strains examined formed a well-defined phylogenetic group showing high interspecies homology (92.4 to 98.1% sequence homology). Consistent with a previous DNA homology study (1), which indicated a degree of relatedness between nalidixic acid-resistant *Campylobacter* strain VC187 (i.e., *C. laridis* NCTC 11352) and *C. jejuni*, the homology data in Table 1 suggest that *C. laridis* is more closely related to *C. jejuni*. The sequence data for *C. fetus* subsp. *fetus* (Fig. 1) were compared with ca. 400 nucleotides of sequence for *C. fetus* subsp. *venerealis* (data not shown). There was less than a 1% sequence difference between the two subspecies (99.2% homology). The error frequency of dideoxy sequencing methods is ca. 1% (17), and therefore the sequences of the two subspecies of *C. fetus* are considered to be identical, indicating that there is probably no genetic basis for classification to the subspecies level, as suggested previously by DNA homology studies (1, 23). However, as convincingly argued by Roop et al. (23), there are practical epidemiological reasons for maintaining these taxa. *C. pylori* was clearly distinct from all other *Campylobacter* species tested, showing a reduced interspecies homology (84.6 to 85.5%).

The partial 16S rRNA sequence data were also compared with sequence data from representative species of 8 of the 10 currently defined eubacterial groups, and the homology data obtained with published sequences are presented in Table 1. Similar homology values were obtained from a comparison of *Campylobacter* species with unpublished sequence data (kindly provided by C. R. Woese) for representatives of the radio-resistant, sulfur-dependent, and spirochete major linkages. In contrast to the high interspecies homology observed among *Campylobacter* species, it is obvious that *Campylobacter* species do not have a close affiliation with any of these phylogenetic groups. The 10 major groups have also been defined on the basis of signature analysis of T1 oligonucleotide catalogues (28). With the *Campylobacter* sequences available, the results of a signature analysis also indicated that the *Campylobacter* species do not belong to any of the currently defined eubacterial groups (data not shown). From these results, we conclude that *Campylobacter* species form a distinct eubacterial phylogenetic group which has not been described previously. A similar observation has been made in a related study with a more limited set of sequence data (P. P. Lau, B. Debrunner-Vossbrinck, B. Dunn, K. Miotto, M. T. McDonell, D. M. Rollins, C. J. Pillidge, R. B. Hespell, R. R. Colwell, M. L. Sogin, and G. C. Fox, submitted for publication).

The homology data in Table 1 confirm that *C. pylori* lies outside the main *Campylobacter* group. The distance between *C. pylori* and the true *Campylobacter* group is at the intergenus rather than the intragenus level. Indeed, *Thiovulum* species and *Wolinella succinogenes* (Lau et al., submitted) are as closely related to the true *Campylobacter* group as *C. pylori* is (Table 1). The tree analysis more clearly illustrates this point.

A diagrammatic representation of the phylogenetic tree is shown in Fig. 2. From this figure and from the data in Table 1, it is clear that *Campylobacter* species are no more closely related to any of the purple photosynthetic bacterial branches than the purple photosynthetic bacterial branches are to one another (*Escherichia coli* versus *Agrobacterium tumefaciens*). This result is significant since the alpha (represented in Table 1 by *A. tumefaciens*), beta (represented by *Pseudomonas testosteroni*), and gamma (represented by *E. coli*) subdivisions of this phylogenetic group each include at least one gram-negative spiral organism (29), which traditionally have been grouped with *Campylobacter* species. The depth of the *Campylobacter* branch in the tree precludes the attachment of any significance to slight variations in the

distance to the other major groups. This result also means that the identification of an organism which might be expected to have closely related biochemical properties to those of *Campylobacter* species will require the accumulation of sequence data from additional organisms. As the tree indicates, the most closely related organisms currently available in the sequence data base are *C. pylori, W. succinogenes*, and *Thiovulum* species. Since *C. pylori* is no more closely related to other *Campylobacter* species than is *Thiovulum* sp. (Table 1), it is our conclusion that the pyloric organism should not be considered a *Campylobacter* species. Although the phylogenetic analysis indicates that *C. pylori* is more closely related to *W. succinogenes* than it is to the campylobacters (90% versus ca. 85% similarity, respectively), the *C. pylori/W. succinogenes* separation is probably sufficient to merit separate genera. The accumulation of 16S rRNA sequences from additional *Wolinella* species would be useful in clarifying this point.

As isolation techniques improve and interest in diverse ecologies grows, problems in the classification of new microorganisms similar to that described above will have to be solved. By combining a comprehensive morphological description of the new species, along with a comparative analysis of partial 16S rRNA sequence data, it should be possible to provide an unambiguous definition of each new organism. This information should be invaluable in providing well-founded direction to further studies of comparative biochemistry and microbiology.

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