**Campylobacter hyoilei** Alderton et al. 1995 and **Campylobacter coli** Véron and Chatelain 1973. Are Subjective Synonyms

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The taxonomic affiliation of **Campylobacter hyoilei** was reevaluated by examining a variety of phenotypic and genotypic criteria. Whole-cell protein electrophoresis and a comparison of 66 phenotypic characters revealed that reference strains of **C. hyoilei** were indistinguishable from **Campylobacter coli** strains. These data were confirmed by a DNA-DNA hybridization level of 67% between the type strains of the two species. Several species-specific assays based on PCR amplification or probe hybridization further substantiated that **C. coli** strains and **C. hyoilei** strains are indistinguishable. It is therefore concluded that **C. hyoilei** and **C. coli** represent the same species and that the former name should be regarded as a junior synonym of the latter name.

The taxonomic structure and relationships of the family **Campylobacteraceae** have been unravelled by an integrated polyphasic approach (40, 41, 44, 49). As in many other lineages of the class **Proteobacteria**, results from rRNA homology experiments formed the framework used to revise the classification and nomenclature of campylobacters (17, 31, 36, 41, 44). Assignment of taxa to the genus **Campylobacter** and to the related genera **Aerobacter** and **Helicobacter** is primarily based on rRNA similarity data, in accordance with the present view of classification (namely, that natural relationships between bacteria [55] should be reflected where possible). Although definitely among the most valuable taxonomic tools, rRNA sequence analysis is not the appropriate method for resolving every problem in bacterial classification. The resolution of 16S rRNA sequence analysis for closely related species (organisms sharing more than 97% of their 16S rRNA sequences) has been shown to be low (5, 11, 35). For such organisms, DNA-DNA hybridization experiments are essential for species demarcation, and guidelines have been presented by an ad hoc committee (54). However, the interpretation of DNA-DNA hybridization results is often not straightforward as the threshold values for species delineation vary among taxa and data obtained in different laboratories or with different hybridization methods often do not correlate (46).

In the present study, we evaluated the criteria used to assign 12 porcine isolates to the new species **Campylobacter hyoilei** (1). Remarkably, strains classified as **C. hyoilei** and **Campylobacter coli** were reported to produce exactly the same band patterns in several DNA probe hybridization assays, and the phenotypic data used to differentiate **C. hyoilei** from **C. coli** were at variance with other published data (1, 3, 27).

**MATERIALS AND METHODS**

**Strains used.** The strains used and their sources are listed in Table 1. Bacteriological purity was checked by plating and examining living and Gram-stained cells.

**Polyacrylamide gel electrophoresis of whole-cell proteins.** All of the strains examined were grown on Mueller-Hinton agar (catalog no. CM 337; Oxoid, Ltd., Basingstoke, United Kingdom) supplemented with 5% (vol/vol) horse blood and were incubated at 36 to 37°C in a microaerobic atmosphere containing approximately 5% O2, 3.5% CO2, 7.5% H2, and 84% N2.

Whole-cell protein extracts were prepared from three **C. hyoilei** strains and from type or reference strains of all **Campylobacter** species and of representative **Aerobacter** and **Helicobacter** species. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously (32). A densitometric analysis, normalization and interpolation of the protein profiles, and a numerical analysis were performed by using the GelCompar software package, version 4.0 (AppliChem, Koertrijk, Belgium). The profiles were recorded and stored on a personal computer. The similarity between pairs of traces was expressed by the Pearson product moment correlation coefficient: data are presented below as percentages of similarity for convenience.

**Phenotypic analysis.** For phenotypic testing, nine strains were examined, six of which were type or reference strains. The strains examined were **C. hyoilei** CCUG 33450T, CCUG 33769A, and CCUG 33780A, **C. coli** CCUG 11283T, LGM 9779, CDC D112, CDC D126, and CDC D134, and **Campylobacter jejuni** subsp. jejuni CCUG 11264. The identities of **C. coli** CDC D112, CDC D126, and CDC D134 were established previously by quantitative DNA-DNA hybridization, and these strains exhibited 73 to 93% DNA homology to the **C. coli** type strain in reassocation experiments performed at both 50 and 65°C (18).

Strains were grown on 5% (vol/vol) calf blood agar for 3 days under microaerobic conditions, as described previously (24). A total of 66 phenotypic characters were determined for the **C. hyoilei** strains by using methods described previously (14, 19, 22-26). The test for nitrite reduction was amended; the base medium consisted of heart infusion broth (Difco). The remaining 65 tests comprise **C. hyoilei** strains (Table 2) were performed on two separate occasions, and freshly prepared media were used to evaluate test reproducibility.

**DNA-DNA hybridization experiments.** The degree of DNA-DNA binding, expressed as a percentage, was determined spectrophotometrically by the initial renaturation rate method of De Ley et al. (10). Each value given below is the average of the values from at least two hybridization experiments. Values of DNA binding of 30% and less do not represent significant DNA homology. The total DNA concentration was about 39 μg/ml, and the optimal renaturation temperature in 1X SSC (0.15 M NaCl plus 0.015 M sodium citrate) was 63.0°C.

**GTPase gene assay.** DNA was isolated as described previously (12). The PCR mixture (total volume, 100 μl) consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl2, 0.01% gelatin, and 0.1% Triton X-100. Decoyribonucleotide triphosphates were used at a final concentration of 0.2 mM, and 0.25 U of **Super Taq** DNA polymerase (Sphaero Q, Leiden, The Netherlands) was added. The test for nitrite reduction was amended; the base medium consisted of blood heart infusion broth (Difco Laboratories, Ltd., East Molesey, England) and not heart infusion broth (Difco). The remaining 65 tests comprise a comprehensive identification scheme for campylobacteria (27); two tests of this scheme, tests for growth at 30°C and on buffered charcoal-yeast medium, were not used. Previously described tests (1) and or tests identified in this study (see below) as being useful for differentiating **C. hyoilei** from **C. coli** were used to examine each of the 11 strains included in these experiments. These tests (Table 2) were performed on two separate occasions, and freshly prepared media were used to evaluate test reproducibility.

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TABLE 1. Strains examined

<table>
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<tr>
<th>Strain*</th>
<th>Other designation(s)*</th>
<th>Source</th>
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<tbody>
<tr>
<td>Arcobacter cryaerophilus LMG 7536T</td>
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<td>Aborted bovine fetus</td>
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<td>Arcobacter nitrojigilis</td>
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<td>(Northern Ireland)</td>
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<td>Arcobacter niyaerophilus</td>
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<td>Campylobacter coli CDC D126</td>
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<td>Helicobacter pylori LMG 7559T</td>
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</table>

* ATCC, American Type Culture Collection, Rockville, Md.; CCUG, Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; LMG, Laboratorium voor Microbiologie Gent Culture Collection, Universiteit Gent, Ghent, Belgium; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; SVS, Statens Veterinaere Serumlaboratorium, Copenhagen, Denmark.

TABLE 2. Differential characteristics for the strains of C. hyoilei, C. coli, and C. jejuni subsp. jejuni examined

<table>
<thead>
<tr>
<th>Strain</th>
<th>H₂S-TSI test</th>
<th>Nitrite reduction*</th>
<th>Alpha-hemolysis</th>
<th>Basic fuchsin test</th>
<th>5-Fluorouracil test</th>
</tr>
</thead>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C. hyoilei CCUG 33769A</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. hyoilei CCUG 33770A</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C. coli LMG 9799</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>C. jejuni CCUG 11284T</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

* All strains were found to reduce nitrate with the method used.

+ positive; - negative; I, test irreproducible upon repeat testing; ND, not determined.
RESULTS

Whole-organism protein electrophoresis. The whole-organism protein patterns of C. hyoilei strains were compared with those of type or reference strains representing all species of the genera Arcobacter, Campylobacter, Helicobacter, and Wolinella. Figure 2 illustrates the numerical comparison of the whole-cell protein patterns of the type strains or well-established reference strains of all Campylobacter species, including three C. hyoilei strains and reference strains of Arcobacter and Helicobacter species. In the resulting dendrogram which was based on a comparison of the complete protein patterns, the C. hyoilei strains clustered near the type strain of C. coli.

A large number of numerical analyses were subsequently performed; in these analyses we included different sets of reference strains and field isolates present in the database, as described previously (47) (data not shown). C. coli and C. hyoilei strains always clustered closely together.

Phenotypic analysis. Close agreement between our results and the results described previously for C. hyoilei (1) was noted, with two important exceptions. First, we did not find that strain CCUG 33770A produced hydrogen sulfide in triple sugar iron (TSI) agar, even when the period of incubation was extended to 4 days (Table 2). Furthermore, none of the C. hyoilei strains that we examined demonstrated the ability to reduce nitrite, although all grew profusely in the test medium. The results described above were consistent when experiments were repeated.

In addition to the interstrain variation in the H₂S-TSI reaction described above, C. hyoilei strains differed in the ability to produce DNase and in the ability to grow on crystal violet- and Janus green-containing media (CCUG 33770A gave positive results in the latter three tests). Furthermore, microscopic examination of Gram-stained bacteria revealed characteristic S forms only in slides of CCUG 33450T and CCUG 33769A preparations, although all cultures contained cells defined as vibrioid (small curved rods). In the remaining 58 tests from the identification scheme of On et al. (27) used, C. hyoilei strains proved to be homogeneous and gave results characteristic of C. coli in all but three tests (alpha-hemolysis and growth on basic fuchsin- and 5-fluorouracil-containing media). The reproducibility and discriminatory potential of these tests (in addition to differential features defined by Alderton et al. [1]) were thus examined by using type and reference isolates. The data in Table 2 show that unequivocal differentiation between C. hyoilei and C. coli was not achieved.

DNA-DNA hybridization experiments. DNA-DNA hybridization experiments were performed with the type strains of C. coli, C. hyoilei, and C. jejuni. The DNA-DNA binding level between the C. hyoilei and C. coli type strains was 67%, while the DNA-DNA binding level measured between the C. hyoilei and C. jejuni type strains was 35% (the standard errors for these values were about 5%).

GTPase gene assay. DNAs from C. hyoilei CCUG 33450T, CCUG 33769A, and CCUG 33770A were analyzed by amplification of the GTPase gene fragment and Southern blotting. The hybridization pattern typical of C. coli strains was obtained for all three isolates, and no cross-hybridization was detected with probes specific for C. jejuni or other species. In addition, the sequence of the 156-bp PCR fragment obtained from C. hyoilei LMG 15882T was identical to sequences obtained from multiple C. coli reference strains (51).

glyA gene assays. In both glyA gene assays, C. hyoilei CCUG 33450T was detected with the same specificity as the C. coli type strain (Fig. 1). In the two separate Southern blot hybridizations, the C. coli-specific oligonucleotide and C. coli CCUG 11282T glyA fragment probes hybridized strongly to the PCR-amplified glyA fragment of C. hyoilei CCUG 33450T (Fig. 1).
Both probes detected C. hyoilei CCUG 33450T and C. coli CCUG 11283T with equal intensities (Fig. 1). The C. jejuni and C. lari-specific oligonucleotide and gflA fragment probes did not hybridize to C. hyoilei CCUG 33450T (data not shown). C. jejuni-C. coli multiplex PCR assay. The three C. hyoilei strains and strain LMG 9799 were identified as C. coli strains. In the multiplex PCR assay, C. coli strains typically produce a 364-bp amplification product, while a 773-bp amplification product is detected with C. jejuni strains (50).

**DISCUSSION**

In the present study, we reexamined the taxonomic affiliation of C. hyoilei by using a variety of phenotypic and genotypic criteria. Each analysis clearly indicated that C. hyoilei should not be considered a distinct species, and we propose that strains of C. hyoilei should be regarded as C. coli strains.

**Identification of campylobacters by whole-organism protein electrophoresis.** It is well-established that numerical analysis of whole-organism protein electrophoresis gels is an excellent tool for species level identification of campylobacters and related bacteria (7–9, 13, 22, 28–30, 32, 42, 43, 45, 47–49, 52). As in many other bacteria (6), a congruence was demonstrated between protein pattern similarity and level of DNA-DNA hybridization, which is the standard for species delineation (21, 32, 42, 45, 49); strains with highly similar whole-cell protein patterns belong to the same species. The presence of a variable dense-band region in Campylobacter species often divides species into different protein electrophoretic types, and this region is regularly omitted in numerical analyses to obtain clusters of highly related strains (i.e., strains exhibiting high levels of DNA-DNA binding). This has been illustrated in detail for Campylobacter species important in veterinary medicine, including C. coli (45). In practice, strains are routinely identified by performing several numerical analyses and changing the number of strains and the region included in the numerical comparison and by visually comparing the protein profiles themselves.

When three C. hyoilei strains which were deposited in international culture collections were examined, most analyses revealed that these strains fell into the C. coli cluster or into one of the C. coli clusters (depending on the number of strains included and their variable regions), suggesting that these strains did not represent a new species but belonged to C. coli.

**Phenotypic analysis.** The close phenotypic resemblance of C. hyoilei to C. coli was noted in the original description of the former species (1). Nonetheless, Alderton et al. suggested that C. hyoilei could be differentiated from C. coli by the ability of the former to produce hydrogen sulfide in TSI medium and by the ability to reduce nitrite (1). However, strains of C. coli giving positive reactions in the H2S-TSI (3, 27; this study) and nitrite reduction (3) tests have been noted previously. In addition, the reproducibility of the nitrite reduction test is questionable since we failed to observe this trait in any of the C. hyoilei strains studied. The influence of age of the test medium on the outcome of the H2S-TSI test for C. coli is also known (3) and may account for the observation that approximately 40% of C. coli preparations yield irreproducible results in this test (as cited by On et al. [27]). We used only freshly prepared TSI medium (at most 3 days old) in this study, and two of three C. hyoilei strains and two of six C. coli strains gave positive results. These data agree with previously published data for C. coli (27).

There are considerable problems associated with comparing campylobacterial phenotypic data obtained by different methods (20). The use of standardized, well-described tests has been recommended for both identification (20) and description (37) of campylobacterial taxa. The reproducibility of such tests is also clearly important (24, 25), but is often overlooked.

We examined C. hyoilei with an extensive set of standardized phenotypic tests which have been used to characterize all known Campylobacter species and subspecies (27). Although these tests demonstrated some potential to discriminate between C. hyoilei and C. coli, one test (tolerance to basic fuchsin) was unreliable, and the remaining tests (alpha-hemolysis and tolerance to 5-fluorouracil) proved inadequate, especially when the results were compared to the results obtained with C. coli isolates whose identities had been determined by quantitative DNA-DNA hybridization (Table 2).

**DNA-DNA hybridization studies.** The level of DNA-DNA binding between the C. hyoilei and C. coli type strains was reported to be 36%, and the DNA base ratio of the former strain was 35 ± 1.5 mol% (1). In order to verify this hybridization value in relation to our data, we hybridized DNA of the C. hyoilei type strain with DNAs of the type strains of C. coli and C. jejuni. We found levels of DNA-DNA binding of 67% between the C. hyoilei and C. coli type strains and 35% between the C. hyoilei and C. jejuni type strains. The initial renaturation rate methods has been used to study DNA-DNA hybridization levels within and between several Campylobacter and Arco bacter species, including Arco bacter butzleri, Arcobacter cryaerophilus, Arcobacter nitrofigilis, Arcobacter skinneri, C. coli, Campylobacter concisus, C. fetus, C. hyointestinalis, Campylobacter mucosals, and Campylobacter sputorum (21, 32, 39, 42, 45, 49). The levels of DNA-DNA hybridization within these species were generally greater than 65%; the only exceptions were A. cryaerophilus and C. concisus (21, 32, 39, 42, 45, 49). The latter two species, however, are known to comprise multiple genotypic (and phenotypic) subgroups (42, 49). The levels of DNA-DNA hybridization between different species were generally less than 25% (21, 32, 39, 42, 45, 49); the only exceptions were C. hyointestinalis and C. fetus (significant levels of hybridization between these species were also reported by other workers [46]). We concluded that these results confirm the protein electrophoretic data and indicate that strain CCUG 33450T and the taxon which it represents belong to C. coli.

Our DNA-DNA hybridization results are thus at variance with those of Alderton et al. (1). The problems encountered when DNA-DNA hybridization results obtained by different methods are compared have been discussed in detail previously (46). We used the initial renaturation rate method developed by De Ley and coworkers (10) to study inter- and intraspecific relationships of several Campylobacter species and obtained consistent results which correlated with protein electrophoretic results and a variety of other phenotypic markers. This integrated genotypic and phenotypic approach has been successfully used to study relationships of numerous gram-positive and gram-negative bacteria (46).

The reasons for the discrepancy between our results and the results of Alderton et al. (1) are not clear. The methods used by Alderton et al. included a dot hybridization technique which was originally described as a semiquantitative method (16). Some of the strains used in their study were mislabeled, while no strain numbers were provided for several others. A possible explanation for the low level of DNA-DNA binding between the C. coli and C. hyoilei type strains in the study of Alderton et al. (1) is that the conditions for hybridization were too stringent. It is not contradictory that the remaining strains classified as C. hyoilei all exhibited high levels of DNA-DNA binding with the type strain, as these strains were all isolated from pigs with porcine proliferative enteritis and therefore
could represent a particular adapted sublineage of C. coli. Indeed, certain strains of C. hyointestinalis isolated from porcine gastric mucosae have been shown to be especially highly related based on their DNA-DNA homologies (21), and similar observations have been reported in other genera (53).

**16S rRNA sequence analysis.** The following levels of 16S rRNA similarity were reported previously (1): 99.7% for the C. hyoilei and C. jejuni subsp. doylei type strains; 99.6% for the C. hyoilei and C. jejuni subsp. jejuni type strains; and 98.5% for the C. hyoilei and C. coli type strains. It has been documented that the resolution of 16S rRNA sequence analysis for closely related species is low and that for species level identification comparisons of similarity levels greater than 97% are not appropriate (11, 35). For instance, a representative urease-positive thermophilic campylobacter strain (CCUG 18267 = LMG 7791 = NCTC 11845), which has been reported by Alderton et al. to share 99.2% of its 16S rRNA sequence with the type strains of C. jejuni subsp. doylei, C. jejuni subsp. jejuni, and C. lari, exhibits a significant DNA-DNA binding level (67%) with the type strain of C. lari (39), but not with the type strains of the C. jejuni subspecies. Protein electrophoretic analysis of this strain clearly identifies it as C. lari (29, 47), as does a numerical analysis of phenotypic test results (26). A similar situation occurred in the present study; the higher level of 16S rRNA similarity between the C. hyoilei and C. jejuni type strains compared to the level of similarity between the 16S rRNA sequences of the C. hyoilei and the C. coli type strains did not correlate with overall DNA homology as expressed by DNA-DNA hybridization values.

**PCR and probe-based identification assays.** The technological burst in molecular biology has resulted in several genome-oriented identification methods for campylobacters (see reference 20 for a review). Alderton et al. (1) reported that C. hyoilei strains were indistinguishable from C. coli strains in their DNA probe hybridization assays. In Southern blots obtained by using a 6.1-kb insert from the C. jejuni genome as the probe and genomic DNA digested with three different restriction enzymes, identical patterns were always detected for C. hyoilei and C. coli strains. In our analysis, we included C. hyoilei strains in several identification protocols based on known or novel genes or on randomly generated DNA fragments. In each of the different approaches, C. hyoilei strains always reacted like typical C. coli strains.

**Conclusion.** DNA reassociation studies currently represent the "gold standard" for species delineation (35, 54). Strains that exhibit approximately 70% or greater DNA-DNA relatedness should be assigned to the same species (54). Furthermore, it has been recommended that genomically distinct taxa should not be formally named unless they can be identified by some phenotypic property (38, 54). On the basis of both of these fundamental criteria, we submit that the taxonomic standing of C. hyoilei as a distinct species cannot be justified. All of our other data clearly support this view. It is conceivable that the C. hyoilei strains represent a variant of C. coli that is highly adapted for the porcine enteric tract with pathological consequences for the animal. Thus, we suggest that the epithet of C. hyoilei could be retained as an infraspecific designation. We recommend that further studies be undertaken to clarify whether an infraspecific designation can be validated by virtue of differential biochemical and/or pathological behavior in such strains. Until such data are available, we propose that C. hyoilei Alderton et al. 1995 should be regarded as a junior synonym of C. coli Véron and Chatelain 1973.

**ACKNOWLEDGMENTS**

We thank Dirk Dewettinck, Bart Hoste, and Dorette Koster for excellent technical assistance. We thank M. A. Nicholson, C. M. Patton, and B. Swaminathan (Centers for Disease Control and Prevention, Atlanta, Ga.) and E. Falsen (Culture Collection of the University of Göteborg, Göteborg, Sweden) for kindly providing strains. P.V. is indebted to the Fund for Scientific Research-Flanders (Belgium) for a position as a postdoctoral fellow.

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