Campylobacter Species Identification Based on Polymorphism of DNA Encoding rRNA

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Total DNA from five *Campylobacter* species was digested with a mixture of *Xho*I and *Bgl*II restriction endonucleases and analyzed by Southern hybridization by using a probe complementary to the DNA coding for the 16S rRNA. Each of the *Campylobacter* species, including *C. jejuni*, *C. coli*, *C. laridis*, *C. fetus*, and *C. upsaliensis*, displayed a characteristic pattern. Although some bands may be common to different species, the simplicity of the hybridization pattern enabled us to discriminate among the different species of *Campylobacter*.

In industrial countries, *Campylobacter coli* and *Campylobacter jejuni* are major agents of diarrheal diseases. *C. coli* and *C. jejuni* have been considered two separate species on the basis of DNA homology (1, 14, 18, 24, 35). The test currently performed to distinguish between these two species is based on hippurate hydrolysis (13, 29), although different studies have pointed out problems associated with this test (14, 15, 34).

Most epidemiological studies have been based on serological methods (20, 25), biotyping (2, 16, 19, 29), plasmid analysis (3, 4, 32), and restriction endonuclease DNA analysis (5, 6). Recently, different methods based on rRNA (8, 9, 17) or DNA encoding rRNA (rDNA) composition have been proposed for organism identification. Grimont and Grimont (11) suggested that the restriction pattern of rDNA genes might provide useful information for species and type strain descriptions. Using total *Escherichia coli* 16S and 23S RNAs as probes in Southern blot experiments, they observed one or several rDNA restriction patterns, depending on the degree of divergence, within a bacterial species.

The nucleotide sequence of rRNA from organisms of the three kingdoms appears to be very conserved in some regions of the 16S RNA and less conserved in surrounding regions (10, 36). In the study reported here, we used a short synthetic probe hybridizing to a very conserved region of the 16S rDNA in order to reveal the *Xho*I-*Bgl*II polymorphism existing between the rDNA of different *Campylobacter* species.

While this work was in progress, Romaniuk and Trust (27) published a work based on a similar approach to identify *Campylobacter* species. Our results will be discussed with special reference to their work (27) and to that of Grimont and Grimont (11).

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The reference strains *C. jejuni* I Lior 4, *C. coli* Lior 1, and *C. laridis* Lior 34 were received from Y. Glupczynski (Brussels, Belgium). *C. fetus* subsp. *fetus* NCTC 10842, *C. jejuni* NCTC 11168, *C. coli* NCTC 11366, *C. coli* Lior 8, and *C. coli* Lior 44 were supplied by J. P. Butzler and H. Goossens (Brussels, Belgium). *C. jejuni* NCTC 11848, *C. laridis* NCTC 11352, and *C. upsaliensis* NCTC 11541 were received from F. Megraud (Bordeaux, France). *C. jejuni* A4646 CDC

Atlanta and *C. coli* CCUG 11283 were received from J. Dekeyser (Brussels, Belgium). The 13 reference strains were as follows: *C. fetus* subsp. *fetus* NCTC 10842, strain 253; *C. jejuni* I Lior 4, strain 106; *C. jejuni* NCTC 11848, strain 208; *C. jejuni* NCTC 11168, strain 255; *C. jejuni* A4646 CDC Atlanta, strain 260; *C. coli* Lior 1, strain 108; *C. coli* Lior 44, strain 211; *C. coli* Lior 8, strain 212; *C. coli* NCTC 11366, strain 256; *C. coli* CCUG 11283, strain 261; *C. laridis* Lior 34, strain 116; *C. laridis* NCTC 11352, strain 195; and *C. upsaliensis* NCTC 11541, strain 170.

One hundred clinical isolates, received from G. Wauters (University Hospital Saint Luc, Brussels, Belgium), were included in this study. Out of them, 93 were isolated from stools, grew at 42°C, and were resistant to cephalothin. They were identified as *C. jejuni*, *C. coli*, or *C. laridis* by total DNA-DNA hybridization following the principle of the differential spot blot test of Totten et al. (34). Seven other strains were isolated from blood samples. Of these, six grew at 25°C and were classified as *C. fetus*. The seventh strain isolated from blood grew at 42°C and was classified as *C. coli* by total DNA-DNA hybridization.

The usual culture medium for *Campylobacter* spp. was brain heart infusion agar (GIBCO Laboratories, Madison, Wis.) supplemented with 3 g liter⁻¹ of yeast extract (GIBCO) and 10% defibrinated horse blood (Ecobio, Genk, Belgium). This medium is currently referred to as *Campylobacter* medium. Plates were incubated for 24 to 72 h at 37°C in a microaerophilic atmosphere generated by the Anaerocult C system (E Merck AG, Darmstadt, Federal Republic of Germany).

NaCl (0.15 M) was used to suspend *Campylobacter* cells scraped from plates. Bacterial counts were determined by measuring the optical density at 600 nm. One A₆₀₀ unit was determined to correspond to 2 × 10⁹ cells ml⁻¹.

Total DNA preparation. Bacteria were cultivated on two plates of *Campylobacter* medium. Cells were collected and suspended in 50 mM Tris hydrochloride-50 mM EDTA-50 mM NaCl (pH 8.5) (THES). The cells were washed and suspended in THES supplemented with 10% sucrose. RNase (Serva, Heidelberg, Federal Republic of Germany) and lysozyme (Serva) were added to final concentrations of 90 µg ml⁻¹ and 1 mg ml⁻¹, respectively. The suspension was incubated for 90 min at 37°C. A 10% sodium lauryl sulfate solution in THES was added to a final concentration of 4.4%, and incubation was prolonged at 37°C for 20 min. The lysate was subjected to two chloroform-octanol (96:4) extractions, one phenol extraction, and one chloroform-isoamyl alcohol

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hol (99:1) extraction. DNA was precipitated by the addition of 2 volumes of ethanol and 0.1 volume of 7.5 M ammonium acetate. The DNA precipitate was resuspended in distilled water and stored at -20°C .

DNA concentration was estimated by comparing the fluorescence intensity of the sample DNA against a standard of lambda DNA (Bethesda Research Laboratories, Gaithersburg, Md.) on an agarose electrophoresis gel stained with ethidium bromide.

Southern blot analysis. Total DNA (1.5 μg) was digested with 15 U of the relevant restriction endonucleases (Pharmacia, Uppsala, Sweden) according to the supplier's specifications.

DNA fragments were separated by 0.8% agarose (SeaKem; FMC BioProducts, Rockland, Maine) gel electrophoresis (21). *Hind*III-digested bacteriophage lambda DNA was used as molecular weight marker; 1 μg of unlabeled marker was mixed with the equivalent of 7,500 dpm of ^{32}P -labeled marker. Labeling was done with T4 DNA polymerase (21).

Prior to transfer on a nylon membrane (Hybond-N; Amersham, Little Chalfont, United Kingdom) as described by Southern (12, 30), the DNA fragments were depurinated and denatured as recommended by the membrane's supplier. The membrane was then washed, irradiated as described by the manufacturers, and prehybridized for 2 h at 50°C in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% dehydrated skim milk (Gloria; Nestlé, Brussels, Belgium), 2% sodium dodecyl sulfate (Serva) and 20 μg ml^{-1} of heat-denatured calf thymus DNA (Serva). Labeled probe was added to a final activity of 10^6 dpm ml^{-1} . After 16 h of incubation at 50°C , the membrane was washed three times for 15 min at 50°C in $2\times$ SSC–1% sodium dodecyl sulfate. The membrane was air dried and autoradiographed (21).

Probe synthesis and labeling. Oligodeoxynucleotide was synthesized on a Applied Biosystems synthesizer model 380A via the solid-phase phosphoramidite method (7, 22, 28).

The oligonucleotide probe was end labeled essentially as described by Thein and Wallace (33), except that only 15 pmol of [γ - ^{32}P]ATP (3,000 Ci mmol^{-1} ; Amersham) was used for the labeling of 15 pmol of probe. After the labeling step, the reaction mixture was diluted to 100 μl with H_2O and the probe was purified by passage through a Sephadex G-50 column (Pharmacia).

Typing by total DNA-DNA hybridization. Typing by total DNA-DNA hybridization was done by the method of Totten et al. (34), but DNA (1 μg) rather than bacteria, was spotted onto the membranes. Total DNA from strains *C. jejuni* I Lior 4, *C. coli* Lior 8, and *C. laridis* Lior 34 was radiolabeled with [α - ^{32}P]dATP (3,000 Ci mmol^{-1} ; Dupont, NEN Research Products, Boston, Mass.) to a specific activity of 10^8 dpm μg^{-1} by using a nick translation kit (Bethesda Research Laboratories). The unincorporated nucleotides were eliminated by passage through a Sephadex G-50 column (Pharmacia). Hybridization conditions were as described for Southern blot analysis except that hybridizations and washings occurred at 63°C .

RESULTS

Selection of the probe. The approach we followed was to select a probe hybridizing to a very conserved region of rDNA from *Campylobacter* species and to look for a restriction enzyme (or a combination of such enzymes) displaying

a polymorphism among the different species of *Campylobacter*. The probe was chosen to hybridize to a sequence conserved between *E. coli* and *C. jejuni* with the assumption that all species of *Campylobacter* would hybridize to it (26, 36). The nucleotide sequence of this probe (ORJ16Sa) was 5'-TGATCCGACCGCAGGTTCTCCTA-3'. This 23-mer probe was complementary to the 3' end of the 16S RNA of *C. jejuni* and hybridized to the sequence corresponding to nucleotides 1511 to 1533 in 16S RNA from *E. coli* (26, 36). The oligonucleotide was synthesized and was shown to hybridize specifically to 16S RNA in a Northern (RNA) blot experiment (data not shown). Specific hybridization of the probe to the 3' end of 16S RNA was further confirmed by using the probe as a primer for sequencing of *C. jejuni* I Lior 4 rRNA by the chain termination method (17 and data not shown).

Analysis of 13 reference strains. DNA from the 13 reference strains was digested with a variety of restriction enzymes and analyzed by Southern blot hybridization. Restriction endonuclease *Bgl*II (data not shown) and the combination of *Bgl*II and *Xho*I allowed us to discriminate between the different species of *Campylobacter*, including *C. coli*, *C. jejuni*, *C. laridis*, *C. fetus*, and *C. upsaliensis*. The probe did not contain any restriction sites for these two enzymes.

Four *C. coli* reference strains (*C. coli* Lior 8 and Lior 44 [Fig. 1A], *C. coli* NCTC 11366, and *C. coli* CCUG 11283 [data not shown]) presented the same pattern characterized by a unique band of 0.8 kilobases (kb). The fifth reference strain (*C. coli* Lior 1; Fig. 1A) presented an additional 3.7-kb band.

C. laridis Lior 34 and *C. laridis* NCTC 11352 (Fig. 1A) presented the same pattern characterized by a unique 2.4-kb band.

C. upsaliensis NCTC 11541 (Fig. 1A) presented two bands of about 24 and 0.83 kb. The high-molecular-weight band appeared to be less intense than the 0.83-kb band. This could be due to a lower transfer efficiency of large DNA fragments than of small DNA fragments or to a lower sequence homology.

C. fetus subsp. *fetus* NCTC 10842 (Fig. 1A) featured a pair of bands at the level of 6 kb. The two bands could only be individualized when the DNA load was lower (Fig. 1B).

The four *C. jejuni* reference strains presented three bands, suggesting the presence of at least three copies of the 16S rDNA in *C. jejuni*. *C. jejuni* I Lior 4 (Fig. 1A), *C. jejuni* NCTC 11168 (data not shown), and *C. jejuni* A4646 (data not shown) featured the same pattern, consisting of bands at 4.6, 11, and 15 kb. *C. jejuni* NCTC 11848 (Fig. 1A) displayed a different pattern characterized by three bands of 3.7, 6.3, and 11 kb. This nitrate-negative *C. jejuni* strain, described by Steele et al. (31), was considered a subspecies of *C. jejuni* by Owen and Dawson (23).

Analysis of 100 clinical isolates of *Campylobacter*. The rDNA *Xho*I-*Bgl*II restriction-fragment-length polymorphism was assessed for 100 clinical isolates identified as *C. jejuni*, *C. coli*, *C. laridis*, or *C. fetus* as described in Materials and Methods.

Out of the nine *C. coli* strains tested, eight presented the same pattern as the reference strains Lior 8, Lior 44, NCTC 11366, and CCUG 11283 (Fig. 1B). One strain (strain 205; Fig. 1B) displayed an additional band of 2.4 kb that was also found in the pattern of the *C. laridis* reference strains. Total DNA-DNA hybridization identified this strain as *C. coli*. The fact that the growth of strain 205 was inhibited by nalidixic acid further confirmed that this strain was not contaminated

FIG. 1. rDNA *Xho*I-*Bgl*II restriction-fragment-length polymorphism in *Campylobacter* spp. (A) Reference strains. (B) *C. coli*, *C. laridis*, and *C. fetus* isolates. (C) *C. jejuni* isolates. Numbers at the sides of the autoradiograph refer to the fragment sizes (in kilobases), and numbers at the top refer to the strain numbers with the corresponding reference assignments. Lanes from two different gels have been rearranged for the sake of clarity. *Hind*III-digested lambda DNA was used as a molecular weight marker (lane M).

by a *C. laridis* strain. None of the human isolates had the hybridization patterns of *C. coli* Lior 1. This result suggests that *C. coli* Lior 1 is not a good representative of the *C. coli* isolated in Western Europe.

The *C. laridis* isolated showed a 2.4-kb band, as in the reference strains (Fig. 1B).

The six *C. fetus* strains tested displayed a pair of bands at the level of 6 kb, as in reference strain NCTC 10842 (Fig. 1B and data not shown).

In contrast with *C. coli*, *C. fetus*, and *C. laridis*, in which the patterns appeared to be very conserved, a rather high polymorphism occurred within the patterns produced by the *C. jejuni* isolates (Fig. 1C and data not shown). However, the patterns of the 84 *C. jejuni* strains tested shared common features: a band at the level of 4.6 kb and two larger bands. The latter were not always separated on the 0.8% agarose gels but were readily apparent on 0.4% agarose gels (data not shown).

None of the human isolates had the hybridization pattern of *C. jejuni* NCTC 11848 and *C. upsaliensis* NCTC 11541. This is not surprising, since all our clinical isolates were resistant to cephalothin whereas *C. upsaliensis* and nitrate-negative *C. jejuni* are known to be susceptible (31; K. Sandstedt and I. Ursing, 14th Int. Congr. Microbiol., abstr. no. P.B8-17, 1986).

DISCUSSION

A Southern blot analysis using a probe complementary to the 3' end of the 16S rRNA enabled us to discriminate among the five species of *Campylobacter*, i.e., *C. jejuni*, *C. coli*, *C. laridis*, *C. fetus*, and *C. upsaliensis*.

This kind of approach was first proposed by Grimont and Grimont (11), who used total 16S and 23S rRNAs as probes. These probes hybridized with many fragments of DNA and permit leading strain discrimination and, hence, can be useful in epidemiological studies. It is, however, difficult to

analyze these patterns in order to identify *Campylobacter* isolates at the species level. Our method gives a simpler pattern, allowing easy discrimination among the commonly found species of *Campylobacter*. However, it does not necessarily allow one to distinguish individual strains. The two methods should therefore be considered complementary.

Our approach is very similar to that of Romaniuk and Trust (27). Our observation that *C. jejuni* contains at least three copies of 16S rDNA is in perfect agreement with the results of these authors. They used as a probe a 17-mer oligonucleotide complementary to a unique sequence present in the 5' domain of the 16S rRNA of several species of *Campylobacter*. This probe seems to be *Campylobacter* specific but the sensitivity of the probe was so far only tested on nine strains from four different species. In their study, the hybridization pattern did not always allow discrimination between *C. jejuni* and *C. coli*.

Our method was evaluated on 100 clinical isolates. All the *Xho*I-*Bgl*II restriction patterns appeared to be species specific and, therefore, allowed for speciation of *Campylobacter* isolates. *C. coli* could be recognized by its conserved 0.8-kb hybridizing fragment. In some cases, *C. coli* had an additional band of either 3.6 (*C. coli* Lior 1) or 2.4 (*C. coli* 205) kb. *C. laridis* had a unique 2.4-kb hybridizing fragment, and *C. fetus* showed a doublet at 6 kb. *C. upsaliensis* had two hybridizing fragments: one at 0.83 kb and one faint band of a size larger than 24 kb. With *C. jejuni*, we observed a rather high degree of polymorphism, but a general profile could be recognized: three hybridizing fragments of sizes larger than 4.6 kb.

We think that hybridization methods involving rDNA offer new prospects in clinical bacteriology. The progress foreseen towards the automation of DNA extraction and subsequent Southern blot analysis could make rDNA restriction-fragment-length-polymorphism analysis an interesting

method for *Campylobacter* species identification and epidemiology.

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