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Speciation of Thermotolerant *Campylobacter* Isolates Involved in Foodborne Disease by Means of DNA Restriction Analysis and Molecular Probes

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The molecular identification of several strains of *Campylobacter jejuni* and *Campylobacter coli* involved in foodborne disease was carried out by investigating the restriction profiles of their chromosomal DNA and by DNA/DNA hybridization. Cleavage with *Eco*RV allowed the visualization of a 3 kb DNA fragment characteristic of *C. jejuni*, whereas restriction with *Cla*I allowed the identification of a 9.3 kb DNA fragment, also characteristic of *C. jejuni*, and a DNA duplet of 9.5−10 kb, specific to *C. coli.* Restriction analysis with enzyme *Bgl*II allowed the visualization of DNA fragments of 3.5, 4, and 6.7 kb, characteristic of *C. jejuni. C. jejuni* subsp. *doylei* strains investigated shared a higher genetic homology among themselves—as determined by DNA/DNA hybridization—than with *C. jejuni* subsp. *jejuni*. A DNA probe, initially designed by Korolik et al. (Korolik, V.; Coloe, P. J.; Krishnapillai, V. *J. Gen. Microbiol.* **1988**, *134*, 521−529), including a DNA fragment encoding an antigenic membrane protein of 31.5 kDa in *C. jejuni*, when used as probe, allowed the specific identification of all strains of *C. jejuni* through the detection of strong hybridization signals in two *Bgl*II DNA fragments of 2.3 and 2.5 kb, which were not observed in *C. coli.* Cleavage of chromosomal DNA with *Bgl*II—either alone or coupled with probing assays with specific probes—proved to be a valuable tool for the speciation of *Campylobacter* isolates involved in foodborne disease.

KEYWORDS: Foodborne disease; *Campylobacter*; DNA analysis; RFLP; hybridization

INTRODUCTION

Certain species of the genus *Campylobacter*, mainly the thermotolerant emerging pathogens *C. jejuni* and *C. coli*, are etiologic agents of human enteric disease of foodborne origin throughout the world (*1*−*3*). Most foodborne infection episodes are sporadic, but only 1% of them are associated with epidemic outbreaks (*4*). Whereas conventional methods of phenotyping of campylobacters afford valuable information for the investigation of epidemic outbreaks, the epidemiology of sporadic foodborne infections is sometimes difficult to appraise with such tools. Thus, phenotypic analysis of campylobacters is sometimes complicated by nonspecific cross-reactions at immunological level—during serotyping, by a lack or a weak expression of certain reporter genes—during biotyping, or by the horizontal transmission of genes involved in antimicrobial resistance—which complicates resistotyping, to name but three conventional phenotyping tools traditionally employed for the identification and differential characterization of campylobacters (*5*). During the past decade, the limitations of such conventional typing techniques have prompted researchers to develop new and more robust typing methods based on DNA technology. Such genotyping methods afford a higher resolution power, allowing the distinction between closely related strains of *C. jejuni* and *C. coli* involved in foodborne disease even in the case of strains that share very similar phenotypic profiles (*6*, *7*). Accordingly, nucleic acid-based detection techniques are becoming increasingly common as alternative tools for the screening of samples in the search for selected target microorganisms (*8*). Among such techniques, genotyping by means of investigation of the restriction fragment length polymorphism (RFLP) of chromosomal DNA has been reported to be an efficient method for the identification of *C. jejuni* and *C. coli* (*7*, *9*, *10*). Together with DNA restriction, hybridization analysis has proved to be a complementary genotyping tool, affording lower detection limits than Polymerase Chain Reaction (PCR) (*11*). Other advantages of hybridization are that this technique seldom yields false-positives derived from the presence of residual amounts of DNA or dead cells (*12*) and that the technique is not as affected by substances present in complex biological samples which might inhibit the activity of DNA polymerase, thereby complicating PCR-based methods (*13*). Hybridization studies between *Campylobacter* strains have thus provided deeper knowledge about both the phylogeny of these emerging foodborne pathogens (*7*, *14*, *15*) and their taxonomic status (*10*).

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C. jejuni subsp. *doylei* is one of the two subspecies into which the species *C. jejuni* is currently divided. On and Holmes reported that the identification of certain strains of this subspecies was difficult and that such strains kept a significant phylogenetic distance with respect to *C. jejuni* subsp. *jejuni* (*5*). The low number of isolates of *C. jejuni* subsp. *doylei* reported to date with respect to *C. jejuni* subsp. *jejuni* has meant that the former subspecies has not been extensively studied.

The purpose of the present work was to study the application of two robust genotyping tools (RFLP of chromosomal DNA and DNA/DNA hybridization) to *C. jejuni* and *C. coli* strains involved in foodborne disease. Four strains of *C. jejuni* subsp. *doylei* were also considered. The usefulness of a DNA probe integrated in the recombinant plasmid pMO2005 (*10*) was also evaluated for the differential characterization of *C. jejuni* and *C. coli*.

MATERIALS AND METHODS

Isolation of Thermotolerant *Campylobacter* spp. and Media. Stool samples were obtained from patients affected by foodborne disease over a one-year period. The presence of *Salmonella*, *Shigella*, enterotoxigenic *Escherichia coli* (ETEC), *Vibrio*, rotavirus, and parasites was investigated using standard laboratory methods (*16*). Selective isolation of thermotolerant *Campylobacter* strains was carried out on Campylosel (BioMérieux, Marcy l'Etoile, France) at 42 °C under a selective microaerophilic atmosphere (gas-generating kit, Oxoid Ltd., London, U.K.). Presumptive campylobacters obtained under selective conditions were subjected to the following tests: morphology, Gram stain, production of oxidase (Mast Diagnostics Ltd., Merseyside, U.K.), and production of catalase. Confirmation studies were carried out by a specific immunological system for campylobacters (Meridian Diagnostics, Cincinnati, OH). Columbia blood agar (BioMérieux) and Brucella agar (Difco Laboratories, Detroit, MI) were used for the subculture and maintenance of the campylobacters, as described elsewhere (*17*).

Phenotyping of *Campylobacter* Strains. Hippurate hydrolysis was investigated according to the method of Harvey (*18*). Biotyping studies including tests to elucidate the assimilation of carbon sources and the production of specific enzymes were carried out by means of a standardized API CAMPY system (BioMérieux) equipped with APILAB PLUS software, as previously described (*17*). Antimicrobial resistance was investigated by both a microdilution technique and standardized agar disk diffusion, as previously described (*19*, *20*). The average agreement between both methods was >90%. Laboratory strains of *C. jejuni* and *C. coli* were kindly given by Dr. Joaquín Rodríguez (Hospital Cristal-Piñor, Orense, Spain) and used as references.

Purification of Chromosomal DNA and RFLP Analysis. Bacterial lysis was achieved by using the method of Korolik et al. (*10*), following the modifications described elsewhere (*7*). Purification of chromosomal DNA was specifically achieved—thus minimizing plasmid contamination—by ultracentrifugation in a cesium chloride/ethidium bromide gradient. For this purpose, a VTI65 rotor (Beckman-Coulter, London, U.K.) was employed on a JA-21 ultracentrifuge (Beckman-Coulter). Ultracentrifugation was carried out at 65000 rpm for 16 h. The DNA upper band, composed of chromosomal DNA, was recovered from the gradient tubes, ethidium bromide was removed by butanol extraction, and each DNA extract (~0.5 mL) was dialyzed overnight at 4 °C against 1 L of sterile distilled water.

All purified DNA extracts were subjected to restriction analysis with enzymes *Bgl*II, *Cla*I, and *Eco*RV (all of them from Amersham Biosciences, Uppsala, Sweden). These enzymes were selected from a pool of eight enzymes evaluated in a preliminary study, as previously described *(7)*. Restriction assays were carried out with 2−5 μg of DNA and 2 μL of each restriction enzyme in a suitable volume of restriction buffer. Following restriction, the digested DNA samples were processed by 0.7% agarose gel horizontal electrophoresis, as previously described, and visualized by ethidium bromide staining in a transilluminator at 320 nm.

Hybridization Studies. Genotyping of *Campylobacter* strains was also carried out by DNA/DNA hybridization. Processing of gels prior to capillary transfer was carried out as follows: depurination of gels was carried out for 15 min in 0.25 M HCl; in-gel DNA denaturation was carried out for 30 min in 1.5 M NaCl + 0.5 M NaOH; neutralization was carried out for 30 min in 1.5 M NaCl + Tris-HCl, pH 7.5. Capillary transfer of DNA from the gels to Hybond-N+ nylon membranes (Amersham Biosciences) by Southern blotting was carried out following the method of Southern (*21*) as previously described (*7*). SSC (20×) was used as transfer buffer. Once transferred, DNA was fixed to the membranes with 0.4 M NaOH. Single-stranded DNA (ssDNA) probes were obtained by heating at 100 °C for 5 min. The non-radioactive ECL gene detection kit (Amersham Biosciences) was used for labeling and detection purposes. Chromosomal ssDNA from selected *C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, and *C. coli* strains was used as probes. In addition, plasmid pMO2005, including a DNA fragment from *C. jejuni* encoding a 31.5 kDa membrane protein in *C. jejuni* (*10*), was also used as a DNA probe. The DNA fragment from *C. jejuni* was recovered from the agarose gel after restriction of the recombinant plasmid pMO2005 with endonuclease *Bam*H1 (Amersham Biosciences) and was purified by means of the QIAEX kit (Qiagen, Washington, DC). Probe quantification, aimed at evaluating the efficiency of labeling and optimizing probe concentrations, was carried out by performing serial dilutions of the probe and determining the highest dilution at which probe was still detected. These experiments indicated that the total amount of ssDNA used as probe should be 200 ng. All hybridizations were carried out in commercial hybridization buffer (Amersham Biosciences) for 16 h at 42 °C. The membranes were washed twice at 42 °C with a buffer containing either 0.5× or 0.1× SSC, for low- or high-stringency conditions. The washing of membranes under high-stringency conditions implies that only the ssDNA probes which have hybridized with a nearly identical ssDNA sequence in the membrane blot would remain fixed to the blot after washing. On the contrary, when low-stringency conditions of washing are used, the ssDNA probes that hybridized to related, but not identical, ssDNA sequences in the blot, also remain fixed to the membrane blot. Finally, the membrane was covered twice with a secondary wash buffer composed of 20× SSC; these secondary washes were carried out at room temperature in all cases.

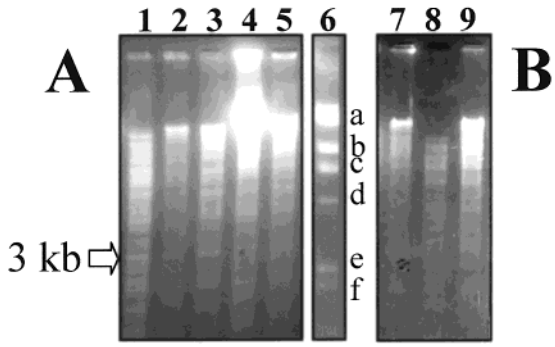

Figure 1. Restriction analysis of chromosomal DNA of (A) *C. jejuni* subsp. *jejuni* and (B) *C. coli* strains with endonuclease *Eco*RV: (A) Lane 1, strain RSC-18; lane 2, strain RSC-88; lane 3, strain RSC-83; lane 4, strain RSC-82; lane 5, strain RSC-79; lane 6, *Hin*dIII-restricted DNA from lambda bacteriophage (band a, 23.1 kb; band b, 9.4 kb; band c, 6.6 kb; band d, 4.3 kb; band e, 2.3 kb; band f, 2.0 kb). (B) Lane 7, strain RSC-68; lane 8, strain RSC-89; lane 9, strain RSC-90.

RESULTS

Identification of *C. jejuni* and *C. coli* by Restriction Analysis and DNA/DNA Hybridization. The RFLPs of the chromosomal DNA specifically purified from *C. jejuni* subsp. *jejuni* and *C. coli* were obtained with endonucleases *Eco*RV, *Cla*I, and *Bgl*II. Thus, cleavage with *Eco*RV of chromosomal DNA from all strains of *C. jejuni* subsp. *jejuni* investigated yielded a characteristic DNA fragment of 3 kb (**Figure 1A**), whereas this specific fragment was not observed in the *C. coli* strains studied (**Figure 1B**). These results agree with those

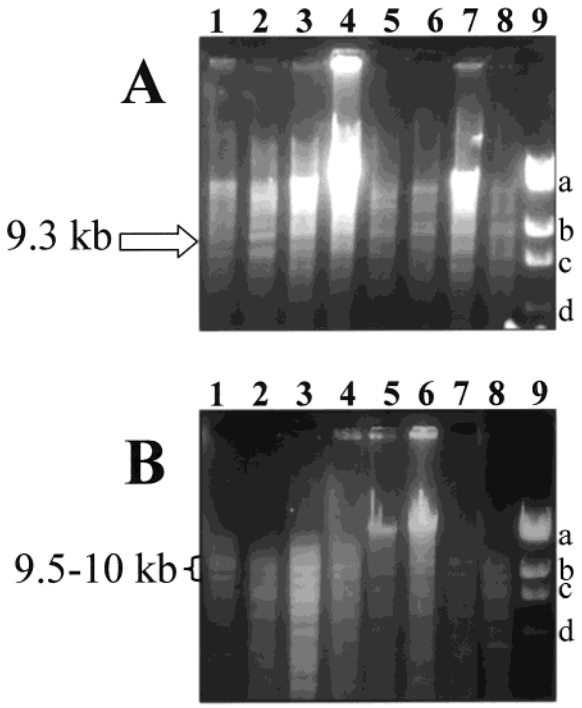

Figure 2. Restriction analysis of chromosomal DNA of (A) *C. jejuni* subsp. *jejuni* and (B) *C. coli* strains with endonuclease *Cla*I: (A) Lane 1, strain RSC-88; lane 2, strain RSC-83; lane 3, strain RSC-82; lane 4, strain RSC-79; lane 5, strain RSC-59; lane 6, strain RSC-58; lane 7, strain RSC-45; lane 8, strain RSC-41; lane 9, *Hin*dIII-restricted DNA from lambda bacteriophage (band a, 23.1 kb; band b, 9.4 kb; band c, 6.6 kb; band d, 4.3 kb). (B) Lane 1, strain RSC-42; lane 2, strain RSC-47; lane 3, strain RSC-49; lane 4, strain RSC-51; lane 5, strain RSC-89; lane 6, strain RSC-90; lane 7, strain RSC-95; lane 8, strain RSC-22; lane 9, *Hin*dIII-restricted DNA from lambda bacteriophage (band a, 23.1 kb; band b, 9.4 kb; band c, 6.6 kb; band d, 4.3 kb).

obtained by Korolik et al. (*10*). Cleavage with *Cla*I yielded a specific DNA fragment of 9.3 kb in all of the strains of *C. jejuni* subsp. *jejuni* investigated (**Figure 2A**), whereas none of the *C. coli* strains tested showed that DNA fragment, although the latter species displayed a characteristic DNA duplet of DNA in the 9.5−10 kb range (**Figure 2B**). This duplet seemed to be characteristic of *C. coli* and was not observed in any of the *C. jejuni* subsp. *jejuni* strains tested. Cleavage with enzyme *Bgl*II also allowed the visualization of characteristic restriction fragments for *C. jejuni* subsp. *jejuni* strains (**Figure 3A**), absent in *C. coli* (**Figure 3B**). Thus, characteristic DNA fragments of 3.5, 4, and 6.7 kb were observed in the *C. jejuni* subsp. *jejuni* strains tested (**Figure 3A**).

The grouping of *Campylobacter* strains as belonging to either *C. jejuni* subsp. *jejuni* or *C. coli* was also carried out by means of DNA/DNA hybridization. Strong hybridization signals were observed in all of the *C. jejuni* subsp. *jejuni* strains tested when chromosomal ssDNA from strains of *C. jejuni* subsp. *jejuni* was used as probes (**Figure 4B**). In contrast, no significant hybridization signal was observed in any of the *C. coli* strains when these were probed with chromosomal ssDNA from *C. jejuni* subsp. *jejuni* (**Figure 5B**). It should be mentioned that the detection of hybridization signals was carried out under low-stringency conditions, which emphasizes the fact that even under low-specificity conditions the strains belonging to each species could be successfully identified.

Investigation of *C. jejuni* subsp. *doylei*. Four of the 102 campylobacters isolated during this study, which comprised a

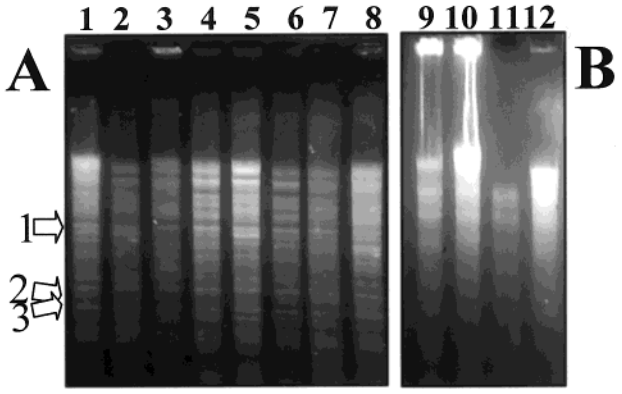

Figure 3. Restriction analysis of chromosomal DNA of (A) *C. jejuni* subsp. *jejuni* and (B) *C. coli* strains with endonuclease *Bgl*II: (A) Lane 1, strain RSC-45; lane 2, strain RSC-58; lane 3, strain RSC-59; lane 4, strain RSC-79; lane 5, strain RSC-82; lane 6, strain RSC-83; lane 7, strain RSC-18; lane 8, strain RSC-27. (B) Lane 9, strain RSC-90; lane 10, strain RSC-89; lane 11, strain RSC-68; lane 12, strain RSC-51.

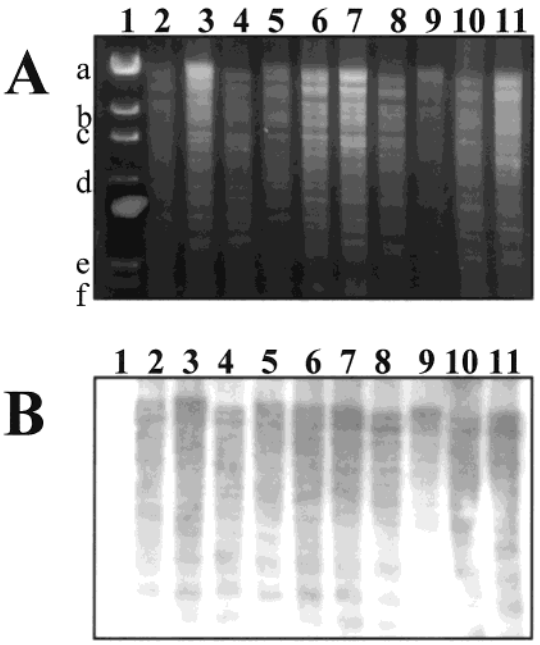

Figure 4. Genetic homology among strains of *C. jejuni* subsp. *jejuni*: (A) Lane 1, *Hin*dIII-restricted DNA from lambda bacteriophage (band a, 23.1 kb; band b, 9.4 kb; band c, 6.6 kb; band d, 4.3 kb; band e, 2.3 kb; band f, 2.0 kb); lanes 2–11, restriction with endonuclease *Bgl*II of 10 strains belonging to *C. jejuni* subsp. *jejuni*. (B) Lanes are as in (A). Hybridization analysis: probe consisted of ssDNA from strain *C. jejuni* subsp. *jejuni* RSC-45 (lane 3); blot processing after hybridization was carried out under low-stringency conditions; time of exposition of autoradiography film was 5 h.

one-year period, were phenotypically identified as belonging to the species *C. jejuni* subsp. *doylei*. The phenotypic features that distinguished this subspecies with respect to *C. jejuni* subsp. *jejuni* and *C. coli* are shown in **Table 1**. It can be observed that none of the four strains of *C. jejuni* subsp. *doylei* reduced nitrates, a feature previously known to be helpful for distinguishing this subspecies from *C. jejuni* subsp. *jejuni* and *C. coli*. Additionally, the four strains of *C. jejuni* susbp. *doylei* did not produce γ-glutamyl transferase, a feature that allowed their differentiation with respect to *C. jejuni* subsp. *jejuni* biotype 2. The three phenotypic features that allowed the differentiation of *C. jejuni* subsp. *doylei* with respect to *C. coli* were (i) their

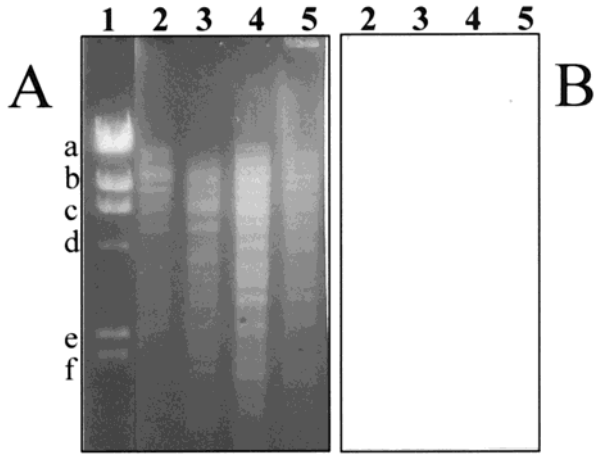

Figure 5. Lack of homology between strains of *C. coli* and *C. jejuni* subsp. *jejuni*: (A) Lane 1, *Hin*dIII-restricted DNA from lambda bacteriophage (band a, 23.1 kb; band b, 9.4 kb; band c, 6.6 kb; band d, 4.3 kb; band e, 2.3 kb; band f, 2.0 kb); lanes 2–5, restriction with endonuclease *Cla*I of four strains belonging to *C. coli*. (B) Lanes are as in (A). Hybridization analysis: probe consisted of ssDNA from strain *C. jejuni* subsp. *jejuni* RSC-79; blot processing after hybridization was carried out under low-stringency conditions; time of exposition of autoradiography film was 5 h.

Table 1. Phenotypic Differences Observed between the Four Strains of *C. jejuni* Subsp. *doylei* with Respect to *C. jejuni* Subsp. *jejuni* and *C. coli*[a]

phenotype	*C. jejuni* subsp. *doylei* ($n = 4$)	*C. jejuni* subsp. *jejuni* biotype 1 ($n = 79$)	*C. jejuni* subsp. *jejuni* biotype 2 ($n = 9$)	*C. coli* ($n = 10$)
nitrate reduction	0	92.4	77.7	100
hippurate hydrolysis	100	97.4	100	0
GGT production	0	0	100	0
L-arginine arylamidase production	0	1.3	0	50
propionate assimilation	0	0	0	50

[a] Results are expressed as percentages of strains that proved to be positive for each test. n = number of strains tested.

lack of production of L-arginine arylamidase; (ii) their inability to assimilate propionate; and (iii) their ability to hydrolyze hippurate. All three features were found in the four strains of *C. jejuni* subsp. *doylei*, whereas none of the *C. coli* strains hydrolyzed hippurate and only half of the strains of *C. coli* investigated produced L-arginine arylamidase or assimilated propionate. Biotyping and resistotyping studies clearly indicated that the four strains of *C. jejuni* subsp. *doylei* were different among themselves at the phenotypic level, as can be observed in **Table 2**. This result confirms that none of these strains were multiple isolates of the same strains or were involved in the same outbreak.

Figure 6A shows the restriction profiles obtained when several strains of *C. jejuni* subsp. *jejuni* biotype 1, *C. jejuni* subsp. *jejuni* biotype 2, and *C. jejuni* subsp. *doylei* were cleaved with *Bgl*II. No specific DNA restriction fragment was observed for *C. jejuni* subsp. *doylei*, which could help in the differentiation of this subspecies with respect to *C. jejuni* subsp. *jejuni*. Similar results were obtained when cleaving with *Cla*I and *Eco*RV. The following step consisted of probing several strains of *C. jejuni* with labeled ssDNA from *C. jejuni* subsp. *doylei* strain RSC-34, with a view to elucidating whether this technique would allow the discrimination of this subspecies with respect to *C. jejuni* subsp. *jejuni*. Strong hybridization signals were obtained

Table 2. Phenotypic Differences Observed among the Four Strains of *C. jejuni* Subsp. *doylei*

test	strain of *C. jejuni* subsp. *doylei* RSC-34	RSC-36	RSC-43	RSC-44
succinate assimilation	−	+	−	+
malate assimilation	−	+	−	+
citrate assimilation	−	+	−	−
cefazoline	R[a]	S	R	S
nalidixic acid	R	S	S	S
ciprofloxacin	R	S	S	S
norfloxacin	R	S	S	S
pefloxacin	R	S	S	S
tetracycline	R	S	R	S

[a] R = resistant; S = sensitive.

with all 10 strains studied when detection was carried out under low-stringency conditions (**Figure 6B**). By contrast, when detection was more selective and was carried out under high-stringency conditions, specific hybridization was preferentially observed between the probe and each of the four strains of *C. jejuni* subsp. *doylei* included in the blot (**Figure 6C**), this indicating the higher homology among these four strains than with respect to the other six strains of *C. jejuni* subsp. *jejuni* studied. It should be stressed that *C. jejuni* subsp. *jejuni* strain RSC-18, which was found to be one of the two hippurate-negative *C. jejuni* subsp. *jejuni* strains isolated in this study, showed detectable cross-reaction with the DNA probe from *C. jejuni* subsp. *doylei* RSC-34, a result that was not observed in any of the hippurate-positive *C. jejuni* subsp. *jejuni* strains.

Usefulness of the DNA Probe Excised from pMO2005. The DNA fragment formerly isolated from *C. jejuni* by Korolik et al. (*22*) was recovered from plasmid pMO2005 after cleavage with *Bam*H1. This fragment was used as a DNA probe against blots containing DNA fragments from our strains of *C. jejuni* and *C. coli*, cleaved with *Bgl*II. As can be seen in **Figure 7B**, all of the strains of *C. jejuni* yielded two strong hybridization signals, corresponding to DNA fragments of 2.3 and 2.5 kb in all 92 isolates of *C. jejuni* (**Figure 7B**). These results agree with those obtained by Korolik et al. (*22*). However, those authors reported that in some strains of *C. jejuni* the hybridization signals of 2.3 and 2.5 kb were not visualized and were replaced by a strong hybridization signal of a DNA fragment of 4.8 kb, probably caused by a point mutation in the restriction site recognized by *Bgl*II (*10*). The results obtained with our strains clearly pointed to the absence of polymorphism in the 4.8 kb DNA fragment in our strains and showed that this fragment is efficiently cleaved by *Bgl*II, leading to two smaller DNA fragments of 2.5 and 2.3 kb that contain, in the case of strains of *C. jejuni*, DNA sequences homologous to the ssDNA probe excised from pMO2005.

DISCUSSION

The chromosomal DNA restriction assays described in this work allowed the identification of DNA fragments specific to *C. jejuni* not present in *C. coli* and vice versa. This technique has been successfully employed in the typing of *Campylobacter* strains by other authors (*10*, *23*, *24*). Whereas Korolik et al. (*10*) has previously reported the presence of some of the above-mentioned specific bands obtained after cleavage with *Eco*RV and *Cla*I, to our knowledge this is the first time that the usefulness of cleaving with *Bgl*II has been considered. Thus, this endonuclease allows the identification of DNA fragments of 3.5, 4, and 6.7 kb, specific to *C. jejuni*. Although the results obtained with *Eco*RV agreed with those described by Korolik

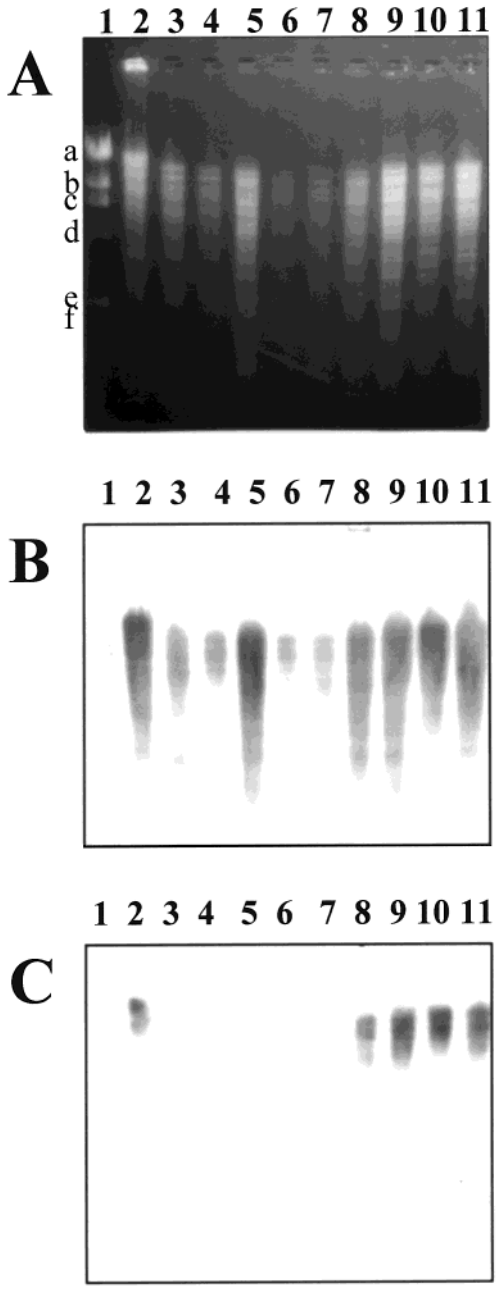

Figure 6. Restriction and hybridization analysis of *C. jejuni* subspecies: (A) Lane 1, *Hin*dIII-restricted DNA from lambda bacteriophage (band a, 23.1 kb; band b, 9.4 kb; band c, 6.6 kb; band d, 4.3 kb; band e, 2.3 kb; band f, 2.0 kb); lanes 2–11, restriction with endonuclease *Bgl*II of strains belonging to *C. jejuni* subsp. *jejuni* biotype 1 (lanes 2–4), *C. jejuni* subsp. *jejuni* biotype 2 (lanes 5–7), and *C. jejuni* subsp. *doylei* (lanes 8–11). (B) Hybridization analysis: probe consisted of ssDNA from strain *C. jejuni* subsp. *doylei* RSC-34 (lane 8); all lanes are as in (A); blot processing after hybridization was carried out under low-stringency conditions; time of exposition of autoradiography film was 5 h. (C) Hybridization analysis: probe consisted of ssDNA from strain *C. jejuni* subsp. *doylei* RSC-34 (lane 8); all lanes are as in (A); blot processing after hybridization was carried out under high-stringency conditions; time of exposition of autoradiography film was 5 h.

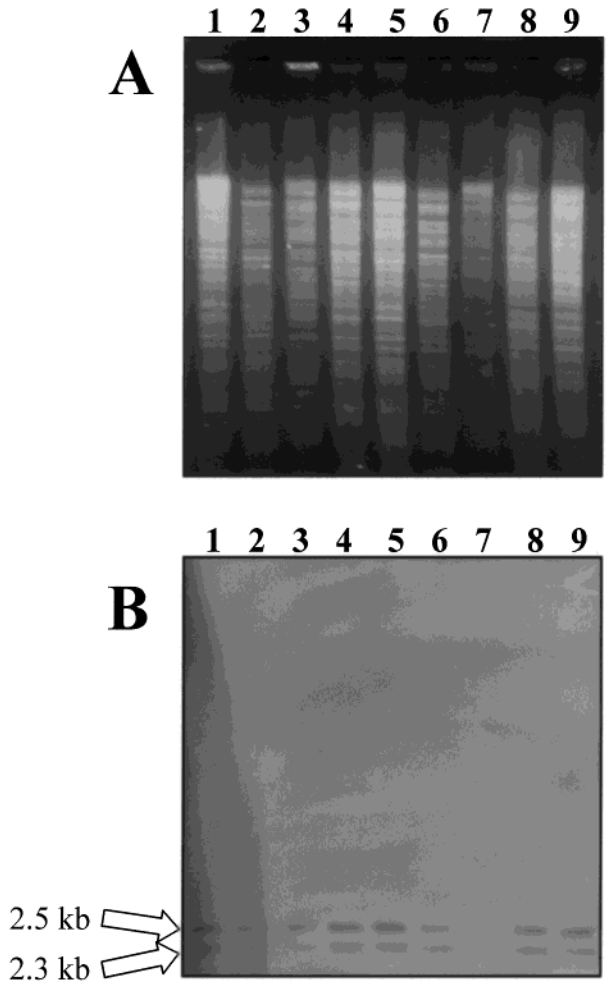

Figure 7. Specific detection of *C. jejuni* by hybridization of *Bgl*II restriction fragments: (A) *Bgl*II restriction fragments of *C. jejuni* and *C. coli* strains; lane 1, strain RSC-45; lane 2, strain RSC-58; lane 3, strain RSC-59; lane 4, strain RSC-79; lane 5, strain RSC-82; lane 6, strain RSC-83; lane 7, strain RSC-88; lane 8, strain RSC-18; lane 9, strain RSC-27. (B) Detection of specific DNA fragments of *C. jejuni* with the DNA probe excised with *Bam*H1 from plasmid pMO2005; all lanes are as in (A); blot processing after hybridization was carried out under low-stringency conditions; time of exposition of autoradiography film was 5 h.

et al. (*10*), the identification of a characteristic DNA band of 9.3 kb after cleavage of chromosomal DNA from strains of *C. jejuni* with *Cla*I has not been previously reported.

The second goal of this work was to investigate four strains of *C. jejuni* subsp. *doylei*, with a view to defining, if possible, specific DNA fragments that could help in the differentiation of this taxon with respect to *C. jejuni* subsp. *jejuni*. No specific DNA fragment was observed for *C. jejuni* subsp. *doylei* that could help to distinguish this subspecies from *C. jejuni* subsp. *jejuni* after cleavage with enzyme *Cla*I, *Eco*RV, or *Bgl*II. In contrast, although the analysis of the RFLPs did not afford much valuable information for differentiation purposes, hybridization analyses of the four *C. jejuni* subsp. *doylei* strains revealed that all four strains were much more closely related among themselves than with respect to all of the strains of *C. jejuni* subsp. *jejuni* tested. Moreover, specific hybridization of *C. jejuni* subsp. *doylei* was observed when detection was carried out under high-stringency conditions. The probe, consisting of labeled chromosomal ssDNA from *C. jejuni* subsp. *doylei* strain RSC-34, could be useful in rapid dot-blot identification assays for confirmation of the accurate grouping of the strains of *C. jejuni* into each of the two subspecies included in this species. Upon investigating the genus *Campylobacter* by means of numerical analysis of phenotypic features, On and Holmes (*5*) described a significant phylogenetic distance between *C. jejuni* subsp.

doylei and *C. jejuni* subsp. *jejuni* and reported some difficulties in the elucidation of the phylogenetic position of *C. jejuni* subsp. *doylei* as well as other species included in superfamily VI (*25*), according to their rRNA data. The present work confirms that the genetic relatedness among the four strains of *C. jejuni* subsp. *doylei* described in this work is much higher than that found among that taxon and either *C. jejuni* subsp. *jejuni* biotype 1 or *C. jejuni* subsp. *jejuni* biotype 2, according to DNA/DNA hybridization under high-stringency conditions.

The present study also included the employment of a specific DNA probe, previously isolated by Korolik et al. (*10*, *22*), for speciation purposes. That DNA fragment was useful in the speciation of the strains tested as belonging to either *C. jejuni* or *C. coli*. As described above, this probe hybridized with *Bgl*II-restricted DNA fragments of 2.5 and 2.3 kb only in the case of strains of *C. jejuni*. An approach based on this work, considering a dot-blot DNA/DNA hybridization strategy with blots containing cells of *C. jejuni* and *C. coli*, might be useful for identification purposes.

In sum, analysis of the RFLPs of *C. jejuni* and *C. coli* with different restriction enzymes allowed the identification of specific DNA fragments that may be helpful in the differential characterization of each species. The results suggest the remarkable usefulness of *Bgl*II for achieving this purpose. *C. jejuni* could be easily distinguished from *C. coli* by hybridization, even under low-stringency conditions. Moreover, hybridization studies allowed us to observe a higher genetic homology among the four strains of *C. jejuni* subsp. *doylei* isolated at our laboratory, with respect to the strains of *C. jejuni* subsp. *jejuni* tested. Finally, when used as a DNA probe, the DNA fragment isolated from *C. jejuni* by Korolik et al. (*10*, *22*) proved to be a valuable tool for the purpose of identifying *C. jejuni*.

ABBREVIATIONS USED

kb, kilobases; kDa, kilodaltons; PCR, Polymerase Chain Reaction; RFLP, restriction fragment length polymorphism; SSC, 15 mM sodium citrate + 150 mM sodium chloride, pH 7.0; ssDNA, single-stranded DNA.

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