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Clonality of *Campylobacter sputorum* bv. paraureolyticus determined by macrorestriction profiling and biotyping, and evidence for long-term persistent infection in cattle

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SUMMARY

Eighteen strains of *Campylobacter sputorum* bv. paraureolyticus (isolated over a 12-month period from seven dairy cows contained in a single herd) were examined by resistotyping, and macrorestriction profiling using pulsed field gel electrophoresis (PFGE). The resistotypes of these strains were identical, although repeat testing indicated resistance to metronidazole was not a reliable trait for typing purposes. Five *Sma*I-derived genotypes were identified among the 18 strains. In 5 of 7 cows, isolates obtained from the same animal, but from different time periods, were genotypically indistinguishable, indicating persistence of infection. Macrorestriction profiles of 5 strains representing the 5 *Sma*I genotypes and 8 other strains of *C. sputorum* from various sources, were prepared using 4 endonucleases (*Sma*I, *Sal*I, *Bam*HI and *Kpn*I). The only other strain of *C. sputorum* bv. paraureolyticus examined (a Canadian isolate from human faeces), was found to have a *Sma*I macrorestriction profile identical with one of the five clones isolated from the cattle. Moreover, *Sal*I and *Bam*HI profiles of all bv. paraureolyticus strains were similar, while digestion with *Kpn*I was not observed. By contrast, the seven strains of *C. sputorum* bv. sputorum yielded various macrorestriction profiles with all the enzymes used, and features distinguishing the two biovars studied could be identified. This study indicates that *C. sputorum* can persist in cattle for at least 12 months and exhibits a clonal population genetic structure.

INTRODUCTION

Campylobacters are regarded as the most common bacterial cause of foodborne gastroenteritis worldwide [1]. The species most commonly isolated from such infections are *Campylobacter jejuni* subsp. *jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* [1, 2]. However, many other species of *Campylobacter* (and of the related genera *Arcobacter* and *Helicobacter*) have been associated with human diarrhoea and other diseases [1, 3], and it has been suggested that such taxa may be significantly under-reported as a consequence of deficient isolation [4] and identification [3] procedures. Indeed, a diverse range of campylobacterial species in human diarrhoea, and various samples from poultry and cattle are detected where appropriate isolation and identification methods are used [5–11]. However, epidemiological studies of all but the aforementioned four *Campylobacter* species are rare.

We described previously an unusual catalase-negative, urease-positive *Campylobacter* sp. that was frequently isolated from cattle faeces [9]. This group was subsequently found to represent a novel biovar of *C. sputorum*, for which the name *C. sputorum* bv. paraureolyticus has been proposed [12]; two other *C. sputorum* biovars (bv. fecalis and bv. sputorum) are also extant [12]. Like many *Campylobacter* spp., the

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species is considered to be a commensal of the animals in which it can be found (cattle, sheep and pigs). In humans, it has been isolated from the oral cavity, and has been associated with several diseases including diarrhoea [6, 12–15]. It has been suggested that the zoonotic potential of *C. sputorum* requires re-evaluation [12]. An improved understanding of the epidemiology of *C. sputorum* would therefore be valuable in itself, and possibly contribute towards a better understanding of the epidemiology of other *Campylobacter* spp. in cattle.

One notable feature of campylobacteriosis is the seasonality of infection: that is, outbreaks of human campylobacter infection peak in spring and autumn [16], with a similar trend observed in numbers found in cattle [17]. The reason for this seasonal trend is unclear. Moreover, the potential for survival of a given strain in the host animal (or in its surroundings) is unknown and thus it is not possible to determine if these seasonal peaks, in animals at least, are due to recrudescent infection or infection with a new strain introduced to the herd.

The initial aim of the present investigation was to investigate the seasonality of *C. sputorum* bv. paraureolyticus in a single herd of cattle and to characterize subsequently strains with conventional biotyping and macrorestriction profiling, a high-resolution genotyping method proven to be an effective molecular epidemiological tool for several *Campylobacter* species [18–20]. As a consequence of the results obtained, we broadened our genotyping investigations to include other *C. sputorum* strains from geographically diverse sources. The results are used to analyse the epidemiology, and population genetics, of this species. The implications for macrorestriction profiling as a molecular epidemiological tool for campylobacteria are also discussed.

MATERIALS AND METHODS

Isolation and identification of *C. sputorum* bv. paraureolyticus strains

Strains were isolated from the rectal contents of a single herd of dairy cows using the methods described previously [9]. Ten cows from which *C. sputorum* bv. paraureolyticus was first isolated in the summer of 1995 (see [9]) were re-examined in the winter (if available) and/or in the subsequent summer. Six cows were examined in each of the three distinct seasons; two were retested in the winter period only and the remaining two animals were reexamined solely during the second summer. All campylobacter-like isolates were identified using the scheme described by Bolton and colleagues [21]; *C. sputorum* bv. paraureolyticus strains were defined by reactions in urease, catalase and triple sugar iron agar tests as described previously [12].

Resistotyping

Resistotyping was carried out using the Mast Camp Bioring (MID/CAMP, Mast Laboratories, Bootle, UK) according to the manufacturer's instructions. The resistotyping system consists of paper disks with six arms, with tips impregnated with the following agents: tetracycline (T, 18 μg); nalidixic acid (NA, 30 μg); metronidazole (MZ, 5 μg); 2,3,5, triphenyl tetrazolium chloride (TTC, 1000 μg); 5-fluorouracil (FUR, 80 μg); sodium arsenite (SAR, 30 μg). Strains were grown at 37 °C on 5% sheep blood agar under microaerobic conditions for 48–72 h, and a suspension subsequently made in 0·1% sterile peptone water (Lab M, UK) equivalent to McFarland standard no. 1. A 1·5 ml aliquot of the suspension was dispensed onto the surface of a 90 mm plate containing blood-free charcoal-based campylobacter biotyping agar (Mast, DM606). Excess fluid was carefully removed and the surface of the plates allowed to dry thoroughly. A resistotyping disk was placed centrally on each plate, lightly pressing down the tips to ensure full contact with the agar. The plates were incubated microaerobically at 37 °C and results were read after 1 and 2 days incubation. Zones of inhibition less than 12 mm diameter were classified as resistant and a score value of 1, 2 or 4 was given depending on the zone size, using the manufacturer's guidelines. Zones of inhibition greater than 12 mm diameter were assigned as sensitive and therefore scored as zero in the resistotyping scheme. The scheme provides a two-digit resistotype for each organism, with the sum of the scores for the first three tests representing the first digit and the remaining three the second digit, respectively. Resistotyping was performed on two different occasions, using freshly prepared media and reagents.

Macrorestriction profiling by pulsed-field gel electrophoresis (PFGE)

DNA-containing agar blocks were prepared for *C. sputorum* and *C. hyointestinalis* (used as a standard reference) strains using methods described previously

Table 1. *Variety of* Campylobacter *spp. isolated from a single herd of cattle over a 1-year period*

Cow	Species* isolated in		
	First summer†	Winter†	Second summer†
73	*C.s.*b.p.	*C.j.*	*C.h.*
651	*C.s.*b.p.	*C.s.*b.p.	*C.s.*b.p. and *C.j.*
308	*C.s.*b.p.	*C.s.*b.p. and U.C.	*C.s.*b.p.
86	*C.s.*b.p.	*C.h.*	*C.s.*b.p.
61	*C.s.*b.p.	*C.j.*	*C.s.*b.p. and *C.j.*
31	*C.s.*b.p.	*C.s.*b.p.	*C.s.*b.p.
150	*C.s.*b.p.	n.t.	*C.s.*b.p.
179	*C.s.*b.p., *C.h.* and U.C.	n.t.	*C.s.*b.p. and *C.j.*
90	*C.s.*b.p.	*C.h.*	n.t.
579	*C.s.*b.p.	*C.j.*	n.t.
Total % positive			
*C.s.*b.p.	100	37·5	87·5
All campylobacters	100	100	100

* *C.s.*b.p., *C. sputorum* bv. paraureolyticus; *C.j.*, *C. jejuni* subsp. *jejuni*; *C.h.*, *C. hyointestinalis*; U.C., unidentified campylobacter; n.t., not tested.

† Samples taken in May–Jun 1995; Jan 1996; and Jun 1996.

[18]. Macrorestriction profiling with each of four restriction enzymes (*Sma*I, *Sal*I, *Bam*HI [Amersham Life Sciences Ltd., Birkerød, Denmark] and *Kpn*I [Gibco BRL Ltd., Paisley, UK]) was performed using the methods and electrophoresis conditions described by On and Vandamme [18] (*Sma*I and *Sal*I digests) and On and colleagues [19] (*Bam*HI and *Kpn*I digests).

RESULTS

Isolation and prevalence of *C. sputorum* bv. paraureolyticus from cattle over a 1-year period

The *Campylobacter* species recovered from the 10 cows over the study period are summarized in Table 1. Of the 10 cows initially known to harbour *C. sputorum* bv. paraureolyticus in the summer of 1995, seven proved positive for this taxon when re-examined in the following winter and/or summer (1996) periods. Cows from which *C. sputorum* bv. paraureolyticus was recovered in winter proved positive when re-examined during the summer of 1996. Whilst the winter carriage of *C. sputorum* bv. paraureolyticus was reduced among the cattle examined at this time, the overall percentage of campylobacter carriage was unchanged (Table 1).

Resistotyping of bovine *C. sputorum* bv. paraureolyticus isolates

Eighteen strains were isolated from 7 of the 10 cows within the herd over the 12-month study period (Table 2). All 17 strains gave the same resistotype pattern on each of the two occasions they were tested. However, on the first occasion all strains appeared to be resistotype 20, whereas on the second screening all were resistotype 60. This difference was due to the metronidazole test giving a resistance score on the first occasion and a negative result on the second. No such variability was observed for the other tests, with strains resistant only to nalidixic acid.

Macrorestriction profiling by PFGE

Initial genotyping studies (using endonuclease *Sma*I) were performed on the aforementioned 17 strains (Table 2), among which 5 macrorestriction profiles (MRPs) were observed (Fig. 1). Indistinguishable MRPs were observed for sequential isolates from 4 of the 7 cows examined (31, 651, 86 and 150), whilst the strain obtained in the summer of 1995 differed from the subsequent winter and second summer (1996) isolates from cow 308. Strains isolated approximately 1 year apart from cows 61 and 179 gave distinct MRPs. However, the overall level of genotypic diversity among the herd isolates was limited: all strains shared common bands of ca. 250, 150, 100, 30 and 20 kb, with bands of > 630 and 325 kb exhibited by 15 and 13 strains respectively. Moreover, the MRP of isolate BU 179-C (isolated in June 1996) was indistinguishable from a strain from another animal obtained from this herd one year earlier (BU 73-A, Fig. 1; also see Atabay and colleagues [9]).

Table 2. *Sources of* C. sputorum *strains examined by biotyping and/or macrorestriction profiling*

Biovar designation	Strain number*	Source	Date of isolation
Paraureolyticus	BU31-A	Cow no. 31, UK	May–June 1995
Paraureolyticus	BU31-B	Cow no. 31, UK	January 1996
Paraureolyticus	BU31-C	Cow no. 31, UK	June 1996
Paraureolyticus	BU308-A	Cow no. 308, UK	May–June 1995
Paraureolyticus	BU308-B	Cow no. 308, UK	January 1996
Paraureolyticus	BU308-C	Cow no. 308, UK	June 1996
Paraureolyticus	BU651-A	Cow no. 651, UK	May–June 1995
Paraureolyticus	BU651-B	Cow no. 651, UK	January 1996
Paraureolyticus	BU651-C	Cow no. 651, UK	June 1996
Paraureolyticus	BU61-A	Cow no. 61, UK	May–June 1995
Paraureolyticus	BU61-C	Cow no. 61, UK	June 1996
Paraureolyticus	BU86-A	Cow no. 86, UK	May–June 1995
Paraureolyticus	BU86-B	Cow no. 86, UK	June 1996
Paraureolyticus	BU150-A	Cow no. 150, UK	May–June 1995
Paraureolyticus	BU150-C	Cow no. 150, UK	June 1996
Paraureolyticus	BU179-A	Cow no. 179, UK	May–June 1995
Paraureolyticus	BU179-C	Cow no. 179, UK	June 1996
Paraureolyticus	BU73-A	Cow no. 73, UK	May–June 1995
Paraureolyticus	LMG 11764	Human diarrhoea, Canada	1991
Sputorum	CCUG 9728	Human oral cavity, USA	1982
Sputorum	CCUG 11290	Bull semen, Belgium	1954
Sputorum	CCUG 24261	Sheep foetus, Sweden	1979
Sputorum	LMG 6447	Bull semen, Belgium	1953
Sputorum	LMG 11765	Human, blood, Canada	1990
Sputorum	LMG 14261	Human pus, Spain	1993

* BU, Bristol University (single dairy cow herd); CCUG, Culture Collection of the University of Göteborg, Sweden; LMG, culture collection of the Laboratorie voor Mikrobiologie, Ghent, Belgium.

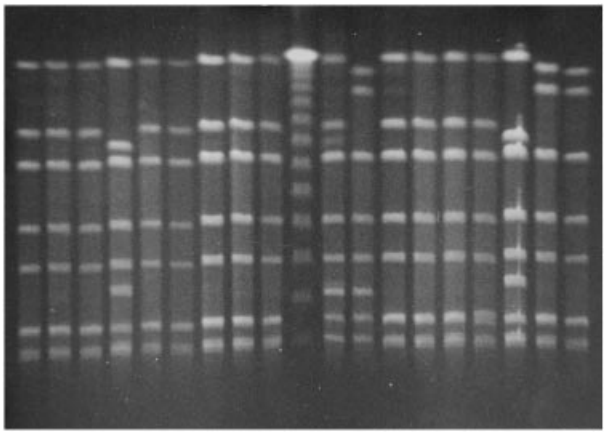

Fig. 1. *Sma*I macrorestriction profiles of 18 *C. sputorum* bv. paraureolyticus strains isolated from a single dairy cow herd over a 12-month period. Lanes: 1, BU 31-A; 2, BU 31-B; 3, BU 31-C; 4, BU 308-A; 5, BU 308-B; 6, BU 308-C; 7, BU 651-A; 8, BU 651-B; 9, BU 651-C; 10, molecular weight standard (λ ladder); 11, BU 61-A; 12, 61-C; 13, BU 86-A; 14, BU 86-C; 15, BU 150-A; 16, BU 150-C; 17, BU 179-A; 18, BU 179-C; 19, BU 73-A. Matching numbers denote that strains were isolated from the same cow, whilst suffixes A, B and C identify the season of isolation (May–June 1995, January 1996 and June 1995 resspectively).

To investigate the genotypic variance of *C. sputorum* in more detail, MRPs derived from each of four endonucleases (*Sma*I, *Sal*I, *Bam*HI and *Kpn*I) were prepared from strains representing each of the five *Sma*I genotypes of *C. sputorum* bv. paraureolyticus and compared with similar MRPs of epidemiologically unrelated type and reference strains of *C. sputorum*, including a human isolate of *C. sputorum* bv. paraureolyticus (LMG 11764). The *Sma*I genotype of LMG 11764 proved indistinguishable from the comparable genotype of strain BU 308-A (Fig. 2*a*), and the common bands noted among *Sma*I-MRPs of *C. sputorum* bv. paraureolyticus (see above) did not appear to be as distinctive or as consistently observed among other *C. sputorum* strains of bovine and human origin (Fig. 2*a*). Similarly, *Bam*HI MRPs of bv. paraureolyticus strains revealed several features characteristic of this taxon, including an absence of bands in the 120–125 kb, and 75–80 kb regions and a dense band at *ca.* 35 kb (Fig. 2*b*). *Sal*I MRPs of *C. sputorum* bv. paraureolyticus comprised a single fragment of *ca.* 450 kb and 1–2 poorly resolved

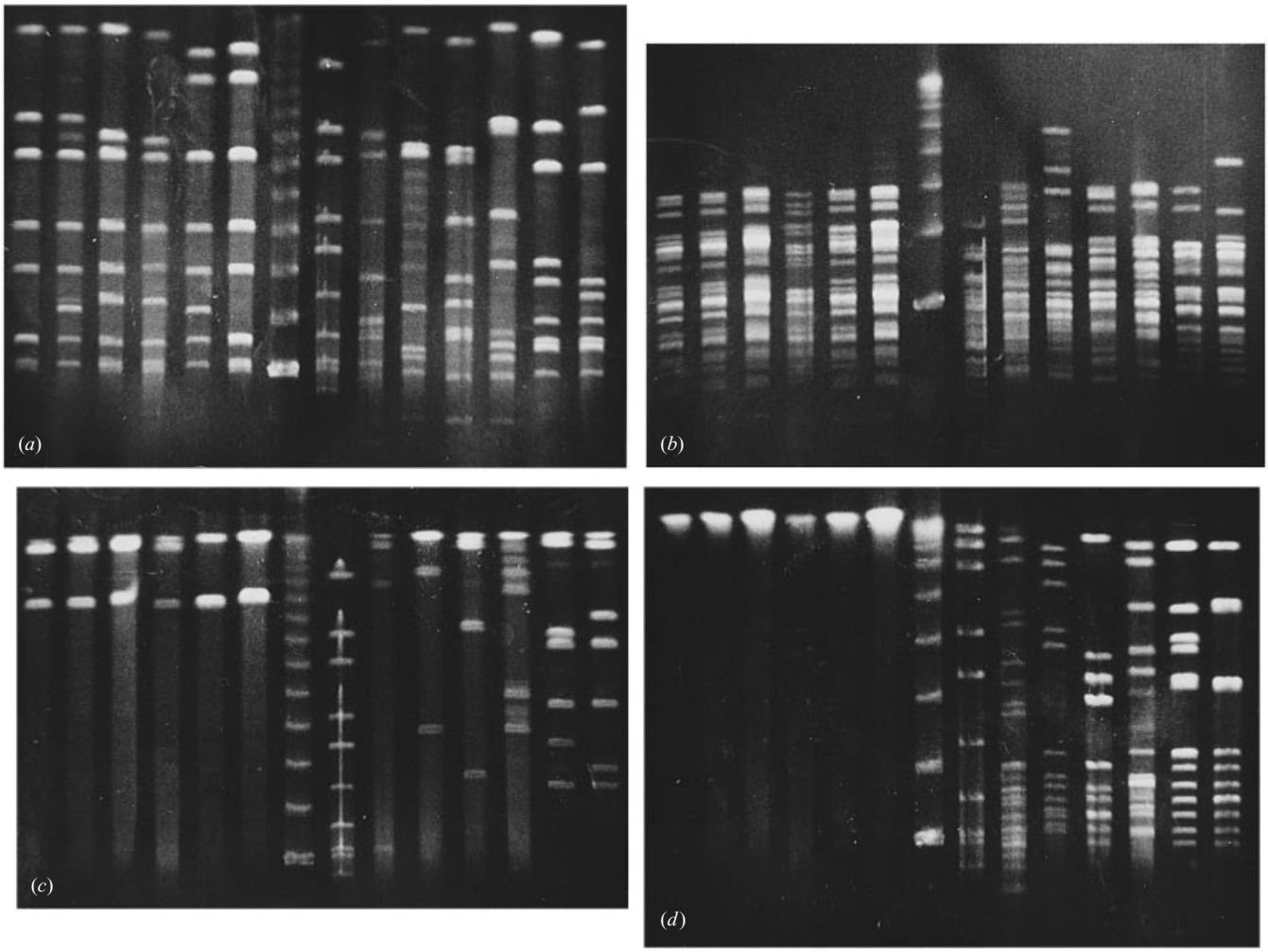

Fig. 2. Macrorestriction profiles of *C. sputorum* bv. paraureolyticus strains (lanes 1–6) compared with those of *C. sputorum* bv. sputorum (lanes 9–14). Polymorphisms are based on digestion with: (*a*), *Sma*I; (*b*), *Bam*HI; (*c*), *Sal*I; and (*d*), *Kpn*I. Lanes (all gels): 1, BU 31-C; 2, BU 61-A; 3, BU 308-A, 4, LMG 11764; 5, BU 61-C, BU 179-C; 7, molecular weight standard (λ ladder); 8, *C. hyointestinalis* subsp. *hyointestinalis* CCUG 14169^{T} (*Sma*I digest used as a reference standard, except on 2B where *Bam*HI is employed for comparative purposes); 9, CCUG 9728^{T}; 10, CCUG 11290; 11, CCUG 24261; 12, LMG 6447; 13, LMG 11765; 14, LMG 14261. ^{T}, type strain.

fragments > 650 kb, in contrast with the *C. sputorum* bv. sputorum strains examined, in which 3–10 bands spanning a comparatively greater molecular weight range were seen (Fig. 2*c*). Digestion of *C. sputorum* bv. paraureolyticus DNA using *Kpn*I was not evident, although *C. sputorum* bv. sputorum isolates were readily differentiated with this enzyme (Fig. 2*d*).

DISCUSSION

Our results strongly suggest that certain strains of *C. sputorum* bv. paraureolyticus can persist in a single cow for at least 1 year. Of the seven cows from which this taxon was reisolated after 1-year period, four yielded isolates from each season showing the same *Sma*I genotype (Fig. 1). Since a previous study indicated the latter genotype to predominate in a representative set of cattle herd isolates [9], we surmise that this genotype is relatively stable, and perhaps well adapted for survival in cattle. In any case, the identification of a single, persistent genotype is convincing for recrudescence, and not cross-infection, to account for some cases of the campylobacter seasonality phenomenon, especially where stable genotypes may be concerned [19].

The limited genotypic diversity of *C. sputorum* bv. paraureolyticus was noted previously, with only three *Sma*I genotypes recognized among 15 strains; fur-

thermore, the pattern of genetic variation suggested a clonal relationship between these genotypes [9]. The results of the present study strongly support this view, but moreover indicate that *C. sputorum* bv. paraureolyticus represents a distinct clonal lineage of the species. All cattle isolates examined were assigned to the same resistotype, despite chronological and genotypic strain differences (Table 2, Fig. 1). However, it was noted that metronidazole resistance was not a wholly reliable trait for typing *C. sputorum* and, since similar findings have been reported for other *Campylobacter* species examined with this system [22], results from this test should be interpreted with caution. Nonetheless, macrorestriction profiling with each of four endonucleases identified many common genotypic characteristics among *C. sputorum* bv. paraureolyticus strains which were either not seen, or highly infrequent, when compared with corresponding analyses of *C. sputorum* bv. sputorum. These distinctions are remarkable, in view of the fact that these biovars are highly related by DNA–DNA hybridization [12] and that strains of both biovars were selected from similar sources. It must also be noted that the human *C. sputorum* bv. paraureolyticus strain examined is chronologically and geographically distinct from the cattle strains (see Table 2), suggesting that our data are not unduly affected by sample bias. Moreover, numerical analysis of both phenotypic tests and whole-cell protein patterns also indicate an especially close relatedness between *C. sputorum* bv. paraureolyticus strains [12].

Our reasoning for a clonal population structure of *C. sputorum* is supported by a number of common features among strains of bv. sputorum, namely an absence of bands in the range *ca.* 85–110 kb in *Bam*HI MRPs (Fig. 2*b*), and the commonality of bands in the regions between *ca.* 300–400 kb and 48·5–97 kb of *Kpn*I MRPs (Fig. 2*d*). These features are absent in bv. paraureolyticus isolates, suggesting that each named biovar of *C. sputorum* may be equivalent to a distinct 'clonal line' of this species. Further research, involving more strains representing the full phenotypic diversity of *C. sputorum* [12] is required to verify this theory. However, it has been suggested that certain phenotypic characters (namely O-antigens) may help to identify strains of *C. jejuni* subsp. *jejuni* that are highly clonally related and, for epidemiological purposes, identical [19]. Thus, given the results of the present study, it is reasonable to postulate that certain biochemical traits expressed by *C. sputorum* may also represent markers of evolutionary descent.

The study of bacterial population genetics is inexorably linked with the subject of molecular epidemiology [23]. It is well known that a range of genetic phenomena can alter the appearance of genotypic profiles [24–25], and several such mechanisms have been described in *Campylobacter* spp. [26, 27]. Multifactoral typing performed by Van der Plas and colleagues [28] led these authors to suggest that extensive genomic rearrangements, and/or genetic exchange in *C. coli* occurred, and that this species at least did not show a clonal population structure. Indeed, natural transformation in the latter species has been suggested as an explanation for some differences in MRPs between outbreak-related and outbreak-associated strains [20]. The importance of the present study in this context is to demonstrate that the population genetics of each *Campylobacter* species should be considered independently, as should criteria for the interpretation of genotypic profiles for epidemiological purposes. Unlike *C. coli*, genotypic diversity in *C. sputorum* does not appear to arise from the uptake of exogenous DNA, since strains could persist in the same host for 12 months, and in the presence of other species (Table 1) without genotypic change. The *Sma*I MRPs of *C. sputorum* bv. paraureolyticus can generally be interpreted using the guidelines described by Tenover and colleagues [25] and differences between strains (using the most common genotype as an index point) explained by between one and three 'genetic events' [25]. However, the presence of an extra band of ca. 98 kb in strain 61 (both first and second summer season isolates) (Fig. 1, lanes 11–12; Fig. 2*a*, lane 5) makes the universal application of the Tenover and colleagues [25] interpretive criteria difficult. Nonetheless, substantial evidence of the clonality of *C. sputorum* is provided by the results of other restriction analyses as well as phenotypic data. The sharing of genotypic and phenotypic properties has long been used as a practical definition of a bacterial clone [29] and in our view remains a logical and valid strategy for interpreting results from genotypic analyses of campylobacters in both population genetic, and epidemiological terms. Indeed, the integration of phenotypic and genotypic data as part of a hierarchical typing system has resulted in the ability to identify clones of *C. jejuni* isolated from diverse sources [19]. The continued practice of such 'polyphasic typing' may therefore prove an invaluable tool for improving our knowledge of the population genetics, and epidemiology, of campylobacters and related taxa.

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