JOURNAL OF BACTERIOLOGY, June 1992, p. 3494–3498
0021-9193/92/113494-05$02.00/0

Vol. 174, No. 11

Physical Map of *Campylobacter jejuni* TGH9011 and Localization of 10 Genetic Markers by Use of Pulsed-Field Gel Electrophoresis

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Received 12 November 1991/Accepted 28 March 1992

The physical map of *Campylobacter jejuni* TGH9011 (ATCC 43430) was constructed by mapping the three restriction enzyme sites *Sac*II (CCGCGG), *Sal*I (GTCGAC), and *Sma*I (CCCGGG) on the genome of *C. jejuni* by using pulsed-field gel electrophoresis and Southern hybridization. A total of 25 restriction enzyme sites were mapped onto the *C. jejuni* chromosome. The size of the genome was reevaluated and was shown to be 1,812.5 kb. Ten *C. jejuni* genetic markers that have been isolated in our laboratory were mapped to specific restriction enzyme fragments. Furthermore, we have accurately mapped one of the three rRNA operons (*rrnA*) and have demonstrated a separation of the 16S and 23S rRNA-encoding sequences in one of the rRNA operons.

In recent years, the use of pulsed-field gel electrophoresis (PFGE) technology has been crucial to the characterization of the genomic structure of organisms such as *Escherichia coli* (24), *Haemophilus influenzae* (16), and *Pseudomonas aeruginosa* (8, 22, 23). In particular, for organisms that have complex growth conditions which present difficulties in isolating mutants (16), or for organisms which do not have well-established gene transfer systems (1), the use of PFGE is especially helpful in the study of their genomic organization and characterization.

Campylobacter jejuni, a gram-negative microaerophilic bacterium, is a major cause of acute enterocolitis in humans (29). Genetic information concerning this organism is limited, and a natural transformation system was reported only recently (30). PFGE is therefore a valuable tool for the analysis and characterization of the chromosome of *C. jejuni*. Chromosomal characterizations with PFGE were recently performed on *C. jejuni* (6, 14, 18), *C. coli* (6, 31), *C. fetus* (6), and *C. lari* (6). In our previous PFGE study (14), we found that the restriction enzymes *Sac*II (5′-CCGCGG), *Sal*I (5′-GTCGAC), and *Sma*I (5′-CCCGGG) cleave the genome of *C. jejuni* into countable fragments which have been useful in the construction of the *C. jejuni* physical map. We report the construction of a physical map of *C. jejuni* TGH9011 with these three restriction enzymes and the mapping of 10 genetic markers on the genome.

MATERIALS AND METHODS

Genomic DNA preparation and restriction enzyme digests. All *C. jejuni* strains used in this study were grown in brucella broth at 37°C under 7% CO_2 for 24 h prior to harvesting. The cells were then centrifuged, washed with 10 mM Tris-Cl (pH 7.5)–1 M NaCl, and incorporated into low-melting-point agarose; then lysis with lysozyme and deproteination by proteinase K were performed by the method of Smith et al. (25).

Before restriction enzyme digestion, the agarose blocks were treated with phenylmethylsulfonyl fluoride and dialyzed with Tris-EDTA (10 mM Tris-Cl [pH 7.5], 1 mM EDTA) buffer. Restriction enzyme digests were typically performed by using a total volume of 100 μl in an appropriate digestion buffer with 30 U of restriction enzyme per μg of DNA. The digestion reactions were then incubated at 37°C (*Sac*II, *Sal*I) or 25°C (*Sma*I) with gentle agitation for 18 h (14).

PFGE. The DNA samples were analyzed by using an LKB 2015 Pulsaphor gel electrophoresis unit and pulse time controller with hexagonal-array electrodes (Pharmacia LKB Biotechnology, Uppsala, Sweden). The samples were loaded onto 1% agarose or 1% low-melting-point agarose and run in 0.5× TBE buffer (14) solution at 10 V/cm. Different pulse times were used for different DNA size ranges as described in our previous study (14). However, to obtain optimal separation of all the restriction enzyme fragments tested, we ramped the pulse time from 5 to 45 s in the course of 18 h (see Fig. 1a). The molecular sizes of each restriction enzyme fragments were analyzed by using lambda DNA concatamers and chromosomes of *Saccharomyces cerevisiae* strains YNN295 (27), AB972 (2), and YPH149 (28).

Southern blot analysis. Southern blot analyses of PFGE samples were carried out by vacuum blotting the gel onto a nylon membrane with a VacuGene apparatus (Pharmacia). The gel was first depurinated in a solution of 0.2 M HCl, denatured in 0.5 M NaOH–0.5 M NaCl, neutralized in 1 M Tris (pH 7.5)–1.5 M NaCl, and finally transferred in the presence of 20× SSC (175.3 g of NaCl per liter, 88.2 g of sodium citrate per liter [pH 7]). Under the hybridization conditions recommended by the manufacturer (Du Pont), the GeneScreen Plus membrane was then incubated with nick-translated radioactive probes.

RESULTS

PFGE of different strains of *C. jejuni*. In recent years, a number of different groups have used PFGE analysis to study the different *Campylobacter* species (6, 14, 18, 31). In our previous study, we tested *C. jejuni* TGH9011, the reference strain for Penner serotype O:3 (ATCC 43430), by using PFGE and found the genome to be about 1,900 kb (14). In this study, we have analyzed the chromosomal DNA of *C. jejuni* 4483 (11) and another *C. jejuni* clinical isolate, strain 7348, in addition to strain TGH9011. Chromosomal DNA preparations from strain TGH9011, 4483, and 7348 were digested with *Sal*I and *Sma*I under different running conditions. Figure 1 shows the PFGE analysis of the three strains under two different running conditions. To obtain the most accurate measurements, we used three different yeast mark-

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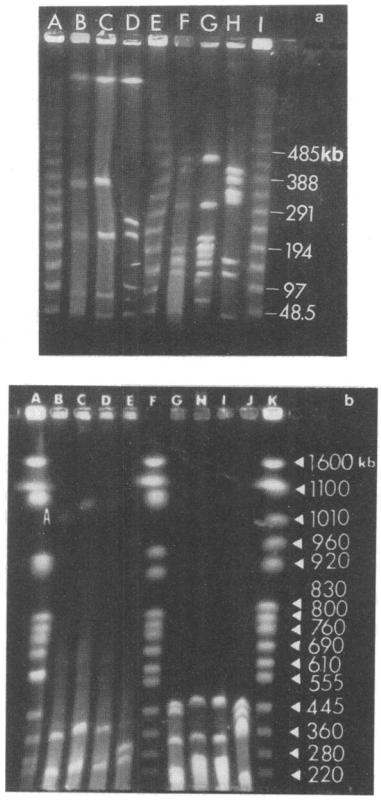

FIG. 1. Chromosomes of three *C. jejuni* strains were analyzed by digestion with *Sal*I and *Sma*I followed by PFGE. (a) The gel was run for 18 h in 1% agarose at a field strength of 10 V/cm with lambda concatamers (increment of 48.5 kb) as size markers. The pulse time was ramped from 5 to 45 s. Lanes: B to D, *Sal*I digests of strains TGH9011, 7348, and 4483, respectively; F to H, *Sma*I digests of strains TGH9011, 7348, and 4483, respectively; A, E, and I, lambda ladders. (b) The gel was run at an 85-s pulse time for 40 h in 1% agarose at a field strength of 10 V/cm. Lanes A, F, and K contain yeast markers AB972, YNN295, and YPH149, respectively; lanes B to E and G to J contain *Sal*I and *Sma*I digests, respectively, of strains TGH9011 (B and G), 7348 (C, D, H, and I), and 4483 (E and J). For convenience, only the molecular sizes of yeast chromosomes of YPH149 and the fragment A of the TGH9011 (lane B) *Sal*I digest are shown.

TABLE 1. Molecular sizes of PFGE fragments from three *C. jejuni* strains

Fragment	Molecular size[a] (kb) of fragments of strain:						
	TGH9011			7348		4483	
	*Sac*II	*Sal*I	*Sma*I	*Sal*I	*Sma*I	*Sal*I	*Sma*I
A	420	1,050	465	1,080	470	1,000	420
B	393	380	420	400	320	280	410
C	210	220	310	230	220	240	370
D	195	71	187	73	190[b]	105	345
E	185	42	160	42	170	68	160
F	165		150		150	42	130
G	120		130		130		46
H	60		46		68		
I	31		1.6[b]				
J	13						
K	6.5						
L	4.3						
M	2.0						
Total	1,804.8	1,763	1,869.6	1,825	1,908	1,735	1,881

[a] The fragment sizes are based on eight independent PFGE runs for strain TGH9011 and three independent PFGE runs for strains 7348 and 4483. The mean and standard deviation (SD) for TGH9011 is 1,812.5 ± 43.9 kb (SD = 2.4%). Means for strains 7348 and 4483 are 1,866.5 and 1,808 kb, respectively. The mean genomic size of TGH9011 was calculated from the total value of the *Sac*II, *Sal*I, and *Sma*I fragments; for strains 7348 and 4483, the calculations were based on the values from the *Sal*I and *Sma*I fragments. Standard deviation = $[\Sigma_i (X_i - \bar{X})^2/N]^{1/2}$, where X_i is genomic size (kb) from determination i and $\bar{X}$ is mean genomic size from N determinations.

[b] Doublet.

ers (2, 27, 28) to measure the largest DNA fragments of each digest; the values are outlined in Table 1.

The three strains show different restriction enzyme patterns with the two restriction enzymes used, but the genomic sizes of all three strains were about 1,800 kb. In addition, we have located an extra 46-kb fragment from the *Sma*I restriction digest of TGH9011, which was not observed in our previous study. This is possibly due to better separation of the restriction enzyme fragments by using running conditions under which the pulse time is increased gradually from 5 to 45 s over the course of 18 h.

Southern hybridization of genetic markers and *Sma*I probes to PFGE fragments. During our genetic characterization of *C. jejuni*, we have isolated and cloned several genetic markers. All the genetic markers used in this study are described in Table 2. Each of the genetic markers was radioactively labeled and subjected to Southern hybridization with PFGE filters containing *C. jejuni* genomic digests with *Sac*II, *Sal*I, and *Sma*I (14). The results of the hybridization are outlined in Table 3. The rRNA probe hybridized to three bands from each of the digests in the PFGE Southern hybridization, whereas all the other genetic markers hybridized with only one band from each digest. Furthermore, the seven largest *Sma*I bands (A to G), were excised from the gel and purified. These DNAs were radioactively labeled and were used as probes for Southern hybridization of the PFGE filters. Figure 2 shows one such Southern hybridization, in which *Sma*I fragment A is hybridizing to *Sac*II fragments A and F and *Sal*I fragment A. The results of these hybridizations are also shown in Table 3.

Construction of the physical map of the *C. jejuni* genome. The basic theory used to construct the physical map of *C. jejuni* with the three restriction enzymes was that if a single DNA probe hybridizes to different fragments from each of the other digests, those hybridizing fragments will be in the same region of the chromosome. For example, the DNA probe used for the *glyA* gene was a 2.3-kb *Hin*dIII fragment from plasmid pCP3b (3), which hybridized to the PFGE *Sal*I B, *Sac*II C, and *Sma*I E fragments (Table 3). This result shows that the three fragments are in the same region of the chromosome. Furthermore, since the 2.3-kb *Hin*dIII fragment of pCP3b does not contain the restriction sites for these three enzymes, the three fragments can be aligned with reference to the *glyA* locus. The same reasoning was used to align additional PFGE fragments that hybridized to the other genetic markers. However, since the genetic markers did not hybridize to all PFGE fragments, the missing regions were filled by observing the hybridization pattern of the labeled *Sma*I fragments to the *Sal*I and *Sac*II fragments (Table 3).

rRNA operon *rrnA* as the reference point in the physical map. Although we can align all the PFGE fragments with respect to each other, a well-characterized reference point on the map would be valuable in orientating the fragments and important in comparing the map with other physical

TABLE 2. *C. jejuni* genetic markers used in this study

Genetic marker	Method of isolation and confirmation	Reference
γ-Glutamyl phosphate reductase (*proA*)	Identified from DNA sequence; confirmed by complementation	16
Argininosuccinate lyase (*argH*)	Identified from DNA sequence; confirmed by complementation and enzyme analysis	9
Flagellin (*fla*)	Hybridization to *C. jejuni* anti-flagellin antibody; confirmed by DNA sequence analysis	12
Serine hydroxymethyltransferase (*glyA*)	Complements *glyA* mutation in *E. coli*	4, 5
Lysyl-tRNA synthetase (*lysS*)	DNA sequence analysis of upstream region of *glyA*	4
16S rRNA probe (1.7-kb *Hin*dIII fragment of pKC28C1)	Hybridization to *C. jejuni* 16S rRNA	9
23S rRNA probe (2.3-kb *Sal*I fragment of pKC28C1)	Hybridization to *C. jejuni* 23S rRNA	9
$tRNA^{Ile}$ and $tRNA^{Ala}$	Sequence analysis of *C. jejuni* rRNA gene intercistronic region	15, 21

maps of *C. jejuni*. We have found that one of the *C. jejuni* rRNA clones that we have tested had several characteristics which are valuable for the construction of the *C. jejuni* physical map. Clone lambda BG51 has a 16-kb *Bgl*II fragment of *C. jejuni* which contains a complete rRNA locus (Fig. 3). This clone not only has an internal *Sal*I site, characteristic of the *C. jejuni* rRNA gene as noted previously (13), but also contains three *Sma*I sites and two *Sac*II sites all congregated in the coding region of the rRNA gene. From the restriction map of BG51, we have learned that the 1.6-kb *Sma*I I fragment is actually a doublet which occurs in tandem inside the structural sequence of the rRNA gene. Furthermore, the two *Sac*II sites within the BG51 specify the 4.3-kb *Sac*II L fragment of *C. jejuni*.

By probing with the 2-kb *Hin*dIII fragment of BG51 located outside the rRNA coding region, we found that the probe hybridizes to the PFGE *Sal*I A, *Sac*II A, and *Sma*I A fragments (Table 3). This means that the internal *Sal*I site of BG51 is the *Sal*I site generating the *Sal*I A and *Sal*I B fragment, the only adjacent *Sal*I fragment which contains the rRNA gene (Fig. 4). We have designated the rRNA gene encoded in clone BG51 as rRNA operon *rrnA* and the internal *Sal*I site of BG51 as point zero in the *C. jejuni* genome. Then all the restriction enzyme fragments starting with the *Sal*I A, *Sac*II A, and *Sma*I A fragments were aligned from point zero.

The resulting physical and genetic map is shown in Fig. 4, which not only shows the order of the restriction enzyme fragments that occurs in *C. jejuni* genome but also gives reliable overlaps of each different restriction fragment. The locations of the genetic markers on the physical map, except for *rrnA*, are mapped anywhere within the length of the smallest restriction enzyme fragments with which they hybridize.

TABLE 3. Hybridization of genetic markers and DNA probes to PFGE fragments

Genetic markers	Positive PFGE fragments		
	*Sal*I	*Sac*II	*Sma*I
γ-Glutamyl phosphate reductase (*proA*)	C	E	C
Argininosuccinate lyase (*argH*)	A	A	A
Flagellin (*fla*)	A	F	B
Serine hydroxymethyl-transferase (*glyA*), lysyl-tRNA synthetase (*lysS*)	B	C	E
16S rRNA probe (1.7-kb *Hin*dIII fragment of pKC28C1)	B	C	E
	D	I	F
	E	G	C
23S rRNA probe (2.3-kb *Sal*I fragment of pKC28C1)	A	L	I1 and I2
	B	D	F
	C	E	C
Lambda BG51 2-kb *Hin*dIII fragment	A	A	A
*Sma*I fragments			
A^b	A^b	A^b, E^b, F	
B	A^b	B, F	
C^{ab}	A^b, C^b, D^a, E^a	E^b, G^a, H	
D	B^{ab}	C^a, D^b	
E^a	B^{ab}, D^a, E^a	C^a	
F^{ab}	B^{ab}, D^a, E^a	D^b, I^a, H	
G	A^b	B	
H	B^{ab}	C^a	

[a,b] DNA fragments containing 16S (*a*) and/or 23S (*b*) rRNA gene (possible cause of cross-hybridization within the same restriction digest).

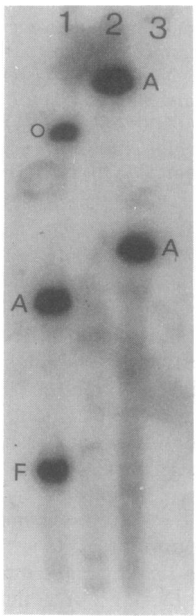

FIG. 2. Southern hybridization of *Sac*II, *Sal*I, and *Sma*I digests of *C. jejuni* TGH9011. The DNA fragments were resolved on PFGE (45-s pulse time at 10 V/cm for 48 h) and probed with the A fragment of the *C. jejuni* TGH9011 *Sma*I digest. *Sac*II, *Sal*I, and *Sma*I digests are loaded on lanes 1, 2, and 3, respectively. The letters correspond to the fragment(s) to which the probe is hybridizing, and the open circle marks the partially digested *Sac*II fragment.

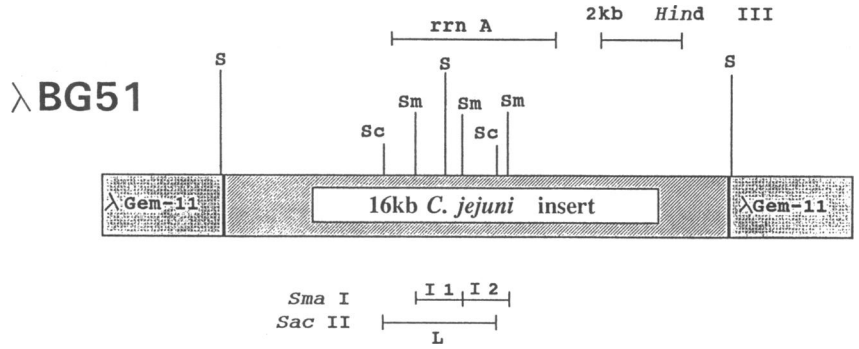

FIG. 3. Restriction enzyme map of the 16-kb *C. jejuni* insert in lambda BG51. *Sal*I (S), *Sac*II (Sc), and *Sma*I (Sm) restriction enzymes sites are shown. Also shown are the *Sma*I I fragment doublet, *Sac*II fragment L, the coding region of the *rrnA* rRNA operon, and the 2-kb *Hin*dIII probe.

As shown in the Table 3, Southern hybridization data with 16S and 23S specific probes indicated a separation of the 16S and 23S rRNA coding regions in the third rRNA operon. The split is approximately 120 kb, the length of *Sac*II fragment G (Fig. 4).

DISCUSSION

This study shows that the genomic size of *C. jejuni* TGH9011 is about 1,812 kb. The previously reported value of 1,900 kb was obtained by using one yeast chromosomal marker (strain nfy 519-16) to calculate the size of the largest fragments from each restriction enzyme digest (14). The value of 1,812 kb obtained in this study is based on three different yeast chromosomal markers. From the PFGE analysis of the *C. jejuni* genome by this study and by other researchers (6, 18), the genomic size of *C. jejuni* seems to range from 1,700 to 1,800 kb. Each strain of *C. jejuni* seems to have its own distinct restriction enzyme pattern with G+C-rich restriction enzymes (6, 14, 18).

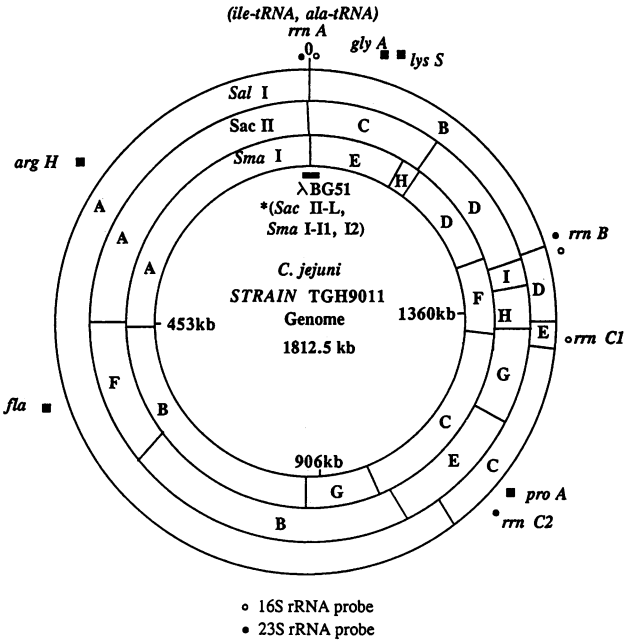

FIG. 4. The physical map of *C. jejuni* TGH9011 with *Sal*I, *Sac*II, and *Sma*I fragments. The 10 *C. jejuni* genetic markers are also shown; except for the *rrnA* and the genes within it, the locations of the genes are based on the set of hybridizing bands (see Table 2). The location of the lambda BG51 clone is marked, and the asterisk marks the location of three small fragments which are contained inside clone BG51.

An interesting characteristic of the *C. jejuni* genomic organization that is evident from our study is the concentration of the G+C-rich restriction enzymes inside the coding region of the structural rRNA gene. Three restriction enzymes, *Sal*I, *Sac*II, and *Sma*I, which occur very rarely in the other regions of the *C. jejuni* genome, are all present in the coding region of the rRNA gene in the lambda BG51 clone (Fig. 3). This observation also seems to be true of the *rrnB* rRNA locus, where the rRNA gene is located in a region of the genome that has all three restriction enzymes *Sac*II, *Sal*I, and *Sma*I (Fig. 4). One explanation for these characteristics is that the structural gene portion of the rRNA genes, which is an essential structural element of the ribosomes (20), is subjected to more sequence conservation than the rest of the *C. jejuni* genome, which has a low G+C content (19). Another explanation might be that the codons which these restriction enzyme sites might specify are rarely used in *C. jejuni*. For example the *Sal*I (GTCGAC) restriction enzyme site, which occurs only five or six times in the *C. jejuni* genome, might specify codons Val (GTC), Asp (GAC), Ser (TCG), or Arg (CGA), all of which are used very rarely in the *fla* (12), *glyA* (5), *lysS* (4), *proA* (17), and *argH* (9) genes of *C. jejuni*. This might explain the possibility that three of the five *Sal*I sites in *C. jejuni* TGH9011 are inside the rRNA structural genes, where the codon restrictions do not apply.

Another interesting observation is the possible separation of 16S and 23S coding regions of the rRNA gene in at least one rRNA operon. Similar results have been observed with another physical map of *C. jejuni* UA580 (26). This observation, although rare, is not unique among eubacteria. Separated 16S and 23S rRNA loci have been observed in *Thermus thermophilus* (10) and *Mycoplasma gallisepticum* (7), where the 16S and 23S rRNA transcripts were transcribed independently from two promoters. We know that at least one rRNA operon has the contiguous 16S-23S structure (*rrnA*). Furthermore, preliminary results suggest that the second rRNA operon (*rrnB*) also has contiguous 16S-23S structure. We are presently trying to isolate rRNA clones consisting solely of 16S or 23S rRNA gene operons, which will establish the presence of independent 16S and 23S rRNA genes.

C. jejuni TGH9011 is the reference strain for Penner

serotype O:3 (ATCC 43430) and is Lior serotype 36 (unpublished data). The physical map of this strain, established in this study, was compared with the map of *C. jejuni* 81116, which belongs to Penner serotype O:6 and Lior serotype 6. The flagellin genes of both strains have been cloned and sequenced and show 78% homology (12). Their genomic sizes differ by about 100 kb. Nevertheless, with these differences, the relative locations of the three rRNA loci and the *fla* genes of these two strains appear to be similar (data not shown). A detailed comparison of the physical and genetic maps of these two strains should be done when more genetic markers are available from strain 81116.

The established physical map of *C. jejuni* TGH9011 with the 10 cloned genes localized should accelerate the genetic studies of this important pathogen. Further addition of cloned genes to the map would enhance the evolutionary studies of *Campylobacter* species.

ACKNOWLEDGMENTS

We thank Don Ye and Stephen Sherer for providing us with the bacterial and yeast strains, respectively.

This work was supported by the Medical Research Council of Canada, the Canadian Foundation for Ileitis and Colitis, and the Ontario Ministry of Health (N. W. Kim is a fellow of the Health Research Personnel Development Program).

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