An improved physical and genetic map of *Campylobacter jejuni* NCTC 11168 (UA580)

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***Campylobacter jejuni* is recognized as the major cause of food-borne gastrointestinal disease in the developed world. To facilitate the molecular genetic analysis of this pathogen, an approximately 18-fold redundant Tropist3 cosmid library was constructed from *C. jejuni* NCTC 11168 genomic DNA. The cosmid library was partially ordered by hybridization to 15 pulsed-field electrophoresis (PFGE) restriction fragments. This analysis confirmed the estimated size of the genome to be 1730 kb, but suggested discrepancies in some regions of the published physical map. The precise locations of two of the three rRNA gene clusters were mapped using a combination of restriction fingerprinting, sample sequencing and riboprobeing. Additionally, 15 further genes were located on the revised map. A more detailed physical and genetic map of *C. jejuni* NCTC 11168 is presented.**

Keywords: *Campylobacter jejuni* NCTC 11168, cosmid library, physical and genetic mapping, PFGE

INTRODUCTION

Campylobacter jejuni is a Gram-negative microaerophilic bacterium, which is a major cause of human enterocolitis world-wide (Ketley, 1997). *Campylobacter* enteritis is characterized by acute abdominal pain and inflammatory diarrhoea. The unpleasant nature of the disease and its increased frequency of reported incidence have resulted in *C. jejuni* being a major health problem which represents a considerable economic burden in industrialized countries. At present, the development of disease prevention and infection control strategies is hindered by a poor understanding of the pathophysiology and genetics of the organism. Methods of genetic analysis that have been used to characterize other enteric pathogens (e.g. transposon mutagenesis and phage transduction) have not been applied successfully to *C. jejuni*. Additionally, little is known about the organization of genes on the chromosome. Physical and genetic maps of bacterial genomes are a useful resource for the genetic characterization of an organism and are of particular interest when the sequence of the entire genome is unavailable. Such maps are also helpful in determining genome plasticity and for the construction of ordered vector libraries.

Variation in genomic maps for different *Campylobacter* species (*C. jejuni* and *C. coli*) as well as for different *C. jejuni* strains have been reported (Taylor *et al.*, 1992). In the related species *Helicobacter pylori*, variability in gene order in different strains is well documented and an important factor in genome diversity (Jiang *et al.*, 1996). Physical and genetic mapping studies of several *C. jejuni* strains, such as UA501 and NCTC 11168 (UA580) (Taylor *et al.*, 1992) and TGH9011 (Kim *et al.*, 1993), have been initiated. However, two different PFGE maps for *C. jejuni* NCTC 11168 have been reported (Taylor *et al.*, 1992; Newnham *et al.*, 1996). This could reflect instability of the genome, or merely an improvement in the interpretation of data. In an attempt to unify genetic and biological research on *C. jejuni*, we have constructed an approximately 18-fold redundant cosmid library which allowed further revision of the published *C. jejuni* NCTC 11168 genome map. The newly constructed map, together with further ordering of the cosmid library, will be a useful resource to aid in sequencing the entire genome and to study the pathophysiology of this important pathogen.

METHODS

Bacterial strains, plasmids and growth. *C. jejuni* NCTC 11168 was grown on blood-free *Campylobacter* selective solid media (Oxoid) in 10% CO₂, 5% O₂ at 37 °C for 3 d.

Abbreviation: DIG, digoxigenin.

Escherichia coli DH5 α MCR used for the library construction was grown at 37 °C in Luria-Bertani broth (LB) or on 1.5% (w/v) LB agar (LA) plates supplemented with 25 μ g kanamycin ml⁻¹. The library was stored at -80 °C in 96-well microtitre plates in LB supplemented with glycerol at 25% (v/v). Colonies for hybridization were transferred from the library onto Hybond-N membranes at a density of 384 per membrane using the Nunc Replication System followed by incubation at 37 °C on top of LA plates with 25 μ g kanamycin ml⁻¹ for 24–48 h.

Construction of cosmid library and DNA manipulation.

Tropist3 cosmid was used for the construction of a genomic library as described previously (De Smet *et al.*, 1993; Henderson *et al.*, 1997). Genomic DNA was partially digested with *Sau*3A, size-fractionated using a 10–40% sucrose gradient, and the 25–50 kb fraction was ligated with *Bam*HI-treated Tropist3. The vector was digested previously with *Sca*I and dephosphorylated. Following ligation the library was packaged into phage lambda particles (Stratagene). After transduction into *E. coli* DH5 α , the cells were plated onto LA plates supplemented with kanamycin. Cosmids were grown in LB containing 50 μ g kanamycin ml⁻¹ in 96-well microtitre plates and cosmid DNA was isolated either from bacteria grown on LA plates (for restriction enzyme fingerprinting) or from 10 ml of overnight liquid cultures (for sequencing) using the QIAprep Spin Miniprep Kit (QIAGEN). Sequencing was performed on an ABI 373 automatic sequencer using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer). The vector-derived primers for cosmid sequencing were: TAATACGACTCACTATAGGG (T7) and GATTTAGGT-GACACTATAG (SP6).

PFGE. DNA-agarose blocks (1% InCert agarose, FMS) were prepared essentially as described by Taylor *et al.* (1992). Bacterial DNA in blocks was digested overnight at 37 °C and 30 °C with *Sal*I or *Sma*I, respectively, using 100 units of enzyme per block in 1 ml enzyme buffer. PFGE in 1% agarose (SeaKem Gold, FMS) was performed using the CHEF DRII system (Bio-Rad) in 0.5 $\times$ TBE buffer, using a switch time of 10–35 s, a voltage of 6 V cm⁻¹ and a run time of 21 h. DNA bands were visualized by ethidium bromide staining, cut and stored at 4 °C.

Hybridization. DNA in blocks after PFGE was fragmented by a mixture of *Sau*3A and *Rsa*I restriction enzymes, extracted using the Prep-A-Gene DNA extraction system (Bio-Rad), and fluorescein-labelled using the Gene Images labelling system (Amersham). This system was also used for labelling of the PCR probes corresponding to 'unique' genes *murD* (CCT-AGTCCAGGTTTTCCACC and GCTTTATGAGTGTAG-TGTAGGG primers), *ungI* (CATCACTCCAGCAAAA-AAGCGG and GGGTAATTTCCCAAAGC primers) and *cdtC* (GCCTTTGCAACTCCTACTGG and CCAAAAGGA-AGTTCATCAGC primers). Labelling efficiency was monitored according to the manufacturer's instructions. PCR probes generated from a 2 kb library using vector-derived primers AK2 (GAGCGGATAACAATTTTCACACAGG) and AK3 (GTAAACGACGCGCCAGTG) were digoxigenin (DIG) labelled according to the procedure described for the DIG High Prime DNA Labelling Kit (Boehringer Mannheim). Cells on the membranes were lysed using alkali and after neutralization the released DNA was immobilized using UV-irradiation. Hybridization for the Gene Images system was performed in Rapid-Hyb buffer (Amersham) at 65 °C overnight, and with DIG-labelled DNA in DIG Easy Hyb buffer at 40 °C overnight. After stringency washes the appropriate detection system (Gene Images or DIG) was used.

Riboprobing. This was performed as described by Bukanov & Berg (1994). Probes specific for the ends of cloned inserts within the cosmids were generated by *in vitro* transcription. Cosmid DNA was digested with either *Hae*III or *Cl*aI for riboprobes from the T7 promoter, or with *Eco*RV from the SP6 promoter. The restriction fragments were purified by phenol/chloroform extraction and ethanol precipitation prior to transcription. The template DNA (1 μ g) was then used in an *in vitro* transcription reaction as recommended by the supplier (Amersham). Template DNA was removed from the fluorescein labelled probe which was then used directly in the library hybridizations. Cosmids hybridizing to the labelled probe were identified using the Gene Images detection system.

Fingerprinting. Cosmid DNA for restriction analysis was isolated from bacteria grown on agar plates. The choice of *Hind*III was based on analysis of limited sequencing information of *C. jejuni* available in EMBL and GenBank. *Hind*III recognition sites were found at a mean frequency of one per 5 kb, which should generate the optimal number of 5–10 fragments on the recombinant cosmids. Cosmids originated from the same hybridization group and sharing several fragments with a total size of at least 5 kb were considered to have overlapping inserts. This was further confirmed by riboprobing analysis.

RESULTS AND DISCUSSION

Cosmid library construction

Initially 1152 colonies were inoculated in LB in 96-well microtitre plates and regrown on three quadra-density filters for hybridization studies. In subsequent experiments 800 cosmids were found to be sufficiently viable for transfer onto nylon filters, resulting in an approximately 18-fold redundant library. *fur*, *flaAB* and *cheY* probes used in the initial hybridization experiment produced on average 15–18 positive colonies, indicating that the library is representative. The insert size was found to be in the range between 5 and 40 kb. The smaller size in some cases was shown to be a result of instability, whereas many cosmids with medium and large inserts appeared to be stable.

Hybridization

A detailed characterization of the cosmid library was performed using hybridization to isolated PFGE fragments. A total of 15 fragments (9 *Sma*I and 6 *Sal*I) was used. As with other bacterial genomes, the *C. jejuni* chromosome may contain repeated sequences (for example IS elements and rRNA genes) which may result in some ambiguities in the interpretation of cosmid clone hybridization. In most cases, however, we found this background to be negligible, probably because the sizes of possible repeats are usually too small compared to the size of a PFGE fragment. Moreover, we only considered the cosmids giving a strong hybridization signal. Some biased distribution of the cloned fragments towards certain regions was detected. For example, fragments that originated from the *Sal*I-6 and *Sma*I-6 fragments (corresponding to the same region on the physical map) were overrepresented. In contrast, no cosmids hybri-

Table 1. Two sets of cosmids hybridizing to the *Sma*I-9 fragment

Cosmid	Hybridization to PFGE fragment:						
	<i>Sall</i> -2	<i>Sall</i> -3	<i>Sall</i> -4	<i>Sma</i> I-3	<i>Sma</i> I-4	<i>Sma</i> I-5	<i>Sma</i> I-9
7G3	+	—	—	—	—	—	+
10B7	+	—	—	—	—	—	+
5E8	+	—	—	—	+	—	+
12F4	+	—	—	—	+	—	+
5B9	+	+	—	—	—	—	+
6G2	+	+	—	+	—	—	+
5H2	—	—	+	—	—	+	+
2A8	—	—	+	—	—	+	+

dized to a *Sma*I-8 fragment. Interestingly, the *Sma*I-1 region linked to *Sma*I-8 was also underrepresented. This could reflect relative instability of some regions of the chromosome cloned in cosmid vectors that may result in gaps during ordering of the cosmid library.

The hybridization results generally agreed with the latest physical map published (Newnham *et al.*, 1996). For example, the cosmids that hybridized to the *Sall*-1 fragment also hybridized to the *Sma*I-1, *Sma*I-7 and *Sma*I-2 fragments, those that hybridized to *Sma*I-4 also hybridized to *Sall*-2, and almost all the cosmids that hybridized to *Sall*-3 also hybridized to *Sma*I-3. Similar overlaps were also found for *Sall*-1/*Sma*I-6, *Sall*-6/*Sma*I-6, *Sall*-5/*Sma*I-6, *Sall*-5/*Sma*I-3 and *Sall*-4/*Sma*I-5 fragments. However, results of hybridization with the *Sma*I-9 fragment contradicted the published map of *C. jejuni* NCTC 11168 (Newnham *et al.*, 1996). This is likely to be the result of ambiguities during construction of the physical map based on partial digestion data (Taylor *et al.*, 1992; Newnham *et al.*, 1996). As several genetic markers are also associated with this fragment, we decided to verify its location by ordering cosmids in this area using hybridization and restriction fingerprinting analyses.

Characterization of the *Sma*I-9 region of the chromosome

Two major sets of cosmids hybridizing to the *Sma*I-9 fragment were found (Table 1). Cosmids from one set hybridized to the *Sall*-2 fragment, whereas cosmids from the other set hybridized to the *Sall*-4 fragment. One explanation for this observation could be that two *Sma*I fragments of a similar size originate from different regions of the genome. However, the intensity of the 39 kb fragment on the PFGE gel corresponds to just one copy of the fragment. Another explanation for this ambiguity is that the *Sma*I-9 region contains highly conserved repeated DNA sequences. According to the most recently published map of the *C. jejuni* genome, the *Sma*I-9 fragment contains an *rrl* gene (Newnham *et al.*, 1996). As three copies of the gene have been found in

different locations on the genome, this would result in hybridization of *Sma*I-9 to cosmids from other areas containing rRNA genes. Therefore, the ends of the cosmids that hybridized to *Sma*I-9 were sequenced. Sequences of three 'unique' genes, *murD*, *ung1* and *cdtC*, identified on these cosmids were used to generate PCR probes. The results showed no cross-hybridization with cosmids originated from other regions of the genome. For example, the *murD* and *cdtC* probes gave only one positive cosmid each from the *Sma*I-5/*Sall*-4 and *Sma*I-4/*Sall*-2 regions, respectively, as expected. The *ung1* probe gave five positives, all from the *Sma*I-4/*Sall*-2 region from where it originated. These results confirm that the repeated sequences, rather than a duplicated fragment, caused the discrepancy. This was further verified by sequencing and restriction analysis (see below).

The overlapping rRNA genes in the two sets of cosmids were used as reference points for ordering. The rRNA genes were identified at the ends of some cosmids (*rrs* at the T7 end of 6G2, *rrl* at the SP6 end of 5B9 and *rrl* at the T7 end of 5C5) by direct sequencing. The overlapping cosmids (2A8, 5H2, 6G2 and 5B9) also contained one or two copies of the identical 1.7 kb *Sma*I fragments. Two copies of 1.7 kb *Sma*I fragments are known to be present in the rRNA gene cluster of *C. jejuni* (z29326.em_ba). The presence of a *Sall* site inside the operon also allows determination of the orientation of the rRNA genes, since inserts in all four plasmids contained this site. This analysis, along with *Hind*III restriction fingerprinting, resulted in the map shown in Fig. 1.

Riboprobng

The derived map was further confirmed in riboprobng experiments using riboprobes generated from ends of the cosmids 5H2, 6G2 and 2D9. The T7 probe generated from 6G2 hybridized to three cosmids from the *Sall*-3/*Sma*I-3 area according to the location of this area shown on Fig. 1, and also to 5H2 and 2A8 cosmids from another area due to repeated sequences of the *rrs* gene. T7 transcripts of 2D9 only gave positive signals with

Fig. 1. (a) An approximately 400 kb fragment of the physical map of the *C. jejuni* NCTC 11168 genome. (b) Fine mapping of the ~60 kb and ~45 kb regions using hybridization, fingerprinting analysis and sequencing of selected cosmids. The inserts that hybridized to the *Sma*I-9 fragment are indicated beneath the genetic map.

Fig. 2. Physical and genetic map of the *C. jejuni* NCTC 11168 genome. New genes and the genes with modified locations (*flaAB* and two *rrs* genes) are indicated in bold and the positions of the other genes corresponding to the previously published map are shown (Taylor *et al.*, 1996). The number of cosmids that hybridized to overlaps of *Sal*I and *Sma*I fragments is shown in the outermost concentric ring. Some of the genes are described in Table 2. The others are: *flaAB*, which encodes A and B flagellar subunits (Z29327.em.ba); *cheY*, which encodes a chemotaxis protein (U62036.em-ba); and *fur*, which is responsible for production of a ferric uptake regulation protein (X78965.em-ba).

cosmids from the *Sal*I-2/*Sma*I-4 area as expected. The T7 probe of 5H2 gave five positive cosmids, all from the *Sma*I-5/*Sal*I-4 area, also in full agreement with the map

shown. On the other hand, the SP6 probe of 5H2 in addition to *Sma*I-5/*Sal*I-4 cosmids also hybridized to 5B9 and several cosmids from the *Sal*I-3/*Sma*I-3 area,

Table 2. Gene orthologues found after sequencing of selected cosmids from the library

No.	Name	Hybridization group	Cosmid size (kb)	Length (bp)	Gene	EMBL/SP Accession number	Gene product	GC content (mol %)
1	8F6-T7	<i>Sall</i> -3/ <i>Sma</i> I-3	16.2	393	<i>ilvC</i> (<i>Bacillus subtilis</i>)	P37253.sp	Ketol-acid reductoisomerase	40
2	1F3-T7	<i>Sall</i> -3/ <i>Sma</i> I-3	13.2	381	<i>fus</i> (<i>Thermus thermophilus</i>)	P13551.sp	Elongation factor G (EF-G)	40
3	1F5-SP6	<i>Sall</i> -3/ <i>Sma</i> I-3	30.7	327	<i>rpoC</i> (<i>E. coli</i>)	P00579.sp	RNA polymerase	43
4	6G2-T7	<i>Sma</i> I-9/ <i>Sall</i> -3/ <i>Sall</i> -2	18.8	438	<i>rrs</i> (<i>C. jejuni</i>)	L14630.em_ba	16S rRNA	51
5	6G2-SP6	<i>Sma</i> I-9/ <i>Sall</i> -3/ <i>Sall</i> -2	18.8	363	<i>hsp90</i> (<i>Borrelia burgdorferi</i>)	P42555.sp	Heat-shock protein	33
6	5H2-SP6	<i>Sma</i> I-9/ <i>Sma</i> I-5/ <i>Sall</i> -4	22.9	372	<i>murD</i> (<i>Bacillus subtilis</i>)	Q03522.sp	UDP-NAc-Mur-Ala- Glu synthetase	44
7	2D9-T7	<i>Sma</i> I-9/ <i>Sall</i> -2/ <i>Sma</i> I-4	14.7	412	<i>frdD</i> (<i>Methanococcus jannaschii</i>)	U67466.em_ba	Fumarate reductase	30
8	2D9-SP6	<i>Sma</i> I-9/ <i>Sall</i> -2/ <i>Sma</i> I-4	14.7	472	<i>lldP</i> (<i>E. coli</i>)	P33231.sp	L-Lactate permease	37
					Hypothetical (<i>Methanococcus jannaschii</i>)	Q57557.sp	Unknown	
						Q60301.sptrem		
9	5E8-T7	<i>Sma</i> I-9/ <i>Sall</i> -2/ <i>Sma</i> I-4	25.7	379	<i>cdtC</i> (<i>C. jejuni</i>)	U51121.em_ba	Cytolethal distending toxin	31
10	12F4-T7	<i>Sma</i> I-9/ <i>Sma</i> I-4	32.3	389	<i>ungI</i> (<i>Pseudomonas aeruginosa</i>)	P29950.sp	Uracil-DNA glycosylase	38
11	5B9-T7	<i>Sma</i> I-9/ <i>Sall</i> -3/ <i>Sall</i> -2	16.2	457	<i>flhF</i> (<i>Borrelia burgdorferi</i>)	L75945.em_ba	Inner membrane protein	30
12	5B9-SP6	<i>Sma</i> I-9/ <i>Sall</i> -3/ <i>Sall</i> -2	16.2	400	<i>rrl</i> (<i>C. jejuni</i>)	Z29326.em_ba	23S rRNA	45
13	5C5-T7	<i>Sall</i> -4/ <i>Sma</i> I-5	14.5	324	<i>rrl</i> (<i>C. jejuni</i>)	U09611.em_ba	23S rRNA	46
14	11E10	<i>Sall</i> -4/ <i>Sma</i> I-5	19.0	375	<i>trpA</i>	P50908.sp	Alpha chain of tryptophan synthase	35

presumably due to the overlap between the two *rrl* genes (Fig. 1).

Use of the library for genetic mapping

To demonstrate applicability of the library for rapid genetic mapping we chose three recently discovered genes possibly related to virulence, *capM*, *neuB* and *fliN*, which were identified during sample sequencing of a random 2 kb pUC18 library (Karlyshev *et al.*, 1997). The *capM* gene orthologue of *Staphylococcus aureus* was shown to be involved in the biosynthesis of capsule polysaccharide (Lin *et al.*, 1994). Although no capsule has been reported for *C. jejuni* *in vitro*, it may be expressed *in vivo*. Alternatively, the *capM* orthologue may be involved in the biosynthesis of other polysaccharide complexes such as LPS. A putative *neuB* gene product is involved in sialic acid synthesis in *E. coli* (Annunziato *et al.*, 1995) and may be important in LPS and flagella biosynthesis in *C. jejuni*, as both of these determinants are sialylated (Guerry *et al.*, 1996; Moran *et al.*, 1996). *FliN* is a component of the flagellar motor switch of *Salmonella typhimurium* and plays an important role in bacterial chemotaxis (Kihara *et al.*, 1989).

The PCR products were DIG labelled and hybridized to the library as described in Methods. Seven cosmids that hybridized to the *capM* probe were found to belong to

the *Sall*-1/*Sma*I-1 hybridization group, eight cosmids from the *Sall*-1/*Sma*I-2 region hybridized to the *neuB* probe, whereas six positive colonies from the *Sall*-4/*Sma*I-5 overlap hybridized to the *fliN* probe. The results (Fig. 2) were verified by PCR on the extracted cosmid DNAs using gene-specific primers.

Other genetic markers were localized by shotgun sequencing of cosmid DNAs using vector-derived primers T7 and SP6. Some of the genes found are presented in Table 2. As shown in Fig. 2, both *rrs* and *rrl* genes were found in the same *Sma*I-9/*Sma*I-3 area, whereas only *rrl* was shown previously in this region (Newnham *et al.*, 1996). A locus involved in peptidoglycan biosynthesis (*murD*) and some potential virulence genes (*flhF*, *cdtC* and *hsp90*) were also identified.

The difference between the previously derived physical map of *C. jejuni* (Newnham *et al.*, 1996) and the one reported here is a clockwise movement of fragments *Sall*-2 and *Sall*-4 by ~ 60 kb. This results in clustering of some *Sma*I, *Sall* and *I-CeuI* sites (which specifically cuts *rrl* genes) in the regions containing rRNA genes. As a result, the 60 kb gap between *rrs* and *rrl* in the *Sma*I-5/*Sall*-4 region has been eliminated. It remains to be determined if the *rrl* and *rrs* rRNA genes in the *Sma*I-6/*Sall*-6/*Sma*I-5 area are interrupted by *gyrB*, as was reported previously. The revised map also shows that since there is no 60 kb gap between the *Sma*I-9/*Sma*I-3 and *Sall*-2/*Sall*-3 junctions, the *rpoB* and *rpoD* genes

should be placed in the *Sma*I-3/*Sal*I-3 overlap. Interestingly, we found another *rpo* gene (*rpoC*) in the same area.

Concluding remarks

An approximately 18-fold redundant cosmid library of *C. jejuni* NCTC 11168 which covered 98% of the genome was constructed and partially ordered. Fine physical mapping and ordering of the cosmids along with sequence analysis allowed correction of the published physical map. The library was shown to be useful for rapid mapping of the genes with known sequences. Some known and previously unknown genetic markers, including those encoding potential virulence factors, were mapped. The data should facilitate analysis of the diversity of *C. jejuni* genome organization and of genes that contribute to disease.

ACKNOWLEDGEMENTS

We thank Victoria Wilson for excellent technical assistance in the cosmid library construction. The work was supported by the BBSRC UK.

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Received 16 July 1997; revised 29 September 1997; accepted 2 October 1997.