Cloning, mutation and distribution of a putative lipopolysaccharide biosynthesis locus in *Campylobacter jejuni*

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A region encoding ORFs with homology to known lipopolysaccharide (LPS) biosynthesis genes was isolated from two strains of *Campylobacter jejuni*. One of the strains produces LPS, but the second strain is reported to produce only lipooligosaccharide (LOS) and therefore lacks the O-chain. The two strains shared six predicted ORFs, but an additional ORF, *orfE*, of unknown function was identified in the LOS-producing strain. Mutation of the shared *wbeE* (*rfbE*) homologue (*orfF*) or deletion of five of the seven genes reduced core reactivity with specific antiserum without affecting O-chain production. Mutation of either the *capD* homologue (*orfG*) or the unique *orfE* had no detectable effect on LOS or LPS production. The presence or absence of *orfE* in 36 isolates of *C. jejuni* did not correlate with LOS/LPS phenotype or serotype. However, after insertion of *orfE* into a LPS-producing *orfE*-negative strain the O-chain ladder was no longer detectable on Western blots. We were not able to disrupt the *wbaP* (*rfbP*) homologue (*orfC*) in *C. jejuni*.

Keywords: *Campylobacter*, lipopolysaccharide, genetics, distribution, mutation

INTRODUCTION

Campylobacter jejuni is the most common cause of foodborne gastroenteritis in the UK (Ketley, 1997); there is also evidence for its involvement in the aetiology of a significant proportion of cases of the neurological disorder Guillain-Barré Syndrome (GBS) (Schwerer *et al.*, 1995; Salloway *et al.*, 1996; Mishu & Blaser, 1993; Moran *et al.*, 1996). Lipopolysaccharide (LPS) and lipooligosaccharide (LOS) constituents of the outer membrane of Gram-negative organisms have been shown to be important virulence factors during bacterial infection conferring, amongst other things, serum resistance, antibiotic resistance and endotoxic properties on the bacterium (Rietschel *et al.*, 1994). The Penner

serotyping system (Preston & Penner, 1987; Penner *et al.*, 1983) of *C. jejuni* is based upon heat-stable antigens, presumed to be LPS/LOS. *C. jejuni* LPS/LOS are also of interest in terms of their potential adherent properties (McSweeney & Walker, 1986) and involvement in the aetiology of GBS (Schwerer *et al.*, 1995; Salloway *et al.*, 1996; Mishu & Blaser, 1993; Moran *et al.*, 1996).

LPS is composed of three regions, lipid A, a core oligosaccharide and an O-chain consisting of repeating oligosaccharide units; LOS lacks the O-chain (Rietschel *et al.*, 1994). *C. jejuni* is capable of producing LOS and LPS with remarkably variable and unusual core sugars (Moran, 1995) and several of these structures have been elucidated (Aspinall *et al.*, 1992, 1993a, b, 1994, 1995; Moran *et al.*, 1991a, b; Moran & O'Malley, 1995; Salloway *et al.*, 1996). In this paper we report the isolation and analysis of part of a putative chromosomal LPS biosynthesis locus from two strains of differing serotypes of *C. jejuni*. In addition, we report the distribution and gene content of the locus in 36 strains of *C. jejuni* from a range of different serotypes.

METHODS

Bacterial strains. The *C. jejuni* strains used to isolate the putative LPS biosynthesis locus were NCTC 11168 and 11351;

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Abbreviations: CAT, chloramphenicol acetyl transferase; GBS, Guillain-Barré Syndrome; LPS, lipopolysaccharide; LOS, lipooligosaccharide.

The GenBank/EMBL/DBJ accession numbers for the *C. jejuni* NCTC 11168 and *C. jejuni* NCTC 11351 clones reported in this paper are AF001497 and AF001498, respectively.

Fig. 1. Chemical structures of the LOS of *C. jejuni* O:2 (NCTC 11168) (Aspinall *et al.*, 1993a) and LPS of *C. jejuni* O:23 (NCTC 11351) (Aspinall *et al.*, 1992, 1993b). Boxes denote regions of ganglioside mimicry. KDO, ketodeoxyoctulosonic acid; L- α -D-Hepp, L-glycero-D-manno-heptose; Neu5Ac, N-acetylneuraminic acid; Glc, glucose; Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; pEtn, phosphorylethanolamine.

the former produces LOS and is from serotype group 2, the latter strain is the type strain of *C. jejuni*, produces LPS and is from serotype group 23. The LPS/LOS from both these serotypes has been characterized chemically (Fig. 1) (Aspinall *et al.*, 1993a, b, 1992). Genetic manipulations were performed using *C. jejuni* NCTC 11168 and NCTC 11828, the latter is an LPS producer of serotype 6 and was used due to the non-transformability of NCTC 11351. Strains for genetic-distribution studies were obtained from various sources and are detailed in Table 1. All strains were confirmed as *C. jejuni* using the PCR identification system described by Gonzalez *et al.* (1997). All *Campylobacter* strains used in this study were cultured on *Campylobacter* serum-free agar (Oxoid) or in Mueller-Hinton broth at 37 °C in microaerophilic conditions (85% N₂, 10% CO₂, 5% O₂).

DNA extraction. DNA was extracted using either the method of Pitcher *et al.* (1989) or an adaptation of the method of Murray & Thompson (1980). Briefly, for the second method, three lawn plates of *Campylobacter* were grown overnight, harvested into 9.5 ml of phosphate-buffered saline and lysed with 2 ml 10% (w/v) SDS. Proteinase K was added to a final concentration of 0.2 mg ml⁻¹ and the mixture incubated at 37 °C for 1 h. Then 1.8 ml 5 M sodium chloride and 1.5 ml 10% (w/v) CTAB (N-cetyl-N,N,N-trimethylammonium bromide) in 0.7 M sodium chloride were added and the mixture incubated for 20 min at 65 °C. An equal volume of 24:1 chloroform/isoamyl alcohol was added and the mixture centrifuged for 20 min at 4 °C. The aqueous layer was removed and the DNA precipitated with 0.7 vols 2-propanol.

Sequence determination. The DNA sequence was determined from a series of nested deletions using the M13 primers (Table 2) in a PCR-based reaction using dye-labelled dideoxy terminators (Perkin-Elmer) and analysed using an ABI 377 sequencer (Perkin-Elmer). Where possible, the new bacterial

polysaccharide gene nomenclature system of Reeves *et al.* (1996) was employed; old names are in parentheses.

PCR primers and conditions. PCR reactions were carried out in a final volume of 10 μ l and consisted of 1 ng template DNA, 2 pmol each primer (Table 2), 0.2 mM dNTPs, 0.5 U *Taq* polymerase using a buffer consisting of 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂. Amplification consisted of 26 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 1 min and extension at 72 °C for 7 min.

Mutation of *C. jejuni*. *C. jejuni* chromosomal mutants can be made by transformation with a mutant construct of the gene of interest in the cloning vector pUC19. pUC19 acts as a suicide vector in *C. jejuni* and can be introduced into the bacterium by electroporation. Subsequent homologous recombination and gene replacement results in the insertion of the mutant gene into the *Campylobacter* chromosome with the loss of the vector.

Transformation of *C. jejuni* was performed using a modification of the method of Wassenaar *et al.* (1993) as follows. Four overnight lawn plates of *C. jejuni* were harvested into 20 ml WE buffer (272 mM sucrose, 15%, v/v, glycerol). The cells were washed twice in WE and resuspended in a final volume of 1 ml WE. Approximately 4 μ g plasmid DNA and 50 μ l electrocompetent *C. jejuni* cells were placed on ice for 10 min, then electroporated using 2.5 kV, 200 Ω and 25 μ F. The bacteria were recovered overnight without antibiotic selection, transferred to appropriate antibiotic-containing plates (50 μ g kanamycin ml⁻¹ or 20 μ g chloramphenicol ml⁻¹) to select for transformants and incubated for 2–5 d at 37 °C.

LPS analysis. Late-exponential phase bacterial cells (1.0 ml; OD₆₀₀ 0.6) were pelleted by centrifugation (3200 g, 20 min, 4 °C). Pellets were resuspended in 200 μ l SDS-PAGE loading buffer (Sambrook *et al.*, 1989) and boiled for 10 min.

Table 1. Characteristics of strains used in genetic distribution studies

ND, Not done, NK, not known, UT, untypeable.

<i>C. jejuni</i> strain	O serotype	Phenotype*	<i>orfE</i>	Source/reference†
NCTC 11168	2	LOS	+	R. Owen ¹
1915/91	2	LOS	+	D. Wareing ²
2561/90	2	LOS	+	D. Wareing
2608/90	4	LOS	+	D. Wareing
27F 155	ND	NK	+	A. Swan ³
18F 5	11	NK	+	A. Swan
2F 8	5	NK	+	A. Swan
4F 182	ND	NK	+	A. Swan
2F 68	50	NK	+	A. Swan
N82	UT	NK	+	Everest <i>et al.</i> (1992)
K85	2	LOS	+	Everest <i>et al.</i> (1992)
G1‡	1	LOS	+	N. Gregson ⁴
G2‡	UT	NK	+	N. Gregson
G3‡	1	LOS	+	N. Gregson
G4‡	UT	NK	+	N. Gregson
F3§	UT	NK	+	N. Gregson
P19	19	LPS	+	N. Gregson
NCTC 11351	23	LPS	–	R. Owen
NCTC 11828	6	LPS	–	T. Wassenaar ⁵
2523/90	2	LOS	–	D. Wareing
306/90	1	LOS	–	D. Wareing
2258/90	23	LPS	–	D. Wareing
21F 185	15	NK	–	A. Swan
21F 242	ND	NK	–	A. Swan
2F 90	UT	NK	–	A. Swan
4F 225	UT	NK	–	A. Swan
8F 169	5	NK	–	A. Swan
B404	ND	NK	–	Everest <i>et al.</i> (1992)
J75	28	NK	–	Everest <i>et al.</i> (1992)
O73	11	NK	–	Everest <i>et al.</i> (1992)
E206	UT	NK	–	Everest <i>et al.</i> (1992)
81-176	23/36	LPS	–	Russell <i>et al.</i> (1989)
16971.94GSH‡	41	LOS	–	A. Lastovica ⁶ , and Prendergast <i>et al.</i> (1998)
28134.94GSH‡	41	LOS	–	A. Lastovica, and Prendergast <i>et al.</i> (1998)
260.94RXH‡	41	LOS	–	A. Lastovica, and Prendergast <i>et al.</i> (1998)
176.83	41	LOS	–	A. Lastovica, and Prendergast <i>et al.</i> (1998)

*Phenotype is predicted from that reported for each O serotype.

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‡ indicates a strain from a GBS patient.

§ indicates strain from Miller-Fisher patient.

Proteinase K was added to a final concentration of 0.5 mg ml⁻¹ and the samples incubated for 2 h at 65 °C.

Tricine SDS-PAGE was performed according to the method of Sprott *et al.* (1994) with the following alterations. Final concentrations of 15 or 18% (w/v) acrylamide, 0.4% (w/v) ammonium persulphate and 0.04% (v/v) TEMED (*N,N,N',N'*-tetramethylethylenediamine) were used for the running gel; 0.5% (w/v) ammonium persulphate and 0.2% (v/v)

TEMED were used for the stacking gel. Proteinase K-treated samples were boiled for 5 min, 10 µl loaded per well of a 16 cm gel and electrophoresed at 15 mA per gel for 16 h. Gels were either silver stained (Tsai & Frasch, 1982) or gel to nitrocellulose transfers were made. Primary antibody was used at a dilution of 1/2500 for O:6 antiserum or 1/5000 for O:2 antiserum and detected using the Bio-Rad Immun-Star Western blotting kit.

Table 2. PCR primers used in this study

Primer	Sequence (5'–3')	Target	Reference
JEJ1	CCTGCTACGGTGAAAGTTTTGC	<i>ceuE</i>	Gonzalez <i>et al.</i> (1997)
JEJ2	GATCTTTTTGTTTTGTGCTGC	<i>ceuE</i>	Gonzalez <i>et al.</i> (1997)
COL1	ATGAAAAAATATTTAGTTTTTGCA	<i>ceuE</i>	Gonzalez <i>et al.</i> (1997)
COL2	ATTTTATTATTGTAGCAGCG	<i>ceuE</i>	Gonzalez <i>et al.</i> (1997)
M13F	GTAAAACGACGGCCAGT	pUC19	Yanisch-Perron <i>et al.</i> (1985)
M13R	AACAGCTATGACCATG	pUC19	Yanisch-Perron <i>et al.</i> (1985)
XR2	CGCGGATCCACTATATAGTGAAC	<i>orfE</i>	This study
XR1	CCACCCCAAAGGCGAAGG	<i>orfE</i>	This study
CAPDF2	GGAAGATCTAGTGGAGATATCCG	<i>orfG</i> (<i>capD</i> homologue)	This study
CAPDR2	GGAAGATCTCAAAGAAAAAGCCAG	<i>orfG</i> (<i>capD</i> homologue)	This study
XR3	GGAAGATCTAAATGCAATTTACATCC	<i>orfE-orfD</i>	This study
CATF1	TAGTGGTCGAAATACTCTTTTCGTG	CAT	This study
STTF1	TATCTACGCTTACTTATTGGCT	<i>orfA</i>	This study
WBBOF1	GCGCCTAGGGTGCCGAAGATGAATACACGC	<i>orfB</i> (<i>wbbO</i> homologue)	This study
WBAPF1	CGGTGATTTTAATCACTGC	<i>orfC</i> (<i>wbaP</i> homologue)	This study
NEUDF1	GCGCCTAGGGGGCTTGTGTGAAGATGTGGC	<i>orfD</i> (<i>neuD</i> homologue)	This study
NADHF1	CCACCCCAAAGGCGAAGG	<i>orfE</i>	This study
WBEEF1	CAAAGCAATTATATAGCAC	<i>orfF</i> (<i>wbeE</i> homologue)	This study
CAPDR1	CGGAATATCTCCACTAAATCTC	<i>orfG</i> (<i>capD</i> homologue)	This study

RESULTS

Cloning the putative LPS-biosynthesis locus

C. jejuni chromosomal libraries from *C. jejuni* NCTC 11168 and 11351 strains were constructed by ligating *Bcl*I-restricted chromosomal DNA and pUC19 restricted with *Bam*HI. Clones of interest were identified from both strains using a ³²P-labelled 800 bp DNA fragment encoding part of a *wbaP* (*rfbP*) homologue, derived from *C. jejuni* NCTC 11828 (a gift from B. Fry and B. van der Zeijst, Utrecht, The Netherlands). A single positive plasmid clone, confirmed to be representative of the chromosomal locus by Southern hybridization (data not shown), was selected from each strain. The plasmid from NCTC 11168 was designated pAWL61, and that from NCTC 11351, pAWL31.

Sequence analysis

The insert of pAWL61 was 7082 bp and encoded five complete and two partial ORFs, whereas the pAWL31 insert was 6478 bp and lacked one of the ORFs present in pAWL61. Restriction enzyme profiles of both plasmid clones were determined and the ORFs identified (Fig. 2). The predicted amino acid sequences from the seven ORFs were used to identify homologous proteins using the EMBL database and the BLAST program (Altschul *et al.*, 1990). Six of the predicted protein sequences showed homology to proteins known to be involved in polysaccharide biosynthesis in other microbes (Table 3). However, the predicted protein sequence from the additional gene in pAWL61 (*orfE*) demonstrated only very low homology with any proteins in the database.

Distribution and organization of the putative LPS locus in *C. jejuni*

Variation in the sugar composition of O-chains within a species is often reflected by gene insertions, deletions or mutations within the biosynthesis locus (Schnaitman & Klena, 1993). Variation at the putative LPS biosynthesis locus of *C. jejuni* was investigated using specific primers (Table 2) in a PCR-based approach. Amplification was performed from a reverse anchor primer, CAPDR1, specific for *orfG*, to each of a series of forward primers for the other six genes. This approach was applied to both *C. jejuni* NCTC 11168 and NCTC 11351 chromosomal DNA to confirm the integrity of the PCR approach (Fig. 3, Table 1).

A further 34 strains of *C. jejuni* were analysed using the same approach as used for NCTC 11168 and NCTC 11351, but no additional variation was seen within the locus. Of these 34 strains, 16 produced an identical PCR profile to *C. jejuni* NCTC 11168 and 18 strains produced an identical profile to *C. jejuni* NCTC 11351, lacking *orfE* (Table 1). The presence or absence of *orfE* was confirmed by Southern hybridization (data not shown). Serotyping of the strains (Table 1) revealed that the presence or absence of *orfE* did not correlate with serotype or LPS/LOS phenotype. Seven strains from GBS patients were included in the screening, four contained *orfE* and three did not (Table 1).

Mutational analysis

Mutation of *C. jejuni* strains was carried out by electroporation using pUC19-based plasmids as described above in Methods. *C. jejuni* NCTC 11351 proved to be untransformable and therefore NCTC 11828 was

Fig. 2. Restriction map and ORFs (arrows) of clones pAWL61 and pAWL31. The insert of pAWL61 is derived from *C. jejuni* NCTC 11168 and is 7082 bp in length, pAWL31 is derived from NCTC 11351 and is 6478 bp in length. *orfA* and *orfG* are incomplete. The dashed line indicates the region where the two clones differ; white triangles represent positions of mutations described in the text; half arrows indicate positions and direction of primers used in the genetic distribution studies.

Table 3. Analysis of predicted protein sequences from the ORFs of pAWL61

ORF	Size (bp)	Protein with highest homology*	Proposed function	P†/identity
<i>orfA</i> (incomplete)	1996	<i>Saccharomyces cerevisiae</i> STT3 (S64024) (Yoshida <i>et al.</i> , 1995)	Oligosaccharyl transferase subunit	2×10^{-7} /19%
<i>orfB</i>	1131	<i>Campylobacter hyoilei</i> WbbO (RfbF) (X91081) (Korolik <i>et al.</i> , 1997)	Galactosyl transferase	2.8×10^{-218} /80%
<i>orfC</i>	603	<i>Campylobacter hyoilei</i> WbaP (RfbP) (X91081) (Korolik <i>et al.</i> , 1997)	UDP-galactose phosphate transferase	4.6×10^{-132} /95%
<i>orfD</i>	588	<i>E. coli</i> NeuD (I55145) (Annunziato <i>et al.</i> , 1995)	Acetyl transferase	1×10^{-25} /29%
<i>orfE</i>	654	<i>Anopheles quadrimaculatus</i> NADH dehydrogenase (L04272) (Mitchell <i>et al.</i> , 1993)	NADH dehydrogenase	0.06/19%
<i>orfF</i>	1161	<i>Vibrio cholerae</i> WbeE (RfbE) (S28471) (Strocher <i>et al.</i> , 1995)	Dideoxyhexose synthesis	3×10^{-69} /31%
<i>orfG</i> (incomplete)	909	<i>Staphylococcus aureus</i> CapD (U10927) (Lin <i>et al.</i> , 1994)	Capsule synthesis	3×10^{-17} /27%

* The predicted protein sequences were analysed using the GenBank/EMBL database and the BLASTP analysis program (Altschul *et al.*, 1990). Old names of proteins are in parentheses. Alphanumeric figures are the protein accession numbers in the GenBank/EMBL database.

† The probability score (P) gives an indication of the confidence with which the identification was made; low numbers indicate high probability.

Fig. 3. PCR profiles of *C. jejuni* NCTC 11168 and NCTC 11351. Six specific PCR products are seen corresponding to the seven known genes with *C. jejuni* NCTC 11168 (a), whereas five bands only are seen with *C. jejuni* NCTC 11351 (b). The lack of product in lane 5 correlates with the absence of *orfE*. The amplicons in lanes 1–4 with NCTC 11351 are approximately 600 bp smaller than those with NCTC 11168, also correlating with the absence of *orfE*. In both parts: lane 1, *orfA*–G; lane 2, *orfB*–G; lane 3, *orfC*–G; lane 4, *orfD*–G; lane 5, *orfE*–G; lane 6, *orfF*–G; lane 7, λ HindIII and ϕ X174 *Hae*III molecular-mass markers.

used for the mutational analysis as it is a LPS producer and has an identical genetic complement at this locus to NCTC 11351. A kanamycin-resistance cassette derived

from *Campylobacter coli* (Trieuquot *et al.*, 1985) was used to mutate *orfE*, *F* and *G* in NCTC 11168 and *orfF* and *G* in NCTC 11828. *orfE* and *orfG* were mutated

Fig. 4. *C. jejuni* LPS/LOS profiles. LPS/LOS samples were electrophoresed on an 18% SDS-PAGE gel using the Tricine buffering system of Sprott *et al.* (1994) and subsequently detected by Western blotting using (a) O:2 antiserum against NCTC 11168 and its mutants and (b) O:6 antiserum against NCTC 11828 and its mutants. In both panels lane 1, *C. jejuni* wild-type; 2, *orfF* mutant; 3, *orfG* mutant; 4, *orfE* mutant; 5, large deletion mutant.

using inverse-PCR mutagenesis (Wren *et al.*, 1994) and primers XR1/XR2 and CAPDF2/CAPDR2, respectively (Table 2), whereas *orfF* was mutated by inserting the kanamycin cassette into the *Cla*I site within the gene. Attempts were also made to mutate the *wbaP* homologue, *orfC*, utilizing the *Sma*I site, but no transformants were obtained with either *C. jejuni* strain. Despite this, it proved possible to create a large deletion of the central two *Cla*I bands from the locus, removing the 3' end of *orfA*, all of *orfB*, *C*, *D* and *E*, and the 5' end of *orfF*. One possible explanation may be the toxic build-up of a pathway intermediate when *orfC* alone is mutated, such as occurs in *Escherichia coli galE* mutants when a build-up of UDP-galactose leads to cell lysis in the presence of galactose (Nikaido, 1961).

LOS/LPS samples from the wild-type and mutant strains of *C. jejuni* were analysed by Western blotting; the O-chain ladder of *C. jejuni* is not detectable by silver staining, only by Western blotting. Mutation of *orfG* had no detectable effect on the LOS/LPS profiles of either strain (Fig. 4, lanes 3). Mutation of *orfE* in NCTC 11168 did not alter the LOS profile of this strain (Fig. 4a, lane 4); the equivalent experiment in NCTC 11828 is not possible as *orfE* is not present. Mutating *orfF* and deleting the central *Cla*I fragments altered the reactivity of the core molecule to antiserum (Fig. 4, lanes 2 and 5). No effect on O-chain production was detected by Western blotting in any of the mutants constructed. The mutants were also analysed by silver staining, but no consistent alteration was detected with any of the mutants (data not shown). Mobility changes were occasionally seen in core molecules (shown in Fig. 4b, lane 2); however, these changes were not consistently observed. The *orfF* mutant and the large deletion mutant also demonstrated an altered morphology when compared with the wild-type; both these mutants were shorter and wider than their parent (Fig. 5).

Analysis of *orfE*

The genetic distribution analysis of the locus in *C. jejuni* confirmed that the only significant variation within the locus was the presence/absence of *orfE*, but this variation did not correlate with serotype or the presence of O-chain. Mutation of *orfE* in the LOS-producing NCTC 11168 had no detectable effect on LOS pheno-

Fig. 5. Electron micrograph of negatively stained NCTC 11168 (a) and *orfF* mutant (b) illustrating that the mutation results in shorter and wider cells (Bars, 1 µm).

type, but this was dependent upon the alteration being detected by the O:2 antiserum and did not indicate what its effect might be on LPS. A strategy was therefore designed to insert *orfE* into an LPS producer to analyse the effect of adding *orfE* to this strain. pCOD2 (Fig. 6) was constructed to facilitate the insertion of an intact *orfE* derived from NCTC 11168 into NCTC 11828 along with an antibiotic-resistance marker, chloramphenicol acetyl transferase (CAT; Wang & Taylor, 1990; Yao *et al.*, 1993). pCOD2 was used to transform

Fig. 6. Schematic diagram of pCOD2 and pCOD4. For pCOD2, the CAT cassette was restricted with *Sma*I and inserted into the *Ssp*I site of a pAWL61 subclone in a three-way ligation.

Fig. 7. LPS/LOS profiles of *C. jejuni* NCTC 11828 with the *orfE* mutations. LPS/LOS samples were electrophoresed on a 15% SDS-PAGE gel using the Tricine buffering system of Spcott *et al.* (1994) and subsequently detected by Western blotting using O:6 antiserum. O-chains are no longer detectable in the LPS sample from *C. jejuni* NCTC 11828 with the insertion of *orfE* (lane 3), disruption of *orfE* within this mutant does not restore detectability of the O-chain (lane 4). In contrast, the presence of an antibiotic-resistance gene in the same position does not affect O-chain production (lane 2). The core reactivity of the LPS samples remains in all cases. Lane 1, *C. jejuni* NCTC 11828; lane 2, *C. jejuni* NCTC 11828::CAT; lane 3, *C. jejuni* NCTC 11828::(*orfE*+CAT); lane 4, *C. jejuni* NCTC 11828::(*orfE*::KAN)+CAT; lane 5, *C. jejuni* NCTC 11168.

C. jejuni NCTC 11828 and transformants were selected using chloramphenicol. The LPS from NCTC 11828 (*orfE* absent), NCTC 11168 (*orfE* present) and the mutant NCTC 11828 containing *orfE* and CAT [NCTC 11828::(*orfE*+CAT)] were analysed by Western blotting using O:6 antiserum (Fig. 7, lanes 1, 5 and 3, respectively). The LPS of the mutant strain consistently showed no alteration in serospecificity, but the O-chain was no longer detectable (Fig. 7, lane 3).

orfE was subsequently disrupted in the NCTC 11828::(*orfE*+CAT) mutant using a kanamycin-resistance-cassette (KAN) insertion in the same manner used to construct the NCTC 11168 *orfE* mutant (see above). NCTC 11828::(*orfE*+CAT) was transformed with

orfE containing the KAN cassette and transformants selected using kanamycin. Production of the O-chain was not detected in NCTC 11828::(*orfE*::KAN)+CAT (Fig. 7, lane 4). To determine the effect of the CAT cassette alone on LPS phenotype at the position of insertion of *orfE* into NCTC 11828, a mutant was created using pCOD4 (Fig. 6). pCOD4 was constructed by inverse-PCR mutagenesis using a primer that anneals to sequence immediately downstream of *orfD* (XR3; Table 2) and a primer that anneals to the start of the CAT cassette (CATF1; Table 2) with pCOD2 as the template. The NCTC 11828::(*CAT*) mutant was found to have a detectable O-chain (Fig. 7, lane 2).

DISCUSSION

A putative LPS-biosynthesis locus was isolated from two strains of *C. jejuni*, one of which is reported to produce LPS and the other LOS. Predicted gene products from the six common ORFs identified showed similarity to proteins known to be involved with polysaccharide synthesis. Mutations in this locus appeared to affect the antigenicity of the core polysaccharide. The only difference between the gene organization of the two loci was the presence of a seventh gene, *orfE*, in the LOS-producing strain. The function of the *orfE* gene product is not known, although it has a low level of homology to several dehydrogenase genes.

The locus we have isolated does not encode all the genes required for LPS or LOS synthesis and therefore, not surprisingly, the presence of plasmid pAWL61 did not confer the production of *Campylobacter*-specific core or O-chain upon the host strain *E. coli* DH5 α . We have evidence indicating the presence of further LPS-biosynthesis genes upstream, but it has not yet been possible to clone the entire region to examine its effect on *E. coli* due to cosmid instability. Our preliminary data (N. J. Oldfield & J. M. Ketley, unpublished results) suggest that genetic variation in LPS-biosynthesis-gene content lies within this uncharacterized region upstream of the locus reported here.

In *Salmonella*, WbaP transfers galactose 1-phosphate to undecaprenol phosphate as an initiation step in the assembly of some O-chain subunits. The presence of a WbaP homologue (OrfC) in both strains would suggest

that a biosynthesis mechanism involving a lipid carrier, similar to that for O-chain synthesis, exists within *C. jejuni*. The lack of an O-chain in NCTC 11168 suggests that the genes either are not expressed or are involved in the biosynthesis of structures other than O-chain, such as core oligosaccharide. The first hypothesis is unlikely due to the inability to create a single *wbaP* mutant; we also have preliminary data (not shown) indicating that the locus is expressed in NCTC 11168. The lack of effect on the O-chain noted when the majority of the genes in the locus were deleted also suggests that the genes are not involved in O-chain synthesis. This observation and the alteration in reactivity to antiserum noted with the core of the *orfF* mutant support the hypothesis that the genes are involved in core synthesis.

The deduced amino acid sequence of OrfF showed homology to WbeE (RfbE; putative perosamine synthase) from *Vibrio cholerae* (Stroeher *et al.*, 1995) and DdhC (RfbH; CDP-4-keto-6-deoxy-D-glucose 3-dehydrase) from *Salmonella typhimurium* (Schnaitman & Klena, 1993), both of which are involved in dideoxyhexose synthesis suggesting that OrfF may also be involved in such a pathway. The relatively high conservation of the locus throughout several serotypes suggests that the region is involved in the synthesis of a common part of LPS/LOS molecules. The only known constituent of the core in a O:2 serotype that contains a dideoxy component is sialic acid. It is also interesting to note the high homology of OrfD to NeuD, a protein thought to be involved in sialic acid capsule synthesis in *E. coli* (Annunziato *et al.*, 1995). The reduced reactivity of antiserum with the core of the LPS and LOS molecules in an *orfF* mutant indicates that OrfF may be involved in core biosynthesis. It is notable that the effect of an *orfF* mutation on core oligosaccharide does not disturb O-chain biosynthesis in NCTC 11828; there are a number of possible reasons for this result. First, the action of OrfF may produce a minor variation in core structure that results in a change in antigenicity but not O-chain linkage. Second, *C. jejuni* may make more than one core structure (although this is not supported by structural studies) which are not all linked to O-chain. Thus, OrfF may be involved in the biosynthesis of an unlinked core region. Third, the O-chain ladder may reflect the production of a capsule-like extracellular polysaccharide (see below) and therefore changes in LOS core polysaccharide may not affect such an 'O-chain' molecule.

There are several explanations for the inability to detect any phenotypic alteration when mutating *orfE* or *orfG*. The polyclonal antiserum used in the Western blot may not detect minor changes in the structure of the LPS. Another possibility is the presence of compensating genes in a second LPS biosynthesis locus. We have used Southern hybridization and PCR to test this hypothesis and have found no evidence of a second locus (data not shown). Cryptic LPS-biosynthesis genes have been documented in *Neisseria* (Robertson *et al.*, 1994; Preston *et al.*, 1996) and *Haemophilus influenzae* (Hood *et al.*, 1996), both of which produce LOS, but not LPS.

Insertional mutagenesis of several genes from both these species failed to alter their LOS profiles.

Given that the locus may be involved in core oligosaccharide biosynthesis, interpretation of our data at the present time is complicated by the fact that the O-chain is no longer visible after the insertion of *orfE* into the LPS producer. Subsequent disruption of this gene by insertion of a KAN cassette does not restore the visibility of the O-chain; however, the presence of the CAT cassette alone did not affect O-chain biosynthesis. Therefore, it is the presence of *orfE* and not the position of insertion that is of importance. The product of the KAN cassette or the orientation of the KAN or CAT cassette are not responsible for the loss of O-chain, as other KAN-insertion mutants (for example in *orfG*) do not have altered O-chain biosynthesis and *orfE*-containing mutants with the KAN and CAT cassettes in both orientations (data not shown) have the same mutant phenotype.

We are currently investigating the role of *orfE* in O-chain production, but our data show that, when inserted into the chromosome of NCTC 11828, the presence of *orfE* can affect LPS/LOS phenotype. If *orfE* can have an effect on O-chain production as suggested by our data, we would have expected to see a correlation between the presence of *orfE* and the LOS phenotype. The absence of such a correlation might reflect the complexity of O-chain biosynthesis, where the presence or absence of many other genes will also affect the production of O-chain and strains of a particular serotype may not have identical O-chain structure.

Extracellular polysaccharides that are not attached to lipid A core structures have been described in *C. jejuni* (Aspinall *et al.*, 1995; Prendergast *et al.*, 1998). It is possible, therefore, that *C. jejuni* also contains genes that encode enzymes involved in the production of (an) other extracellular polysaccharide(s) and produce a lipid-linked polysaccharide, rather than LPS. One group postulated that the Penner serotyping screen is actually a reflection of capsule content (Chart *et al.*, 1996); however, the antigen treatment described by Chart *et al.* (1996) does not rule out the involvement of LPS. Interestingly, part of an ABC-transporter system with closest similarity to genes involved in capsular extracellular-polysaccharide biosynthesis have been identified in *C. jejuni* (A. Karlyshev, personal communication); the mutation of these genes also has an effect on O-chain production in *C. jejuni*. It is possible that in some *C. jejuni* strains (for example, those without *orfE*), the extracellular polysaccharide could also be added to a lipid A core. Such a structure would be analogous to the lipid A-core-linked enterobacterial common antigen (ECA_{LPS}) and lipid A-core-linked K antigen (K_{LPS}) (Whitfield *et al.*, 1997). This may imply that all campylobacters are LOS producers with some strains also containing a polysaccharide structure that is independent of a subset of core oligosaccharide. The genetic analysis presented here does not indicate the nature of the linkage of the O-chain structures observed in some strains of *C. jejuni*.

In conclusion, we have isolated a locus encoding ORFs with homology to bacterial polysaccharide-biosynthesis genes and shown that mutating one of the genes in this locus affects the reactivity of antiserum to the core of LPS, although the mutation of two other genes had no effect. None of these mutants demonstrated an altered O-chain. The locus is distributed throughout *C. jejuni* isolates regardless of serotype; the only variation is the presence of *orfE* which, at present, is of unknown function. Insertion of *orfE* into an LPS-producing *orfE*-negative strain results in the loss of O-chain as seen on Western blots.

NOTE ADDED IN PROOF

A description of the LPS-biosynthesis locus of *Campylobacter jejuni* NCTC 11828 (81116) was recently published by Fry *et al.* (1998).

ACKNOWLEDGEMENTS

This work was funded by the UK Department of Health, and the Biotechnology and Biological Sciences Research Council. We would like to thank the Royal Society for a University Research Fellowship to J. M. K. We gratefully acknowledge B. Fry and B. van der Zeijst, Department of Bacteriology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands for the NCTC 11828 DNA fragment; E. Bolton, Preston Public Health Laboratory, UK and J. Frost, Central Public Health Laboratory, London, UK for antiserum; R. Thwaites, Central Public Health Laboratory, Colindale, London, UK for serotyping; all those who supplied isolates for the genetic distribution studies (listed in Table 1); R. Owen, Central Public Health Laboratory, London, UK for *C. jejuni* NCTC 11168 and NCTC 11351; T. Wassenaar, University of Mainz, Germany for *C. jejuni* NCTC 11828. We would also like to thank J. ten-Brinke, Department of Bacteriology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands for technical assistance; A. Moran, Department of Microbiology, University College Galway, Ireland, for help with the O:41 strains and D. Maskell, Dept of Clinical Veterinary Medicine, University of Cambridge, Cambridge, UK for helpful discussions.

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Received 14 August 1998; revised 12 October 1998; accepted 28 October 1998.