molecular microbiology

Role of capsular modified heptose in the virulence of *Campylobacter jejuni*

1   **Role of capsular modified heptose in the virulence of *Campylobacter jejuni***

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20   **Running title:** Role of modified heptose in virulence of *C. jejuni*.

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22   **Key words:** Campylobacter, capsule, heptose, virulence, chicken, macrophages.

23

24   **SUMMARY**

25

26   The *Campylobacter jejuni* capsular polysaccharide is important for virulence and often

27   contains a modified heptose. In strain ATCC 700819 (a.k.a. NCTC 11168), the modified

28   heptose branches off from the capsular backbone and is directly exposed to the

29   environment. We reported previously that the enzymes encoded by *wcaG*, *mlghB*, and

30   *mlghC* are involved in heptose modification. Here, we show that inactivation of any of

31   these genes leads to production of capsule lacking modified heptose and alters the

32   transcription of other capsule modification genes differentially. Inactivation of *mlghB* or

33   *mlghC,* but not of *wcaG,* decreased susceptibility to bile salts and abrogated invasion of

34   intestinal cells. All mutants showed increased sensitivity to serum killing, especially

35   *wcaG::cat*, and had defects in colonization and persistence in chicken intestine, but did

36   not show significant differences in adhesion, phagocytosis, and intracellular survival in

37   murine macrophages. Together, our findings suggest that the capsular heptose

38   modification pathway contributes to bacterial resistance against gastrointestinal host

39   defenses and supports bacterial persistence via its role in serum resistance and invasion of

40   intestinal cells. Our data further suggest a dynamic regulation of expression of this

41   pathway in the gastrointestinal tract.

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INTRODUCTION

Campylobacter jejuni infections are the leading cause of enteritis worldwide (Rautelin and Hanninen, 2000; Wassenaar and Blaser, 1999). Infected patients can also develop neurological complications such as Guillain-Barre and Miller-Fischer syndromes (Godschalk *et al.*, 2004; Godschalk *et al.*, 2007). Numerous factors contribute to pathogenicity of *C. jejuni* and successful host colonization, including production of toxins and glycoproteins (Guerry *et al.*, 2006; Kakuda and DiRita, 2006; Karlyshev *et al.*, 2004; Szymanski *et al.*, 2002; Vijayakumar *et al.*, 2006) and resistance to bile salts (Lin *et al.*, 2003; Lin *et al.*, 2005). Flagella-mediated motility also contributes to the virulence of *C. jejuni* (Biswas *et al.*, 2007), as it facilitates colonization of the mucus layer covering the intestinal epithelium (Beery *et al.*, 1988; Lee *et al.*, 1986) and subsequent invasion of epithelial cells (Jin *et al.*, 2001; Konkel *et al.*, 2005; Pei and Blaser, 1993; Vijayakumar *et al.*, 2006). Survival of *C. jejuni* within macrophages for several days is also a key virulence factor (Day *et al.*, 2000; Kiehlbauch *et al.*, 1985), and epithelial and macrophage cell damage resulting from invasion may be critical for the inflammatory response elicited by *C. jejuni* infection (Biswas *et al.*, 2000; Fauchere *et al.*, 1986; Manninen *et al.*, 1982; Newell and Pearson, 1984; Newell *et al.*, 1985; Szymanski *et al.*, 1995). Further, the surface expression of lipooligosaccharide (LOS), which in many *C. jejuni* strains mimics human gangliosides, has been associated with autoimmune reactions implicated in the Guillain-Barre and Miller-Fischer syndromes (Godschalk *et al.*, 2007; Mishu and Blaser, 1993; Salloway *et al.*, 1996; Yuki *et al.*, 1994).

C. jejuni also produces a surface capsular polysaccharide (CPS) (Karlyshev *et al.*, 2000; Karlyshev *et al.*, 2001) initially identified as a heat-stable antigen and thought to be

67   a lipopolysaccharide (Preston and Penner, 1989). Mutational studies have demonstrated

68   that CPS is a virulence factor for *C. jejuni*. For example, a non-capsular *kpsE* mutant of

69   strain ATCC 81116 shows reduced adherence and invasion of intestinal epithelial cells

70   (Bachtiar *et al.*, 2007), and a non-capsular *kpsM* mutant of strain ATCC 81-176 has

71   reduced invasion of intestinal epithelial cells, is attenuated in a ferret model (Bacon *et al.*,

72   2001), and has decreased ability to colonize the gut in chickens (Jones *et al.*, 2004). Non-

73   capsular mutants of invasive clinical isolates 84-25 and 84-19 are also more readily killed

74   by serum complement than wild type (Keo *et al.*, 2011).

75   The CPS from strain ATCC 700819 (a.k.a. NCTC 11168) used in this study

76   consists of a phospholipid anchor (Corcoran *et al.*, 2006) and repeating units containing a

77   backbone of β-D-ribofuranose (β-D-Rib*f*), 2-acetamido-2-deoxy-β-D-galactofuranose (β-

78   D-Gal*f*NAc), and α-D-glucopyranuronic acid (α-D-Glc*p*A) amidated with 2-amino-2-

79   deoxyglycerol (GroN) or ethanolamine (EtN) (Fig. 1A). The repeating units also

80   comprise a side branch consisting of a modified heptose: 6-O-methyl-D-*glycero*-α-L-

81   *gluco*-heptopyranose (6-O-Me-D-α-L-*gluco*Hep*p*) (McNally *et al.*, 2007; St Michael *et*

82   *al.*, 2002; Szymanski *et al.*, 2003) or 3,6-O-Me₂-D-α-L-*gluco*Hep*p* (McNally *et al.,* 2007).

83   Addition of O-methyl phosphoramidate (MeOPN) moieties onto heptose and Gal*f*NAc

84   residues has also been reported (McNally *et al.*, 2007; Szymanski *et al.*, 2003). The role

85   of MeOPN modification of CPS in virulence and host colonization is controversial. A

86   contribution to insecticidal activity was demonstrated in one study (Champion *et al.*,

87   2010), but another study showed neither insecticidal activity nor any effect on chicken

88   colonization (van Alphen *et al.*, 2014). The MeOPN modification may increase invasion

89   to epithelial cells and resistance to serum killing (van Alphen *et al.*, 2014) or modulate

90   host immune responses (Maue *et al.*, 2013). The contribution to virulence of the uniquely

91   modified heptoses remains unknown. Due to their branch location in the CPS of strain

92   ATCC 700819, the modified heptose residues protrude into the extracellular

93   environment, becoming the outermost exposed carbohydrates of the CPS.

94   Various heptose derivatives are found in the CPS of *C. jejuni* (Aspinall *et al.*,

95   1992; Aspinall *et al.*, 1995a; Chen *et al.*, 2008; Kilcoyne *et al.*, 2006), *C. coli* (Aspinall *et*

96   *al.*, 1993) and *Burkholderia pseudomallei* (Perry *et al.*, 1995; Reckseidler *et al.*, 2001), in

97   the exopolysaccharide of *C. lari* (Aspinall *et al.*, 1995b), and in the lipopolysaccharide of

98   *Yersinia pseudotuberculosis* (Samuelsson *et al.*, 1974). We previously demonstrated that

99   the modified heptoses play a role in *Y. pseudotuberculosis* virulence (Ho *et al.*, 2008;

100   Kondakova *et al.*, 2008). Variations in the heptose structure (ring configuration and

101   modifications) among different isolates and species suggest specific adaptations of the

102   heptose residue to its function within the polysaccharide. We therefore hypothesized that

103   the modified heptoses and their unusual ring configurations are important to fine tune

104   host-bacteria interactions.

105   Genomic sequence data of strain ATCC 700819 suggest that the *C. jejuni* CPS is

106   related to group II and group III CPS of other Gram-negative bacteria (Karlyshev *et al.*,

107   2000). CPS gene clusters of *E. coli* strains that produce type II and III CPS show a

108   conserved organization of the genes into three regions (Boulnois *et al.*, 1987; Boulnois

109   and Roberts, 1990). Regions 1 and 3 include genes involved in CPS export, such as the

110   gene for the CPS ABC-transporter component KpsM for transport across the inner

111   membrane (Cuthbertson *et al.*, 2010; Whitfield, 2006). The central region 2 comprises

112   genes for the synthesis of serotype-specific sugars and their assembly into repeating units

113   (Roberts *et al.*, 1988a; Roberts *et al.*, 1988b). These features apply to *C. jejuni* strain

114   ATCC 700819 (Fig. 1B). In the CPS cluster of this strain and also in *B. pseudomallei*,

115   region 2 contains genes devoted to the synthesis of GDP-D-*glycero*-α-D-*manno*-heptose

116   (Kneidinger *et al.*, 2001; Parkhill *et al.*, 2000; Reckseidler *et al.*, 2001), suggesting that

117   this compound is likely the precursor for the synthesis of the modified heptose present in

118   their CPS, as demonstrated previously for the 6-deoxy-D-*manno*-heptose in *Y.*

119   *pseudotuberculosis* (Butty *et al.*, 2009).

120   We recently elucidated the biochemical synthesis pathways of the 6-deoxy-D-

121   *altro*-heptose present in *C. jejuni* 81-176, which involved the sequential activity of the

122   C4, C6 dehydratase DdahA (a.k.a. WcbK), a C3 epimerase DdahB (a.k.a. Cjj1430) and a

123   C4 reductase DdahC (a.k.a. Cjj1427) on GDP-D-*glycero*-D-*manno*-heptose (McCallum *et*

124   *al.*, 2011; McCallum *et al.*, 2012). This pathway is under post-translational regulation by

125   the activity of WcaG, a C4 reductase encoded by the CPS cluster itself that reduces the

126   DdahA product, thereby diverting it off the *altro*-heptose synthesis pathway. We also

127   showed additional regulation of the biosynthesis pathway via interactions between WcaG

128   and DdahC. Using this knowledge, we demonstrated the C3, C5 epimerase activity of

129   MlghB (encoded by *mlghB,* a.k.a. *cj1430c*) and the C4 reductase activity of MlghC

130   (encoded by *mlghC,* a.k.a. *cj1428c*) of *C. jejuni* ATCC 700819 (Fig 1B) (McCallum *et*

131   *al.*, 2013). This was achieved by using a hybrid pathway that combined MlghB and

132   MlghC from *C. jejuni* ATCC 700819 with the DdahA dehydratase from *C. jejuni* 81-176

133   (Fig 2) since the putative oxidase MlghA necessary to initiate the pathway in *C. jejuni*

134   ATCC 700819 is unknown. We also showed a C4 reductase activity on the DdahA

135   product for the WcaG enzyme encoded by *wcaG* (a.k.a. *cj1427c*) in strain ATCC 700819

136   (McCallum *et al.*, 2013). The proposed 6-O-methyl-D-*glycero*-α-L-*gluco*-heptose (called

137   thereafter 6OMe-Hep) synthesis pathway is depicted in Fig. 2. Our biochemical work

138   suggests that WcaG, MlghB, and MglhC are required for CPS heptose modification. This

139   agrees with data showing that homologues of *wcaG*, *mlghB*, and *mlghC* are only found in

140   CPS biosynthesis loci of strains whose CPS contains modified heptoses (Karlyshev *et al.*,

141   2005; St Michael *et al.*, 2002), and with the observation that inactivation of *mlghC* results

142   in loss of the modified heptose (St Michael *et al.*, 2002).

143   In this study, we investigated the role of *wcaG*, *mlghB*, and *mlghC* genes from

144   strain ATCC 700819 in CPS synthesis and function. Using a panel of defined mutants,

145   we determined the contribution of each gene to CPS composition and to phenotypes

146   associated with pathogenesis including protection against bile salts, detergents and serum,

147   and bacterial motility. We also tested the contribution of these genes to *C. jejuni*'s

148   adhesion and invasion of intestinal epithelial cells, survival within macrophages, and

149   colonization of the chicken intestine. Our results demonstrate that *wcaG*, *mlghB*, and

150   *mlghC* are required for CPS heptose modification and indicate that the modified heptoses

151   contribute to the pathogenicity of *C. jejuni* ATCC 700819.

152

153   **RESULTS**

154   *Genetic characterization of the capsular heptose biosynthesis mutants*

155

156   Insertional mutants in *wcaG*, *mlghB*, *mlghC*, and *kpsM* genes were constructed as

157   described under *Experimental Procedures*. PCR and Southern blot analyses indicated that

158   each mutant contained the disrupted targeted gene without compromising the surrounding

159   genes (data not shown). One of the *wcaG*::*cat* mutants, named *wcaG*::*cat*Δ, also had a

160   6.1-kb deletion removing genes *cj1421c* to *cj1426c*. This mutant was included in our

161   studies for the following reasons. First, the deletion removes *cj1425c-cj1423c,* which are

162   involved in the synthesis of the heptose precursor (GDP-*glycero-manno*-heptose, Fig.1B)

163   (Karlyshev *et al.*, 2005; Kneidinger *et al.*, 2001; Parkhill *et al.*, 2000; Valvano *et al.*,

164   2002), suggesting that *wcaG*::*cat*Δ should lack heptose in its CPS. Second, the deletion

165   also eliminates the putative heptose methyltransferase *cj1426c* (Fig.1B) (Sternberg *et al.*,

166   2013), thereby ensuring lack of methylation. Third, *cj1422c* and *cj1421c*, which are also

167   deleted, encode enzymes for the transfer of MeOPN to D-α-L-*gluco*Hep*p* and β-D-

168   Gal*f*NAc, respectively (Fig.1B) (Karlyshev *et al.*, 2005; McNally *et al.*, 2007). While

169   MeOPN synthesis genes (*cj1415c-cj1418c*) are intact, the attachment of MeOPN to the

170   CPS should not occur in this mutant. Therefore, *wcaG*::*cat*Δ should produce a CPS

171   backbone devoid of modifications (no heptose, no methylation and no MeOPN), thereby

172   serving as a valuable control in our study. The *kpsM* mutant provided a non-capsular,

173   negative control since KpsM is a critical component of the ABC transporter for CPS

174   export across the cytoplasmic membrane (Silver *et al.*, 2001). Complemented strains

175   were constructed by re-introducing the original genes in the chromosome between the

176   16S and 23S rRNA genes and using the constitutive OmpE promoter as described

177   previously (Karlyshev and Wren, 2005).

178   For all phenotypic analyses, the strains were revived under appropriate antibiotic

179   selection, but the final sub-culture prior to phenotypic analysis was performed in the

180   absence of antibiotic selection so that all strains were grown under the exact same

181   conditions. Bacteria were harvested and suspensions normalized to the same OD_{600nm} to

182   alleviate potential variation arising from different growth rates. CFU measurements

183   performed on bacterial suspensions used as inoculum for phenotypic assays indicated

184   similar viability of all strains used as measured after 24 h of growth (data not shown).

185

186   *Mutants in the heptose modification pathway produce high molecular weight CPS*

187

188   The effect of inactivation of heptose modification genes on CPS synthesis was

189   investigated by SDS-PAGE and silver staining of CPS obtained by hot water/phenol

190   extraction (Westphal and Jann, 1965) and ultracentrifugation. The wild type had high and

191   low molecular weight bands (Fig. 3A) corresponding to CPS and co-extracted LOS,

192   respectively (Karlyshev and Wren, 2001). As expected, the *kpsM* mutant lacked high

193   molecular weight bands while production of LOS was not affected (Fig. 3A). In contrast,

194   all heptose modification mutants formed CPS but *mlghB*::*cat* apparently produced less

195   CPS than the others (Fig. 3A). Western blot analysis (with anti HS:2 Penner serotyping

196   antibodies) of CPS samples obtained by SDS solubilization of total cells and proteinase K

197   digestion of proteins (Hitchcock and Brown, 1983) also showed that all mutants tested

198   produced CPS (Fig. 3B) and the amount produced by *mlghB::cat* was on par with all

199   other strains. The differences in relative amounts seen for *mlghB::cat* by silver staining

200   and Western blotting could relate to slight variations in CPS composition that could affect

201   silver staining (Szymanski et al., 2003) and CPS solubility and recovery in the hot

202   water/phenol extraction method, but compositional data below exclude this possibility.

203   The differences probably rather relate to additional modifications of surface properties in

204   this mutant, which may in turn affect extractability of the CPS by the hot water/phenol

205   method. Together, these results indicate that the absence of heptose or modified heptose

206   does not prevent production of high molecular weight CPS.

207   Slight upwards shifts of CPS bands of the mutants were observed depending on

208   loading amounts and gel composition. Because wild type *C. jejuni* CPS is anchored to the

209   outer membrane via a phospholipid (Corcoran *et al.*, 2006), the band shifts could reflect

210   CPS anchoring to a different lipid carrier, such as lipid A, as seen in *E. coli* K_{LPS} (Jann *et*

211   *al.*, 1992). To test this possibility, CPS samples were treated with phospholipase prior to

212   SDS-PAGE analysis (Karlyshev *et al.*, 2000). This led to disappearance of CPS bands in

213   all strains tested (data not shown), demonstrating that CPS was phospholipid-bound.

214   Also, in contrast to LOS bands, wild type and mutant CPS bands did not react with anti-

215   lipid A antibody (data not shown), confirming that CPS was not covalently linked to lipid

216   A. We conclude that the band shifts represent subtle changes in CPS composition

217   affecting not only the size of CPS units but also their hydrophobicity and ability to bind

218   SDS.

219

220   *The CPS of heptose modification pathway mutants does not contain methylated heptose*

221

222   The composition of hot water/phenol extracted CPS was determined using a sugar

223   analyzer after complete acid hydrolysis, and also using one- and two-dimensional NMR

224   spectroscopy (Tables 1 and 2, Figs. S1 and S2). The data obtained, combined with data

225   reported for *C. jejuni* NCTC 11168 CPS (McNally *et al.*, 2007; St Michael *et al.*, 2002;

226   Szymanski *et al.*, 2003), enabled inferring the CPS structures of wild type and mutant

227   strains (Fig. 4). Sugar analysis revealed the three CPS backbone constituents, Rib,

228   GalNAc (detected as GalN) and GlcA in all strains. The higher than expected Rib/GalN

229   ratios (expected equimolar) are likely due to RNA contamination of the samples. GlcA

230   was not quantitated because it was amidated with EtN or GroN and was poorly released

231   by hydrolysis but NMR spectroscopy analyses confirmed its presence by the

232   characteristic signals for H-1 and C-1 in all strains (Table 2). Without authentic D-

233   *glycero*-L-*gluco*-heptose as standard, the presence or absence of heptose was inferred

234   from signals for H-1 at δ 5.58 and C-1 at δ 98.3, which were observed in ^{1}H and ^{13}C

235   NMR spectra of wild type CPS only (Table 2). Characteristic signals were also detected

236   for 3-O-Me and 6-O-Me groups on heptose in wild type only (Fig S2), confirming that

237   heptose was present and 3,6-di-O-methylated in wild type CPS while it was absent in the

238   CPS from the mutants.

239   NMR spectroscopy did not reveal MeOPN modification in wild type CPS, but

240   MeOPN was readily apparent on GalNAc in *wcaG*::*cat* and *mlghB*::*cat* (Table 2) and

241   traces of MeOPN were found in *mlghC*::*cat*. Consistent with the deletion of MeOPN

242   transfer genes in *wcaG*::*cat*Δ, no MeOPN was detected in its CPS despite the presence of

243   the GalNAc acceptor. Also, while the GlcA residue carried only the EtN substituent in

244   wild type CPS, both EtN and GroN were found in the mutants (Table 1). These

245   differences between wild type and heptose modification mutants could represent a

246   compensatory mechanism whereby amidation of GlcA by GroN and/or MeOPN addition

247   on GalNAc is enhanced when the production of D-*glycero*-L-*gluco*-heptose is impaired.

248   No O-methyl groups were present in the complemented *wcaG* and *mlghC* mutants by

249   NMR spectroscopy, suggesting that incorporation of wild type-like modified heptose was

250   not restored in these strains. The complemented *mlghB* strain was not tested by NMR

251   spectroscopy for lack of sufficient CPS material.

252

253   *Inactivating heptose modification genes causes drastic changes in transcription within*

254   *the CPS gene cluster*

255

256   The compensatory mechanisms inferred from NMR spectroscopy analyses in our mutants

257   and lack of complementation of modified heptose incorporation in the CPS could result

258   from polarity effects or from transcriptional regulation within the CPS cluster. We used

259   quantitative real-time PCR (qRT-PCR) to demonstrate lack of polarity on expression of

260   genes located downstream of the chloramphenicol (*cat*) and kanamycin (*kan*) resistance

261   cassettes used for gene inactivation. Expression of the gene immediately downstream of

262   *kpsM* in *kpsM::kan* or downstream of *wcaG* and *mlghC* in *wcaG::cat* and *mlghC::cat*,

263   respectively was not affected (Fig. 5), indicating that insertion of the *cat* and *kan*

264   cassettes was non-polar. However, in *mlghB::cat*, transcription of *cj1429c* directly

265   downstream of *mlghB* was upregulated ~187-fold. Since the *cat* cassette causes no

266   polarity, we concluded that the upregulation of *cj1429c* transcription is related to

267   inactivation of *mlghB* via a regulatory effect and not a mutagenesis artifact. Such

268   transcriptional regulation implies the existence of promoters within the CPS cluster.

269   *C. jejuni* promoters are different from *E. coli* promoters (Wosten *et al.*, 1998) and

270   difficult to predict. To test for their existence, we measured the transcription levels of

271   genes involved in CPS synthesis and modification by qRT-PCR. By doing so, we could

272   also assess whether inactivation of one step during D-*manno-* to L-*gluco-* heptose

273   conversion had any feedback regulatory effects on other steps or on other CPS

274   modifications (e.g. MeOPN addition, amidation of GlcA, heptose methylation).

275   Measurements were relative to housekeeping gene *cj1537c* (acetyl-CoA synthase) and

276   accounted for primer efficiencies.

277   Expression levels varied considerably among the genes tested, with some too low

278   to be quantitated accurately (e.g. *kpsM*), others on par with the housekeeping control, and

279   others ~15-fold greater than control (Fig. 5A). The various transcription patterns

280   observed indicate that numerous promoters exist within the CPS cluster. To further assess

281   potential feedback regulatory effects of inactivation of one gene on surrounding genes,

282   we analyzed the data as expression ratios between each mutant and the wild type (Pfaffl,

283   2001) (Fig. 5B). This comparison indicated that inhibition of CPS transport by *kpsM*

284   inactivation only had minor effects on heptose modification genes and no effects on the

285   remaining CPS cluster genes tested, and that inactivation of biosynthesis/modification

286   genes had little or no impact on transport functions. However, inactivation of *mlghB* or

287   *mlghC* (responsible for heptose ring configuration switch from D-*manno* to L-*gluco*) led

288   to upregulation of other genes involved in heptose synthesis and modification, including

289   the O-methyl-transferase gene *cj1426c (*2.8- to 9.9-fold). Inactivation of *mlghB* or *mlghC*

290   also strongly upregulated the transcription of *cj1429c* (186.7- and 18.1-fold,

291   respectively), a gene of unknown function. Inactivation of *wcaG* produced different

292   effects, with modest upregulation of heptose modification genes (2.9- to 4.5-fold), no

293   effect on heptose synthesis and 60% reduction of transcription of *cj1429c*. These

294   differences could be due to the previously established regulatory role of WcaG on

295   heptose modification by substrate scavenging and interaction with MlghC (McCallum *et*

296   *al.*, 2011; McCallum *et al.*, 2012; McCallum *et al.*, 2013). Together, the transcriptional

297   data suggest that inactivation of *wcaG*, *mlghB*, or *mlghC* or the absence of their products

298   exert regulatory effects on other CPS modification genes, which may influence the

299   functional properties of CPS.

300   We next assessed whether these regulatory effects were abrogated in our

301   complemented strains, focusing on the *mlghC* and *mlghB* mutants that showed the

302   strongest regulatory effects above. Reintroduction of *mlghC* into the *mlghC* mutant led to

303   60+-1.5 fold up-regulation of *mlghC* compared to wild type, mirrored by 67+-3.8 fold up-

304   regulation of *mlghB* upon its reintroduction into the *mlghB* mutant. While such high

305   transcription levels had no significant effects on transcription of other CPS genes such as

306   *wcaG* and *mlghB* in the complemented *mlghC* strain (with 1.9+-0.3 and 0.4+-0.1 fold

307   compared to wild type, respectively), *wcaG* and *mlghC* were upregulated 5.4+-0.3 and

308   8.5+-0.4 fold compared with wild type, respectively, in the complemented *mlghB* strain,

309   which may affect CPS composition and function. Overall, this analysis indicates that the

310   strong OmpE promoter and internal promoters in the CPS cluster result in significant

311   transcriptional differences in complemented strains, which together with enzymatic

312   regulatory loops (McCallum *et al.*, 2013) may explain the lack of functional

313   complementation of the mutants observed by NMR. Others have also reported previously

314   similar difficulties with complementation upon perturbation of regulatory networks or

315   improper stoichiometry of enzymes involved in polysaccharide synthesis (van Sorge *et*

316   *al.*, 2014). Therefore, in view of these difficulties and since qRT-PCR data demonstrated

317   lack of polarity of the mutations, we did not pursue the characterization of the

318   "complemented" strains.

319

320   *Disruption of heptose modification pathway affects growth rate and decreases motility*

321   *but does not affect autoagglutination*

322

323   When measuring growth rates of wild type and mutants under microaerobic conditions,

324   we observed that the heptose modification mutants grew consistently faster than wild

325   type. The *wcaG*::*cat*Δ mutant, which not only lacks heptose in its CPS but also lacks

326   MeOPN on GalNAc (Fig. 6A), reached the highest final cell density. This suggests that

327   implementation of the heptose modification pathway (configuration switch, and

328   methylation) and of the MeOPN addition pathway imposes an energetic cost. The

329   *kpsM*::*kan* mutant exhibited a longer lag phase before entering exponential phase,

330   suggesting that the accumulation of CPS units or CPS precursors inferred from the qRT-

331   PCR analysis above may be slightly toxic to the bacterium, as observed in comparable

332   mutants of other species (Kroll *et al.*, 1988; Pavelka *et al.*, 1994).

333   We also investigated if alteration of CPS amount or composition in the mutants

334   could affect autoagglutination, which was measured as the decrease of turbidity over time

335   in the upper level of static bacterial suspensions. Autoagglutination occurred in all strains

336   and there was no significant difference between wild type and the heptose modification

337   mutants (Fig. 6B). In contrast, *kpsM*::*kan* showed increased autoagglutination (Fig. 6B)

338   and microscopy revealed that this mutant formed clumps of cells (Fig. 6C). Motility was

339   assessed in soft agar to determine if impaired motility contributed to the strong

340   autoagglutination of *kpsM*::*kan*. This mutant was nonmotile while the heptose

341   modification mutants were motile, albeit at a statistically significantly reduced rate

342   compared to wild type (Table 3). Electron microscopy revealed that the majority of

343   *kpsM*::*kan* cells produced flagella similar to the wild type strain (Fig. 6D), suggesting that

344   loss of motility was due to a defect in flagellar function. Overall, the total lack of motility

345   of *kpsM*::*kan* may contribute to its autoagglutination.

346

347   *Disruptions of heptose modification pathway alters CPS barrier function*

348

349   CPS contributes to resistance to bile salts in many bacteria (Begley *et al.*, 2002; Hsieh *et*

350   *al.*, 2003; Pace *et al.*, 1997). We tested whether CPS and its modified heptose contribute

351   to resistance to bile salts for *C. jejuni*. Wild type and mutant strains were exposed to

352   increasing concentrations of a mixture of cholic and deoxycholic acids, two abundant bile

353   salts in humans. Low concentrations (0-0.25 g/l) of bile salts had little effect on all strains

354   (Fig. 7A). However, higher concentrations (0.5-2.0 g/l) resulted in 80-90% killing in the

355   wild type strain. Interestingly, *kpsM*::*kan* was as susceptible to bile salt killing as wild

356   type, indicating that CPS as a whole and in its wild type structure and composition does

357   not contribute to resistance. Likewise, *wcaG*::*catΔ* and *wcaG*::*cat* had wild type like bile

358   salts susceptibility while *mlghC*::*cat* and *mlghB*::*cat* were more resistant ($p<0.001$) than

359   wild type. These differences in bile salts resistance may reflect subtle differences in the

360   CPS, which may not be apparent in NMR analyses of CPS extracted from the mutants

361   since the mutants had similar NMR profiles, except for a lower MeOPN content in

362   *mlghC*::*cat* and lack of MeOPN in *wcaG*::*catΔ*. The observed differences for *mlghB*::*cat*

363   and *mlghC*::*cat* were specific to bile salts, as no differences in resistance to SDS were

364   observed for these mutants or with any of the others.

365   CPS protects bacteria against killing by serum, including in *C. jejuni* 81-176

366   (Maue *et al.*, 2013). We investigated whether alterations of CPS composition in our

367   mutants would affect resistance to serum killing. The wild type exhibited 40% survival

368   after 90 min in 30% serum, while non-capsular *kpsM::kan* did not survive in 5% serum

369   (Fig. 8A), indicating that wild type CPS protects *C. jejuni* against serum killing. The

370   *wcaG*::*cat* and *wcaG*::*cat*Δ mutants were resistant to up to 5% serum but highly

371   susceptible to concentrations above 20%, while *mlghB*::*cat* and *mlghC*::*cat* displayed

372   intermediate susceptibility, resisting killing up to ~15% of serum, but being killed by

373   more than 30% serum (Fig. 8A). Time-course experiments were performed using 20%

374   serum, a concentration where maximal differences were observed between strains (Fig.

375   8B). The *kpsM*::*kan* and *wcaG*::*cat*Δ mutants were killed very rapidly while *wcaG*::*cat*

376   resisted slightly longer (Fig. 8B). Together, these results demonstrate that heptose in its

377   3,6-O-Me$_2$-L-*gluco* form, as present in wild type, is essential for CPS-mediated protection

378   against serum killing. In contrast, *mlghC*::*cat* showed wild type killing kinetics for the

379   first 45 min, with a slightly lower survival afterwards (Fig. 8B). Since the only difference

380   between the CPS of *mlghC*::*cat* and *wcaG*::*cat* is the lower amount of MeOPN in

381   *mlghC*::*cat*, we conclude that MeOPN is actually deleterious for resistance to serum

382   within the context of heptose-less mutants. This agrees with the observation that wild

383   type does not produce MeOPN under our growth conditions. Unexpectedly, *mlghB*::*cat*

384   resisted killing better than all strains including wild type for about 45 min, but plummeted

385   beyond that time to reach wild type levels at 90 min. Because we could not distinguish

386   the CPS of *mlghB*::*cat* from that of *wcaG*::*cat* by NMR spectroscopy or Western blotting,

387   their different serum resistance may reflect additional differences in surface properties in

388   the *mlghB::cat* mutant.

389

390   *Disruption of heptose modification pathway does not affect interactions with RAW 264.7*

391   *macrophages*

392

393   Several studies have suggested that *C. jejuni* can survive within murine macrophages for

394   several days (Day *et al.*, 2000; Kiehlbauch *et al.*, 1985; Mixter *et al.*, 2003). To test

395   whether the CPS and its modified heptose protect *C. jejuni* against intracellular killing,

396   the CPS mutants were assayed for survival in RAW 264.7 macrophages. Intracellular

397   survival decreased drastically within a few hours and there was no statistically significant

398   difference between wild type and any of the mutants whether the data were analyzed as

399   percent of original inoculum (Fig. S3A) or percent of initial bacterial load (Fig. S3B),

400   Likewise, no significant differences were observed between heptose modification

401   mutants and wild type regarding adhesion and phagocytosis rates (Fig. S3C and S3D).

402   These data indicate that the modified heptose does not protect against adhesion, uptake or

403   clearance by macrophages. A slight anti-phagocytic role could nevertheless be attributed

404   to wild type CPS as *kpsM*::*kan* was engulfed at higher levels than wild type when

405   interactions were allowed to proceed for at least 2 h (Fig S3D).

406

407   *The heptose modification pathway is important for invasion of intestinal epithelial cells*

408

409   Adhesion to intestinal epithelial cells and their invasion are important for *C. jejuni*

410   pathogenicity (Black *et al.*, 1988; De Melo *et al.*, 1989; Fauchere *et al.*, 1986; Konkel *et*

411   *al.*, 1992) and CPS is thought to be essential for these processes (Bachtiar *et al.*, 2007;

412   Bacon *et al.*, 2001). We therefore assessed our mutants for adhesion and invasion with

413   Caco-2 intestinal epithelial cells. The heptose modification mutants adhered at wild type

414   levels, indicating no role of heptose modification in adhesion (Fig. 9A). Differences

415   among heptose modification mutants were observed for invasion, with abrogated invasion

416   for *mlghB*::*cat* and *mlghC*::*cat*, wild type invasion for *wcaG*::*cat*, and increased invasion

417   for *wcaG*::*catΔ.* Finally, *kpsM*::*kan* was more adhesive and invasive than wild type.

418   Phase contrast microscopy showed that it agglutinated on the surface of Caco-2 cells,

419   which could enhance adhesion, while other mutants did not (data not shown).

420

421   *The heptose modification pathway is important for colonization and persistence in*

422   *chicken intestine*

423

424   *C. jejuni* wild type and mutants were tested for their ability to colonize the intestine of

425   two day old chicks. All mutants were significantly impaired for colonization and/or

426   persistence in the chicken intestinal tract compared to wild type, but differences were also

427   observed between the mutants (Fig. 10). Specifically, *wcaG*::*catΔ* and *mlghC*::*cat*

428   demonstrated a median 5.4 log (p<0.0001) and 6.3 log (p<0.0001) reduction in

429   colonization compared to wild type, respectively. In addition, while infection of all

430   inoculated chicks was observed with wild type, *wcaG*::*catΔ* and *mlghC*::*cat* were only

431   isolated from 6 of 11 (54.5%) and 4 of the 12 (25%) inoculated chicks, respectively. In

432   contrast, bacteria were isolated from all chicks infected with *mlghB*::*cat* and *kpsM*::*kan*

433   and in 12 out of 14 chicks inoculated with *wcaG*::*cat*. All these mutants also persisted

434   significantly less than wild type and demonstrated medians of 1.9 log (p=0.0001), 1.1 log

435   (p=0.0231) and 1.8 log (p=0.0095) reduction in colonization when compared to wild

436   type, for *mlghB*::*cat*, *kpsM*::*kan* and *wcaG*::*cat* respectively. The data indicate that in *C.*

437   *jejuni* ATCC 700819, CPS is important but not essential for colonization of the chick

438   intestine. We speculate that underlying adhesins compensate for the lack of CPS.

439   However, when CPS is present, its wild type composition featuring the modified heptose

440   appears optimal for colonization of the chicken intestinal tract.

441

442   **DISCUSSION**

443

444   To assess the role of the role of heptose and its modification in CPS function, we

445   performed a detailed analysis of *C. jejuni* mutants of the *wcaG*, *mlghB*, and *mlghC* genes,

446   which affect the switch of CPS heptose from a D-*manno* to a L-*gluco* form (McCallum *et*

447   *al.*, 2013). Our results showed that these genes play unexpected roles in transcriptional

448   regulation within the CPS cluster. The *C. jejuni* CPS cluster is similar to group II and III

449   CPS clusters of *E. coli*, whereby conserved CPS transport regions 1 and 3 flank the sugar

450   synthesis region 2 (Karlyshev *et al.*, 1999; Whitfield and Roberts, 1999). Region 3 in the

451   *E. coli* K5 CPS cluster has a σ70 promoter (upstream of *kpsM* included in this region)

452   and RfaH-dependent read-through transcription from this promoter into region 2 is

453   essential for CPS production (Rowe *et al.*, 2000; Stevens *et al.*, 1997). If this also applied

454   to *C. jejuni*, genes from regions 2 and 3 would be transcribed at similar levels or with

455   decreasing levels as their distance from the region 3 promoter increases, without

456   differential regulation of gene expression within region 2. However, we observed

457   differential transcription of CPS modification genes in the wild type strain, which is

458   likely important to fine tune the CPS composition as *C. jejuni* passes from one host to

459   another or is exposed to the environment, as the modified heptose, MeOPN and

460   methylation may each enhance resistance to different adverse conditions. The qRT-PCR

461   data imply the existence of internal promoters within regions 2 and 3, which agrees with

462   a recent RNA-seq study (Dugar *et al.*, 2013). Transcriptional start sites were identified by

463   RNA-seq upstream of *cj1445c, cj1444c*, and *cj1425c* to *cj1429c*, which was consistent

464   with our qRT-PCR findings. This RNA-seq study identified a start site for *cj1447c* within

465   *kpsM* in two strains but not in the strain NCTC 11168 used in our study. We show that

466   transcription of *kpsM* can vary without affecting that of *cj1447c*, indicating that the start

467   site for *cj1447c* is also present in strain NCTC 11168.

468

469   *Implications of heptose modification pathway disruption for CPS composition*

470

471   While it had already been shown that a *mlghC*::*cat* mutant produces heptoseless CPS (St

472   Michael *et al.*, 2002), as we also observed in our study, the CPS composition in

473   *wcaG*::*cat*Δ, *wcaG*::*cat* and *mlghB*::*cat* mutants had to be determined. We found no

474   heptose in *wcaG*::*cat*Δ by NMR spectroscopy analysis, as expected from deletion of its

475   heptose biosynthesis genes. Like for *mlghC::cat*, we also found no modified heptose in

476   *wcaG*::*cat* and *mlghB*::*cat*. The lack of even an unmodified heptose in these mutants

21

477   despite their ability to synthesize GDP-D-*glycero*-D-*manno*-heptose is consistent with the

478   generally high specificity of glycosyltransferases involved in CPS assembly.

479   SDS-PAGE analysis indicated that except for *kpsM*::*kan*, CPS production was not

480   abrogated in the mutants, and lack of heptose did not prevent CPS polymerization.

481   Further, all mutants produced comparable amounts of CPS, although lower hot

482   water/phenol extractability of the CPS produced by *mlghB*::*cat* was observed. This

483   difference in CPS extractability along with its enhanced serum resistance despite equal

484   CPS composition and production suggest additional differences in surface properties in

485   this mutant. It is possible that such additional differences could be mediated by the

486   striking ~187 fold upregulation of *cj1429c* that is observed in this mutant only.

487   The upregulation of the putative heptose methyl-transferase gene in all mutants

488   shown by qRT-PCR did not result in incorporation of methyl heptose in the CPS, as

489   shown by NMR spectroscopy. This could indicate that either methylation occurs after the

490   heptose configuration switch or that methylated *manno*-heptose is not recognized as a

491   substrate by glycosyltransferases for incorporation into CPS. NMR spectroscopy analysis

492   also showed that the MeOPN modification was strongly upregulated in heptose

493   biosynthesis mutants, which may affect the biological function of their CPS.

494

495   *Role of modified heptose on barrier function of CPS*

496

497   *C. jejuni* can resist physiological levels of bile salts (0.1-21 mM) (Lin *et al.*, 2003; Lin *et*

498   *al.*, 2005) and has been isolated from the gall bladder and bile (Gerritsen van der Hoop

499   and Veringa, 1993; Udayakumar and Sanaullah, 2009). In *Listeria monocytogenes* and

500   *Vibrio parahaemolyticus*, upregulation of CPS genes upon exposure to bile salts suggests

501   a role of CPS in bile resistance (Begley *et al.*, 2002; Pace *et al.*, 1997). Therefore, we

502   determined whether CPS as a whole, or its modified heptose, contributes to resistance of

503   *C. jejuni* to bile salts. The concentrations tested (0.25 to 2 g/l) amount to ~ 0.6 to 4.8 mM

504   and fall within the physiological ranges previously reported (Campbell *et al.*, 2004; Lin *et*

505   *al.*, 2003; Perez de la Cruz Moreno *et al.*, 2006). The wild type-like susceptibility of

506   *kpsM*::*kan* suggests that wild type CPS does not provide resistance against bile salts, and

507   the enhanced resistance of the *mlghB* and *mlghC* mutants showed that heptose and its

508   modification as present in wild type CPS actually rendered the strain more susceptible to

509   bile salts. Therefore, the cumulative effects of lack of heptose and methylation, and

510   variations in MeOPN, GroN and EtN contents observed in the mutants, could alter the

511   bacterial surface hydrophobicity and confer slight protective function to CPS via

512   enhanced repulsion of bile salts.

513   Serum complement is an essential innate immune defense against pathogens and

514   the sensitivity of *C. jejuni* strain 81-176 to serum is mitigated by its CPS (Maue *et al.*,

515   2013) as also reported for *Salmonella typhi*, *Klebsiella pneumoniae*, and *B. pseudomallei*

516   (Cortes *et al.*, 2002; Hashimoto *et al.*, 1993; Reckseidler-Zenteno *et al.*, 2005). Our

517   *kpsM*::*kan* mutant confirmed an essential role of CPS in protection against serum in our

518   strain, and the heptose modification mutants showed that the modified heptose in its wild

519   type form is essential for protection. Killing of *C. jejuni* by serum involves activation of

520   the classical complement pathway (van Alphen *et al.*, 2014) but we do not know if *C.*

521   *jejuni* CPS and modified heptose interfere with deposition of critical C3 complement

522   component as observed in other pathogens (Vogel *et al.*, 1997; Woodman *et al.*, 2012).

523   As mentioned above, the slower kinetics of killing of *mlghB*::*cat* at low serum

524   concentration may be due to additional modifications in surface properties since no

525   obvious differences were seen in its CPS. Also, we showed a slightly deleterious effect of

526   MeOPN, which increased serum susceptibility in the absence of heptose. Thus the role of

527   MeOPN in serum resistance appears to depend on other CPS components, which may

528   explain prior conflicting results (Maue *et al.*, 2013; van Alphen *et al.*, 2014). Finally, the

529   protective function of CPS against serum killing may depend on other surface

530   components such as LOS since a LOS mutant lacking sialic acid is serum sensitive

531   despite the presence of CPS in strain 81-176 (Guerry *et al.*, 2000; Guerry *et al.*, 2002).

532   All our mutants produce LOS and the serum sensitivity of *kpsM*::*kan* (that produces LOS

533   but no CPS) shows that LOS does not confer protection against serum killing in strain

534   ATCC 700819. This is consistent with prior studies indicating no role of LOS in serum

535   resistance (Bacon *et al.*, 2001; Keo *et al.*, 2011).

536

537   *Role of CPS and modified heptose on interactions with macrophages and epithelial cells*

538

539   In contrast to prior reports (Day *et al.*, 2000; Kiehlbauch *et al.*, 1985; Mixter *et al.*, 2003),

540   we did not observe prolonged survival of *C. jejuni* in murine RAW 267.4 macrophages

541   and wild type capsule production did not affect intracellular survival or initial adhesion

542   but decreased phagocytosis as determined by comparing wild type to non-capsular

543   *kpsM*::*kan*. The sharp increase in phagocytosis of *kpsM*::*kan* coincided with its

544   agglutination and likely resulted from phagocytosis of clumps of bacteria. It could also be

545   due to exposure of adhesins that are masked by the CPS layer in wild type.

546   The heptose-modification mutants showed that heptose modification and MeOPN

547   addition (that also varies in these mutants) played no role in macrophage / bacteria

548   interactions. Thus, the true biological role of CPS modified heptose is not resistance to

549   clearance by murine macrophages. Different effects may be observed with human or

550   avian macrophages, and may explain the host-dependent pathogenic versus commensal

551   character of *C. jejuni*.

552   We showed that *C. jejuni* ATCC 700819 adhered to and invaded Caco-2 cells at a

553   very low rate, consistently with prior reports (Ashgar *et al.*, 2007; Carrillo *et al.*, 2004;

554   Everest *et al.*, 1992; Ganan *et al.*, 2010; Szymanski *et al.*, 1995; Vijayakumar *et al.*,

555   2006). In contrast to what was observed in strain ATCC 81-176 (Bacon *et al.*, 2001), our

556   non-capsular *kpsM*::*kan* mutant showed increased adhesion and invasion of Caco-2 cells

557   (Fig. 9). Unmasking of adhesins and autoagglutination may each contribute to this

558   phenomenon. The presence of CPS, which limits adhesion to low levels, may prevent

559   overt tissue damage that could lead to faster bacterial clearance.

560   Although heptose is essential for adhesion of pathogenic *E. coli* to HeLa cells

561   (Benz and Schmidt, 2001), our data indicate that *C. jejuni* CPS heptose is not important

562   for adhesion to Caco-2 cells. Variable levels of invasion were observed for the heptose

563   modification mutants but did not correlate directly with NMR structural data concerning

564   heptose and MeOPN contents. For example, abrogated invasion for *mlghB*::*cat* and

565   *mlghC*::*cat* could suggest a role for modified heptose in cell invasion, but the wild type-

566   like and enhanced invasion of heptoseless *wcaG*::*cat and wcaG*::*catΔ*, respectively, does

567   not support this. Likewise MeOPN presence or abundance could not be correlated with

568   invasion, although MeOPN contributes to epithelial cell invasion in strain 81-176 (van

569 Alphen *et al.*, 2014). The variations of invasion however correlated well with the levels

570 of transcription of *cj1429c* in the mutants, and may be due to additional surface

571 differences mediated by *cj1429c*. The increased invasion of *wcaG*::*cat*Δ despite its lack

572 of heptose and MeOPN also suggests that additional components contribute to the

573 process. The data nevertheless indicate that interferences with heptose modification affect

574 invasion. Since invasion can promote tissue damage, transepithelial transport and

575 protection against clearance, our data suggest that the heptose modification pathway is

576 important for host colonization and pathogenicity.

577

578 *Role of CPS and modified heptose on colonization of chicken intestinal tract*

579

580 The chick intestinal colonization data with *kpsM*::*kan* showed that CPS of strain 700819

581 is not essential to establish an infection since all chicks were colonized. This agrees with

582 data obtained with a CPS export mutant of strain ATCC 81116 (Bachtiar *et al.*, 2007).

583 These data do not negate a role for CPS in colonization but may reflect the fact that

584 unmasked adhesins could compensate for lack of CPS. In addition, our data with

585 *kpsM*::*kan* showed that CPS was important for persistence since a 1.6 log reduction of

586 caecum load was observed. This attenuation differs from another study which showed

587 that a *kpsM* mutant could not persist at all in the chicken intestine (Jones *et al.*, 2004).

588 The discrepancy could be due to the use of a hypermotile strain (NCTC 11168H) and of

589 2-week-old chickens that present a more mature gut microflora.

590 Flagella-mediated motility enhances intestinal colonization by *C. jejuni* (Biswas

591 *et al.*, 2007; Wassenaar *et al.*, 1997) but is not essential (Biswas *et al.*, 2007; Wassenaar

592   *et al.*, 1993) as confirmed by colonization of all chicks tested by our non-motile

593   *kpsM*::*kan.* The flagellar apparatus also serves as a secretion system for C. *jejuni* invasion

594   antigens (Cia), which facilitate colonization and persistence (Konkel *et al.*, 1999; Konkel

595   *et al.*, 2004). The *kpsM*::*kan* mutant assembled flagella, indicating that it has the

596   apparatus required for Cia secretion and should thus be able to colonize and persist within

597   the intestinal tract as observed. Also, the increased exposure of adhesins in the absence of

598   CPS may compensate for the lack of motility of this mutant.

599   The data obtained for our heptose modification mutants show a role of modified

600   heptose in colonization and persistence. Indeed *wcaG*::*cat*Δ and *mlghC*::*cat* were

601   impaired both in colonization (less chicks colonized than wild type) and persistence

602   (lower load than wild type), while *wcaG*::*cat* and *mlghB*::*cat* showed mostly defects in

603   persistence (most chicks colonized but at lower levels) (Fig. 10). We assume that the

604   effects are due to the modified heptose independently of MeOPN since a MeOPN

605   transferase mutant of strain 81-176 showed wild type-like chicken colonization (van

606   Alphen *et al.*, 2014), although strain-specific differences cannot be excluded. While all

607   heptose modification mutants were less motile than wild type, we exclude that their

608   decreased colonization was due to decreased motility since the *kpsM::kan* data indicated

609   that the contribution of motility to colonization was limited in this model. Also, the

610   bacterial suspensions used for inoculation were adjusted to the same OD to alleviate

611   interferences from different growth rates, and the highest bacterial load was actually

612   recovered with the slowest growing strain (*kpsM::kan*). Thus factors other than growth

613   and motility were critical for gut colonization and persistence in our heptose mutants.

614   Overall, our data indicate that CPS and its heptose affect the efficiency of chicken

615   gut colonization and persistence. It is likely that *C. jejuni* modulates CPS expression and

616   composition *in vivo* at different stages of colonization as seen in other intestinal

617   pathogens such as, for example, the Vi-antigen of *S. typhi* (Jones *et al.*, 2004). This is

618   plausible for *C. jejuni* as expression of CPS is phase variable (Bacon *et al.*, 2001) and co-

619   culture of *C. jejuni* with epithelial cells reduces surface CPS expression, thereby

620   highlighting the dynamic role of CPS in interactions with host cells (Corcionivoschi *et*

621   *al.*, 2009). Our data suggest that suppressed CPS expression during infection exposes

622   surface adhesins that lead to increased agglutination of *C. jejuni*, facilitating adhesion in

623   clumps and increasing invasion. Resumed CPS production and fine-tuning of its

624   composition may favor persistence at other stages of infection, affecting differentially its

625   barrier functions and its effects on intracellular survival. Our discovery of internal

626   promoters that likely allow transcriptional optimization of CPS composition in response

627   to environmental stimuli is in line with this hypothesis.

628   Collectively, the data presented in this study indicate a role of *wcaG*, *mlghB*, and

629   *mlghC* in determining CPS composition and biological function. The mutants

630   demonstrate very different behaviors in terms of CPS barrier functions, interactions with

631   different cell types and motility, which culminate in different outcomes concerning

632   colonization and persistence in the chicken intestinal tract. Overall, fine tuning of CPS

633   composition via modified heptose incorporation and other modifications that seem to be

634   interdependent with heptose modification, allows reaching an optimal configuration that

635   contributes to survival of the bacterium when confronted with various host defenses

636   along the gastrointestinal tract (bile salts, serum and phagocytic cells) and also

637   contributes to persistence of the bacterial population via invasion of intestinal cells. This

638   investigation illustrates the impact that altering CPS composition can have on CPS

639   function and on the virulence of encapsulated bacteria and suggests that the enzymes

640   responsible for the biosynthesis of CPS components such as the modified heptose could

641   be suitable targets for future therapeutic research.

642

643

644   **EXPERIMENTAL PROCEDURES**

645   *Bacterial culture conditions*

646

647   All experiments were carried out with strain NCTC 11168 / ATCC 700819 (HS: 2 Penner

648   serotype). *C. jejuni* was grown in a microaerobic atmosphere (85% N_2, 10% CO_2, and 5%

649   O_2) at 37˚C on Trypticase Soy Agar (TSA) supplemented with 5% sheep blood and 10

650   μg/ml vancomycin and 5 μg/ml trimethoprim as background antibiotics. Where

651   appropriate, the medium was supplemented with 15 μg/ml chloramphenicol or 30 μg/ml

652   kanamycin. Unless stated otherwise, *C. jejuni* was grown overnight from freezer stock on

653   TSA containing appropriate antibiotics, followed by a further growth on TSA containing

654   only the background antibiotics for 20-24 hours. The cells were harvested and normalized

655   to the same OD_{600nm} before use for phenotypic analyses. Viability of the bacterial

656   suspensions used as inoculum for phenotypic assays was assessed by CFU measurements.

657   For transformation, *C. jejuni* was grown on Mueller Hinton (MH) agar (BD, Canada),

658   containing 10 μg/ml vancomycin, 5 μg/ml trimethoprim, 0.05% pyruvate, and 5% heat

659   inactivated Fetal Bovine Serum (Sigma Aldrich). *E. coli* strain DH5α was used for

660   cloning and was grown in Luria Bertani medium. When necessary, 100 μg/ml ampicillin,

661   30 μg/ml kanamycin, or 34 μg/ml chloramphenicol were added.

662

663   *Preparation of knockout constructs*

664

665   The *wcaG* (a.k.a. *cj1427c*), *mlghC* (a.k.a. *cj1428c*), *mlghB* (a.k.a. *cj1430c*) and *kpsM*

666   (a.k.a. *cj1448c*) genes were amplified via Polymerase Chain Reaction (PCR) from

667   chromosomal DNA of *C. jejuni* strain ATCC 700819 using primers *cj1427c* P2/P3,

668   *cj1428c* P2/P3, *cj1430c* P2/P3, and *kpsM* P2/P3 (Table 4). The PCR was performed using

669   Expand Long Range DNA polymerase (Roche) according to Manufacturer's instructions.

670   For *wcaG*, *mlghB* and *mlghC*, the PCR products were cut with BamHI and NcoI and were

671   ligated to a pET23 vector derivative (Newton and Mangroo, 1999) that had been cut with

672   the same enzymes. The *kpsM* gene was cloned into the EcoRV site of pBluescript KS(+),

673   following blunting of the PCR product by T4 DNA Polymerase (Roche, Canada). After

674   transformation of ligation reactions into *E. coli* DH5α and selection on ampicillin, the

675   resulting plasmids (pET/*wcaG*, pET/*mlghC*, pET/*mlghB* and pBluescriptKS/*kpsM*) were

676   extracted and checked by DNA sequencing (Robarts Research Institute, London,

677   Ontario).

678   Disruption constructs were generated by performing inverse PCR on pET/*wcaG*,

679   pET/*mlghC*, pET/*mlghB* using primers *cj1427c* P4/P5, *cj1428c* P4/P5, *cj1430c* P4/P5,

680   which contain KpnI and ApaI sites. The chloramphenicol resistance cassette (*cat*) was

681   amplified from plasmid pRY111 (kindly provided by P. Guerry, Naval Medical Research

682   Center, MD, USA) using primers Catcoli P2 and CatColi P3 which contained ApaI and

683   KpnI sites, respectively. The amplicon was subsequently ligated into the inverse PCR

684   reaction products following ApaI and KpnI digestion. The constructs were introduced

685   into *E. coli* DH5α and transformants were selected on ampicillin and chloramphenicol.

686   The resulting pET/*wcaG::cat*, pET/*mlghC::cat*, and pET/*mlghB::cat*, constructs were

687   extracted and checked by DNA sequencing**.** For the disruption of *kpsM*, inverse PCR was

688   used to amplify pBluescriptKS/*kpsM* with the primers *kpsM* P4/P6 that contain BglII and

689   NcoI sites, respectively. The kanamycin resistance cassette (*kan*) was amplified from

690   plasmid pHel3 (Heuermann and Haas, 1998) using Aph3P1 and Aph3P2, which also

691   contained BglII and NcoI restriction sites. The *kan* cassette was subsequently ligated into

692   the pBluescriptKS/*kpsM* inverse PCR reaction product after BglII/NcoI digestion.

693   Following *E. coli* DH5α transformation, transformants were selected on ampicillin and

694   kanamycin. The resulting pBluescriptKS/*kpsM*::*kan* construct was checked by DNA

695   sequencing.

696

697   *Generation of knockout mutants in* C. jejuni *ATCC 700819*

698

699   The *wcaG*::*cat*, *mlghB*::*cat*, *mlghC*::*cat*, and *kpsM*::*kan* fragments were amplified using

700   gene specific primers P2 and P3 (Table 4) containing NcoI and BamHI restriction sites

701   respectively. *C. jejuni* chromosomal DNA digested with the same restriction enzymes

702   was ligated to either end of the PCR fragments. The DNA was introduced into *C. jejuni*

703   via natural transformation using 0.03% saponin (Nuijten *et al.*, 1989; Vijayakumar *et al.*,

704   2006). Potential transformants were selected on MH agar containing 15 μg/ml

705   chloramphenicol or 30 μg/ml kanamycin, The transformants were checked by PCR using

706   gene specific and antibiotic cassette specific primers, as well as by Southern blotting

707   using digoxigenin (Roche) labeled DNA probes.

708   As an alternative to obtain the *wcaG* mutant, the pET/wcaG*::cat was* methylated

709   *in vitro* as described by others (Donahue *et al.*, 2000). Ten μg of plasmid DNA were

710   treated with a cell-free extract of *C. jejuni* (containing 300-400 μg of proteins) in the

711   presence of 200 nM S-adenosyl methionine (Sigma Aldrich). Methylation was carried out

712   at 37°C for 1 hour in "methylation" buffer (20 mM Tris-acetate (pH 7.9), 50 mM

713   potassium acetate, 5 mM Na$_2$EDTA, 1 mM dithiothreitol (DTT)) in a total volume of 200

714   μl. To obtain a cell-free extract, wild type *C. jejuni* was harvested from five TSA plates

715   and resuspended in 4 ml of "methylation" buffer supplemented with protease inhibitor

716   cocktail (Roche). This was passed through a French pressure cell press (Thermo

717   Scientific) five times. The amount of protein in the cell-free extract was quantitated by

718   Bradford Assay (Bradford, 1976). Following methylation, phenol:chloroform:isoamyl

719   alcohol (in a 25:24:1 v:v:v ratio) was used to purify the DNA which was concentrated by

720   sodium acetate/ethanol precipitation. The plasmid was introduced in *C. jejuni* by natural

721   transformation as above, using 70 μl of cells resuspended at 10E9 cells/ml and 2 μg of

722   DNA in a total volume of 80 μl, with recovery of ~ 8-10 h prior to selection on

723   chloramphenicol.

724

725   *Preparation of complemented strains*

726

727   Complementation was performed by chromosomal integration in the 16S - 23S rRNA

728   region as reported previously (Karlyshev and Wren, 2005). All primers used are listed in

729   Supplementary Table 1. The 16S – 23S rRNA region was amplified from genomic DNA

730   using primers 16SrRNATop and 23SrRNABottom and was inserted into the KpnI/NotI

731   sites of pBluescript KS (+). Clones were obtained using *E. coli* DH5α and selection with

732   ampicillin. Separately, the primer overlap extension method (Heckman and Pease, 2007)

733   was used to fuse each gene of interest to the *ompE* promoter and to a *kan* resistance

734   cassette. The promoter was amplified using primer ompEFor combined with

735   ompERev1427, ompERev1428, or ompERev1430. The genes to be complemented were

736   amplified using primer pairs 1427ForOmpE / 1427RevKan, 1428ForOmpE /

737   1428RevKan, or 1430ForOmpE / 1430RevKan. The *kan* cassette was amplified using

738   primer AphP3 combined with KanFor1427, KanFor1428 or KanFor1430. All fragments

739   were fused together by PCR using standard conditions. The fusions were then inserted

740   into the XbaI site of the 16S–23SrRNA-containing vector from above to generate the

741   final complementation constructs. Clones were selected with ampicillin and kanamycin

742   and sequenced (Robarts Sequencing Facility, London, Ontario). The constructs were

743   transformed into their respective mutants following *in vitro* methylation as reported

744   above. Clones were selected on ampicillin and kanamycin and analysed by PCR to check

745   integration of the constructs.

746

747   *Real-time PCR analyses*

748
749   Messenger RNA was extracted from *C. jejuni* using the GE-Healthcare mRNA midiprep

750   kit, and cDNA synthesis was performed using the Biorad cDNA biosynthesis kit and

751   random hexanucleotides following Manufacturer's instructions. Real-time PCR was

752   performed on a Rotor-Gene 6000 (Corbett Life Science, Canada) using SYBR green

753   Supermix (Biorad) and gene-specific primers (Table 4). The optimal annealing

754   temperature and primer concentrations were determined for each gene. The primer

755   efficiencies were determined from standard curves established using serial dilutions of

756   genomic DNA under optimal annealing temperature and primer concentrations. Negative

757   controls for the reverse transcription did not contain the reverse transcriptase, while

758   negative controls for the real time PCR did not contain template cDNA. The relative gene

759   expression for each gene was calculated using the Pfaffl equation (Pfaffl, 2001):

760     $\text{Ratio} = (E_{\text{target}})^{\Delta\text{CTtarget(WT-mutant)}}/(E_{\text{reference}})^{\Delta\text{CTref(WT-mutant)}}$.

761   In this equation, target refers to the gene being studied and the reference is the

762   housekeeping *cj1537c* gene, which encodes acetyl-CoA synthase (Parkhill *et al.*, 2000). E

763   is the primer efficiency, and C_T corresponds to the number of PCR cycles necessary to

764   observe a fluorescence signal above a set threshold.

765

766   *SDS-PAGE and Western blotting analysis of CPS*

767

768   Total surface carbohydrates (CPS and LOS) were extracted via SDS solubilization of *C.*

769   *jejuni* cells (Hitchcock and Brown, 1983), followed by digestion of proteins by proteinase

770   K treatment. They were separated on a 15% SDS-PAGE 5 cm mini gel and analyzed by

771   Western blotting using Penner serotyping antibody HS:2 (kindly provided by Dr. M.

772   Karmali, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph,

773   Ontario) since strain ATCC 700819 belongs to the HS:2 Penner serotype (Karmali *et al.*,

774   1983; Penner *et al.*, 1983) and CPS contributes to serotype specificity (Karlyshev *et al.*,

775   2000). Detection was performed with a fluorescently labeled anti-rabbit secondary

776   antibody (IRDye 680 Goat Anti-Rabbit, Licor Biosciences) on an Odyssey Infrared

777   Imaging system (Licor Bioscience). Alternatively, CPS was extracted by hot water

778   phenol (see below), ran on a 13 cm long gel and detection was performed by silver

779   staining (Fomsgaard *et al.*, 1990).

780

781   *CPS isolation by the hot water phenol method*

782

783   *C. jejuni* ATCC 700819 wild type and mutants were grown on one hundred TSA plates

784   for 24 hours and harvested in saline. The cells were then inoculated into 10 L of Brucella

785   broth, containing 7.5% heat inactivated horse serum and 25 mM sodium pyruvate to an

786   OD_{600nm} of approximately 0.05. After 24 hours of growth (OD_{600nm} approximately 0.3),

787   the bacteria were spun down at $4200 \times g$ (Avanti J-25I, Beckman-Coulter) for 30 min and

788   the pellets were lyophilized. Purification of CPS was performed by hot water/phenol

789   extraction (Westphal and Jann, 1965). Briefly, 2 g of dry cell pellet were re-suspended in

790   20 ml of MilliQ water pre-heated to 68°C. An equal amount of phenol (Fisher) preheated

791   to 68°C was added to the pellet and sealed in a 50 ml conical tube. The samples were

792   incubated at 68°C for 10 min with rapid stirring. They were allowed to cool to 10°C on

793   ice and centrifuged for 30 min at $6300 \times g$ (Eppendorf 5810R) and 10°C. The aqueous

794   (top) phase was collected, and an equal amount of water was added to the remaining

795   organic phase. The procedure was repeated three times and the aqueous phases were

796   pooled. The aqueous phases were dialyzed (molecular weight cut off 12-14000 Da)

797   against running water for 2-3 days until no phenol remained. The samples were

798   lyophilized and re-suspended in double distilled water. Ultracentrifugation of the samples

799   for 30 hours at 4°C and 110000 × g (Optima Max-XP Ultracentrifuge, Beckman-Coulter)

800   pelleted most of the LOS, while the CPS remained in the supernatant. The samples were

801   lyophilized and re-suspended in 500 μl double distilled water. Treatment with 200 mg of

802   Proteinase K was carried out for 2 hours at 60°C to degrade any remaining proteins. The

803   CPS samples were stored at -20°C until further required.

804

805   *Sugar analysis*

806
807   CPS samples (0.2 mg each) were hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), and the

808   monosaccharides were identified using a Biotronik LC-2000 sugar analyzer (Germany)

809   on a column (15 × 0.4 cm) of a Dionex A×8-11 anion-exchange resin and Ostion LC AN

810   B cation-exchange resin (7.5 cm column each). Sugars were eluted with a stepwise

811   gradient of 0.17 M (20 min) and 0.3 M sodium borate buffer pH 8.0 at 0.5 ml min^{-1} and

812   detected with the bicinchoninate reagent.

813

814   *Nuclear magnetic resonance spectroscopy*

815

816   Samples were deuterium-exchanged by freeze-drying twice from 99.9% D_2O and then

817   examined as solutions in 99.95% D_2O at 30° C on NMR spectra were recorded on an

818   Avance II 600 spectrometer (Bruker, Germany) using internal sodium 3-

819   (trimethylsilyl)propanoate-2,2,3,3-d$_4$ (δ_H 0, δ_C -1.6) or 85% H_3PO_4 (δ_P 0.0) as references

820   for calibration. The ^1H and ^{13}C NMR spectra were assigned partially using two-

821   dimensional $^1H,^1H$ correlation spectroscopy (COSY), total correlation spectroscopy

822   (TOCSY), rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY),

823   ^{1}H,^{13}C heteronuclear single-quantum coherence (HSQC), and ^{1}H,^{31}P heteronuclear

824   multiple-bond correlation (HMBC) experiments, which were performed using standard

825   Bruker software. A mixing time of 100 ms was used in the TOCSY and ROESY

826   experiments, and a 65-ms delay was used for evolution of long-range coupling in the

827   ^{1}H,^{31}P HMBC experiment. TopSpin 2.1 (Bruker) program was used to acquire and

828   process the NMR data.

829

830   *Growth curves assays*

831

832   A 100 ml sidearm flask containing 20 ml MH broth and background antibiotics (pre-

833   saturated with 85% N_2, 10% CO_2, and 5% O_2) was inoculated with *C. jejuni* cells grown

834   on TSA at a starting OD_{600nm} of 0.075. The flask was sealed and incubated with shaking

835   (120 rpm) at 37˚C for up to 48 h. Growth was monitored over time using a Klett

836   Summerson photocolimeter.

837

838   *Motility assays*

839

840   *C. jejuni* was harvested in MH broth and adjusted to OD_{600nm} of 1.0 or 2.0. Motility plates

841   (0.3% agar in MH) were stabbed in triplicate with the wild type or mutants and incubated

842   for 24 h under microaerobic conditions at 37˚C. The diameter of the motility halo was

843   monitored over time.

844

845   *Bile salts susceptibility assay*

846

847   Following growth on TSA as described above, *C. jejuni* was harvested and washed once

848   in saline and re-suspended in saline to an OD_{600nm} of 0.1. Bile salts (50% cholate and

849   50% deoxycholate, Sigma Aldrich) were diluted to the appropriate concentrations (0-2

850   g/l) and 90 µl were aliquoted into the wells of a 96 well plate. Ten µl of the bacterial

851   suspension were added to each well and incubated in microaerobic conditions at 37°C for

852   15 minutes. The samples were washed once in TSB media and serially diluted for CFU

853   counts.

854

855   *SDS susceptibility assays*

856

857   For SDS sensitivity assays, different concentrations (0.002 to 0.03%) of SDS were

858   prepared in TSB (supplemented with vancomycin and trimethoprim). A volume of 170

859   µL of each SDS concentration was added to the wells of a 96 well plate. *C. jejuni* wild

860   type and mutants were grown as previously described and re-suspended in TSB to an

861   $OD_{600\ nm}$ 1.0. To each well, 30 µl of bacterial suspension was added, and control wells

862   were set up which contained no SDS. The plates were incubated at 37˚C with shaking at

863   180 rpm in microaerobic conditions for 15 h, at which point the OD_{600nm} was read. The

864   data were normalized to controls which did not receive any SDS treatment.

865

866   *Serum susceptibility*

867

868   Fresh rabbit blood obtained from two rabbits was allowed to clot at room temperature.

869   The serum was separated from the clot and centrifuged for 15 minutes at 10,000 rpm at

870   4°C to remove remaining blood cells. Half of the serum was inactivated by incubation at

871   56°C for 1 hr. Bacteria grown for 16-20 h on TSA were washed in saline and re-

872   suspended to an OD_{600nm} of 0.1. Serum (inactivated or not) was re-suspended in saline to

873   the appropriate concentration (0% - 100%) and 90 μl were aliquoted into the wells of a 96

874   well plate. To the wells, 10 μl of bacteria were added. The plates were then incubated

875   with shaking (100 rpm) for 1.5 h in microaerobic conditions at 37°C. Following

876   incubation, 100 μl TSB was added to the wells and the samples were serially diluted and

877   plated for CFU counts. For time course experiments, bacterial samples were exposed to

878   20% pooled rabbit serum over 1.5 h.

879

880   *Phagocytosis and survival within macrophages*

881

882   RAW 267.4 murine macrophages (obtained from ATCC) were grown in Dulbeco's

883   Modified Eagles Medium (DMEM) containing 10% heat inactivated FBS, and passaged

884   every 3-4 days to a maximum of 5 passages. Macrophages were seeded at $\sim 2*10^5$ cells

885   per well in 6-well plates, and incubated overnight in DMEM (containing 10% inactivated

886   FBS). They were counted (final counts about 10^6 per well after incubation) and *C. jejuni*

887   bacteria that had been resuspended in tryptic soy (TS) broth and adjusted at the

888   appropriate OD were added at a multiplicity of infection (MOI) of 1:100. After addition

889   of the bacteria, the media composition was 97% (DMEM with 10% FBS) and 3% TS

890   broth. The plates were centrifuged for 1 min at 300 *x g* to synchronize bacteria /

39

891   macrophage interaction. For adhesion experiments, the plates were incubated at 4°C for

892   30 minutes to promote adhesion in the absence of phagocytosis (Peterson *et al.*, 1977),

893   washed with cold PBS five times and lysed in water. Control experiments showed that all

894   strains resisted the water treatment (data not shown). The bacteria were then serially

895   diluted and plated for CFU counts. For the time course of intracellular survival, the

896   macrophages were exposed to the bacteria for 2 h at 37°C in a CO_2 incubator. The cells

897   were then washed three times with PBS and incubated with DMEM containing 225 μg/ml

898   gentamicin for 1 h to kill extracellular bacteria. The macrophages were then washed and

899   incubated in DMEM for up to 3 h. At each time point, the macrophages were washed

900   three times with PBS and lysed in water (20 min). The samples were serially diluted and

901   plated for CFU counts. For infection time course experiments, the macrophages were

902   exposed to the bacteria for various lengths of time. The macrophages were washed in

903   PBS, treated with gentamicin, and lysed in water, as described above. The surviving

904   bacteria were enumerated by CFU counting.

905

906   *Adhesion and invasion of wild type and mutants to Caco-2 cells*

907

908   Caco-2 cells (obtained from ATCC) were grown for 3 days in DMEM containing 25 mM

909   glucose and supplemented with 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino

910   acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin until

911   they formed a confluent monolayer (approximately 650,000 cells per well in 24-well

912   plates) and were fully differentiated. The differentiation state of the cells was determined

913   by measuring the hydrolysis of *p*-nitrophenyl phosphate in a standard colorimetric assay

914   (detection at 405 nm) which reflects the production of alkaline phosphatase and is a

915   hallmark of differentiation (Ferruzza *et al.*, 2012). The cells were infected for 5 h with *C.*

916   *jejuni* at an MOI of 1:100. The bacteria were prepared as indicated for the macrophage

917   experiments. The plates were centrifuged briefly (500 *x g* for 5 min at room temperature)

918   to maximize contact between the bacteria and the cell monolayer. To determine total

919   bacterial cell association (adhering and internalized bacteria), Caco-2 cell monolayers

920   were washed 3 times, lysed with 0.1 % Triton X-100 for 10 min and CFUs were

921   determined by plating serial dilutions. To determine the number of internalized bacteria,

922   the infected monolayers were treated with 200 μg/ml gentamicin for 2 h to kill

923   extracellular bacteria. The cells were then washed and treated as above to determine

924   bacterial viable counts.

925

926   *Autoagglutination assay*

927

928   The autoagglutination assay was performed as previously described (Misawa and Blaser,

929   2000). Briefly, 2 ml of sterile PBS were inoculated with *C. jejuni* at an OD_{600nm} of 1.0. At

930   time points 0, 1 and 2 h, the top 1 ml from each tube was carefully removed and the

931   optical density was read at 600 nm. Live images were acquired using an Axioscope 2

932   (Carl Zeiss) microscope with a 100× oil immersion objective coupled to a Qimaging

933   (Burnaby) cooled charged-coupled device camera.

934

935   *Electron microscopy (EM)*

936

937   EM was performed at the EM facility of the department of Microbiology and

938   Immunology led by Dr. S. Koval with uranyl acetate staining as previously described

939   (Merkx-Jacques *et al.*, 2004).

940

941   *Chicken colonization assays*

942

943   Chicken colonization assays were performed as previously described (Vijayakumar *et al.*,

944   2006). *C. jejuni* was grown on TSA agar containing 5% sheep blood under microaerobic

945   conditions at 42°C for 24 hours. The bacteria were re-suspended in PBS pH 7.4, and

946   adjusted to an OD_{600nm} of 0.35 (approx. 10E9 CFU/ml) and diluted 1:10 in PBS. Two-

947   day-old white-leghorn specific pathogen-free chicks were orally administered 100 µl

948   (10E7 CFU) of either the *C. jejuni* wild type or one of the mutants. Five days later, the

949   chicks were euthanized, the caeca and their contents were harvested, weighed and

950   homogenized. Viable counts were obtained from serial dilutions of samples that were

951   plated on Campylobacter selective medium (CSM) plates (Quélab Inc., Montreal) for 48

952   hours.

953

954   **ACKNOWLEDGEMENTS**

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1380   lipopolysaccharides of *Campylobacter jejuni* isolated from patients with Fisher's
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1382
1383

1384 **Table 1: Sugar and NMR spectroscopy analyses of hot water / phenol extracted CPS**

1385 **from the wild type *C. jejuni* and its isogenic mutants.**

1386 Monosaccharide analysis was conducted with a sugar analyzer after full acid hydrolysis.

1387 Numbers in the columns under "Sugar analysis data" represent the amounts of various

1388 sugars present, after normalization to GalN. A plus (+) or minus (-) in the NMR

1389 spectroscopy data columns represent either the presence or the absence of the specified

1390 component, respectively. GalN = galactosamine (from GalNAc), Rib = ribose, Gal =

1391 galactose (probably from the lipooligosaccharide), GlcA = glucuronic acid, Hep =

1392 heptose, Me = methyl, MeOPN = O-methyl phosphoramidate, EtN = ethanolamine, GroN

1393 = 2-amino-2-deoxyglycerol.

1394

	Sugar analysis data			NMR spectroscopy data				
	GalN	Rib	Gal	GlcA	Hep	3,6-O-Me$_2$ on Hep	MeOPN on GalNAc	Substituent on GlcA
Wild type	1	2.6	0.1	+	+	+	-	EtN
wcaG::*cat*	1	1.2	trace	+	-	-	+	EtN, GroN
mlghC::*cat*	1	3.6	0.15	+	-	-	trace	EtN, GroN
mlghB::*cat*	1	1.4	0.18	+	-	-	+	EtN, GroN
wcaG::*catΔ*	1	0.9	trace	+	-	-	-	EtN, GroN
kpsM::*kan*	1	2.7	trace	Not analyzed				

1395
1396

1397   **Table 2: ^{1}H and ^{13}C NMR data for the CPS isolated from *C. jejuni* wild type and**

1398   **isogenic mutants *wcaG*::*cat*, *mlghB*::*cat*, *mlghC*::*cat* and *wcaG*::*cat*Δ.**

1399   [a]: Data from this study.

1400   [b]: The mutant *mlghC*::*cat* had essentially the same chemical shifts.

1401   [c]: The mutant *wcaG*::*cat* had essentially the same chemical shifts.

1402   [d]: Published data (McNally *et al.*, 2007).

1403   [e]: Values without/with the MeOPN modification at position 3.

1404

Sugar residue	Atom or position	Type	Chemical shift (ppm)	Wild type[a]	Mutants *wcaG*::*cat*Δ [a,b]	Mutants *mlghB*::*cat* [a,c]	NCTC 11168H[d]
Rib	1	CH	δ_H	5.36	5.38	5.38	5.36
			δ_C	106.3	105.9	106.0	106.1
GalNAc	1	CH	δ_H	4.97	4.98	5.03	5.08/5.02[e]
			δ_C	106.1	106.7	106.9	105.1/104.3[e]
	3	CH	δ_H	4.25	4.17	4.76	4.93/4.23[e]
			δ_C	75.4	76.8	81.0	78.8/73.9[e]
	3	MeOPN	δ_H	none	none	3.77	3.77
			δ_C	none	none	55.0	54.6
			δ_P	none	none	13.6	not indicated
GlcA	1	CH	δ_H	5.14	5.22	5.22	5.12
			δ_C	99.0	99.0	99.0	98.8
Hep	1	CH	δ_H	5.58	none	none	5.58
			δ_C	98.3	none	none	97.9
	3	MeO	δ_H	3.63	none	none	3.61
			δ_C	60.8	none	none	60.7
	6	MeO	δ_H	3.56	none	none	3.55
			δ_C	60.8	none	none	59.3

1405
1406

1407

1408   **Table 3. Comparison of motility for wild type and mutant strains.**

1409   The motility of the wild type and mutant strains were measured and compared

1410   quantitatively after stabbing into 0.3% agar and incubation for 48 hours under

1411   microaerobic conditions. The values shown are for the diameters of the motility halos,

1412   measured in centimeters. The mean and standard errors (SE) of three independent

1413   experiments are shown for each strain. In addition, the *p* value, as determined by one-way

1414   ANOVA, is shown. Compared to the wild type, all mutant strains were significantly

1415   reduced in motility.

1416

C. jejuni strain	Diameter of halo (cm) ± SE	*p* value
wild type	3.09 ± 0.09	N/A
wcaG::*cat*	2.35 ± 0.08	< 0.01
mlghC::*cat*	2.77 ± 0.06	< 0.01
mlghB::*cat*	2.73 ± 0.06	< 0.01
wcaG::*cat*Δ	2.38 ± 0.10	< 0.01
kpsM::*kan*	0.00 ± 0.00	< 0.01

1417

1418

1419

1420   **Table 4: List of primers used to construct mutants and perform qRT-PCR analyses.**

1421   When applicable, the restriction sites are indicated in bold letters.

1422

Primer name	Sequence (5' – 3')
Aph3 P1	GA**AGATCT**GATAAACCCAGCGAACCA
Aph3 P2	AG**GGTCCA**TGGAGACATCTAAATCTAGGTAC
CatColi P2	GTC**GGTACC**TTATTTATTCAGCAAGTCTTG
CatColi P3	GTCATC**GGGCCC**TTCCTTTCCAAGTTAATTGC
cj1427c P2	AG**GGTCCA**TGGGCATGTCAAAAAAGTTTTAATTAC
cj1427c P3	GCGTC**GGATCC**TTAATTAAAATTTGCAAAGCGA
cj1427c P4	GTG**GGTACC**TAGAATGAGACTTGA
cj1427c P5	CTGCTA**GGGCCC**ATATTCTGAGATAGG
cj1428c P2	AGGTA**CCATGG**GCATGCAAACAAATTCAAAAATATAA
cj1428c P3	GCT**GGATCC**TCAATTTTGTGTTTTATACCA
cj1428c P4	GTG**GGTACC**TGTAGCTATCTATACGATGC
cj1428c P5	CTGCTA**GGGCCC**CTCAGGATACATATACCCA
cj1430c P2	AGGGT**CCATGG**CAATAGAATTTGATATA
cj1430c P3	GCGTC**GGATCC**TTATCCTTTATTTTTAGTTGCAA
cj1430c P4	GTG**GGTACC**AAATATGGGAAATTCT
cj1430c P5	CTGCTA**GGGCCC**ATTTTAAATAGGTTGG
kpsM P2	A**GGTACC**ATGGTGAGTTATGATTATAGTTTATG
kpsM P3	GCT**GGATCC**TAGATTAATTAACTTTATCATTC
kpsM P4	GA**AGATCT**TGTATTTCCTGTTCATTGC
kpsM P6	AGGTA**CCATGG**AGTTCTAGCAATAAATACATG
RT1425F	ACCACTTGGTACATCCGAATAGGT
RT1425R	TGCGACTATATCTTTATACATACATTG
RT1426F	TTATCGACATAAAGCATCTTGTAAAAAA
RT1426R	ATTGATAATCATCAGCAAGCTAGGAA
RT1427F	CCGCATGCACTTTATCGATCCCA
RT1427R	CTGATATTATTATTCCTCTAGCTGCT
RT1428F	AAATCACCTTGAAATAAATATTCCTCTT
RT1428R	CAAGCGGTTGCTAAATTTTTTAAAGAA
RT1430F	TTTTATAACTTATAATAAATTTCTCCCATT
RT1430R	ATTTGGACAGCTTTTACAGATGAATAT
RT1444F	TGAATCCGAACTAAGTCCTTGATAAA
RT1444R	AGTGGTGGATTCTCAAGGAAATATTT
RT1445F	AAGGTAAATCTATATGTTGCTCTTGAT
RT1445R	ATACATTGATTGCAGCACCAAGATAT
RT1447F	CCTTTATTTAGTGGTGGAAGACATTA
RT1447R	TTATCTCTAGCTGTTAAAGAACCTTG
RTKpsMF	ATTACAAAATAAATACAAAACTCAAGTAAA
RTKpsMR	GTTAGAGAATATCATCATCAAGTTATG
RTAcCoAF	AATGTCTTGATCGTCATATGAAAACAA
RTAcCoAR	TAGCCCCAATCCTTGCACAAGCT

1423

1424   **FIGURE LEGENDS:**

1425   **Fig. 1: Schematic representation of the CPS from *C. jejuni* ATCC 700819 and**

1426   **organization of its CPS gene cluster. Panel A:** The CPS unit comprises 4 sugars,

1427   including a 3-sugar linear backbone (sugars 1-3) that is attached to a phospholipid (PL)

1428   for anchorage in the membrane and a modified heptose (H) branch. The exact structure is

1429   also indicated and denotes additional modifications such as O-methyl phosphoramidate

1430   (MeOPN on GalNAc and heptose) and addition of ethanolamine (EtN) or 2-amino-2-

1431   deoxyglycerol (GroN) on GlcA (Karlyshev *et al.*, 2005; McNally *et al.*, 2007; St Michael

1432   *et al.*, 2002). **Panel B:** Global organization of the CPS gene cluster (Karlyshev *et al.*,

1433   2005; Parkhill *et al.*, 2000) showing the tripartite organization of CPS genes as well as

1434   the genes relevant to this study. Diagram not to scale.

1435

1436   **Fig. 2: Simplified biochemical pathway highlighting the role of enzymes encoded by**

1437   ***wcaG*, *mlghB* and *mlghC* in the synthesis of L-*gluco*-heptose.** Intricacies of this

1438   pathway were described earlier (McCallum *et al.*, 2011; McCallum *et al.*, 2012;

1439   McCallum *et al.*, 2013). This figure only highlights the linear pathway from the *manno*-

1440   heptose precursor to the final product (additional intermediates excluded) as well as the

1441   side branch mediated by WcaG that decreases product formation via substrate

1442   scavenging. R represents OH or OMe. It is not known when methylation by *cj1426c*

1443   occurs along this pathway (as denoted by ?).

1444

1445   **Fig. 3: SDS-PAGE analysis of the CPS produced by wild type and mutants. Panel A:**

1446   CPS extracted by hot water/phenol method was analyzed on a 13 cm long gel with

1447   detection by silver staining. **Panel B:** CPS obtained by SDS solubilization of total cells

1448   and proteinase K digestion of all proteins was analysed on a 5 cm gel. Detection was

1449   performed with anti-HS:2 Penner serotyping antibody (kindly supplied by Dr M.

1450   Karmali). The *wcaG*::*cat* mutant is not represented on panel B as no antibody was

1451   available anymore when the proper mutant was obtained.

1452

1453   **Fig. 4: CPS structures of the wild type strain and the mutants.** The structures are

1454   based on the data presented in Tables 1 and 2 and in supplementary Figures S1 and S2,

1455   and also take into account previously published data for the wild type strain (McNally *et*

1456   *al.*, 2007; St Michael *et al.*, 2002; Szymanski *et al.*, 2003)**.**

1457

1458   **Fig. 5: qRT-PCR analysis of expression of capsular genes in the wild type and**

1459   **mutants. Panel A:** Intra-strain comparison where the level of transcription of each gene

1460   is expressed relatively to housekeeping gene acetyl-CoA synthase in the same strain.

1461   **Panel B:** Inter-strain comparison where data from panel A are expressed relatively to

1462   wild type (Pfaffl, 2001). Cat and Kan denote the antibiotic resistance cassettes used for

1463   gene inactivation. Crosses for *wcaG*::*cat*Δ denote deleted genes. Additional genes deleted

1464   in this mutant are not shown as their levels of transcription were not investigated. Small

1465   arrows indicate promoters inferred from differential transcription of neighboring genes in

1466   each individual data set. The +/- refers to standard error obtained from 2 independent

1467   experiments each including triplicate. Darker and darker shades indicate more and more

1468   deviation from housekeeping levels (Panel A) or from wild type levels (Panel B), red

1469   being for reduced levels and green for higher levels. ND: none detected. NA: not

1470   applicable, used for *kpsM* in *wcaG::catΔ* and *mlghC::cat* where data could not be

1471   calculated since levels were too low in the wild type reference but colors nevertheless

1472   indicate higher transcription than in wild type in both strains, with a 10-fold difference

1473   between *wcaG::catΔ* and *mlghC::cat* as per Panel A.

1474

1475   **Fig. 6: Effect of mutations on growth rates, auto-agglutination and flagellum**

1476   **production. Panel A:** Growth curves in broth under microaerobic atmosphere

1477   determined using Klett's flasks over 24 h. Error bars represent SEM (standard error of the

1478   mean) for 3 independent experiments with 1 reading/flask at each time point. **Panel B:**

1479   Auto-agglutination quantitated by measuring the OD_{600nm} of the top layer of a static

1480   bacterial suspension over time. Error bars represent SEM for 3 independent experiments

1481   each including 3 replicates. The same color scheme applies as in panel A. *: p<0.01 by

1482   one-way ANOVA (with Dunnett's post test). **Panel C:** Phase contrast microscopic

1483   examination of auto-agglutination for *kpsM*::*kan* compared with wild type (100x

1484   magnification). **Panel D:** Electron microscopy analysis of flagella production in wild

1485   type and *kpsM*::*kan* strains with uranyl acetate staining.

1486

1487   **Fig. 7: Effect of mutations on resistance to bile salts and SDS. Panel A:** Resistance to

1488   various concentrations of bile salts. The bacteria were exposed to bile for 15 min under

1489   microaerobic atmosphere at 37°C and serially diluted for enumeration of viable cells by

1490   CFU counting. **Panel B:** Resistance to various concentrations of SDS. The cells were

1491   exposed to SDS for 15 h and cell lysis was assessed by measuring the OD_{600nm}. The

1492   legend is the same in both panels and is shown within panel B. Error bars represent SEM

1493   for three independent experiments, each carried out with triplicates. Data for each replica

1494   were obtained from 2 CFU spots Panel A and 1 OD_{600nm} read for Panel B.

1495

1496   **Fig. 8: Effect of mutations on resistance to serum. Panel A:** Resistance to various

1497   concentrations of serum for 1.5 h under microaerobic atmosphere at 37°C. **Panel B:** Time

1498   course of resistance to 20% serum. For both panels, viability was assessed by CFU

1499   counting. The legend is the same in both panels and is shown within panel A. Error bars

1500   represent SEM. Experiments performed 3 times independently with triplicates within

1501   each experiment and determination of CFU from 2 spots for each replica.

1502

1503   **Fig. 9: Effect of mutations on interactions with epithelial cells.** The bacteria were

1504   centrifuged onto cell monolayers to account for motility defects. **Panel A:** Adherence to

1505   Caco-2 cells measured after 5h incubation, with elimination of unbound bacteria by

1506   washing and CFU counting of bound bacteria. **Panel B:** Invasion of Caco-2 cells

1507   measured after 5 h incubation and after elimination of externally bound bacteria via

1508   gentamicin treatment. Live intracellular bacteria were enumerated via CFU counting

1509   after lysis of epithelial cells with Triton X-100. ND: none detected. Error bars represent

1510   SEM. *: $p<0.05$. **: $p<0.01$ by one-way ANOVA. Experiments performed 3 times with

1511   duplicates within each experiment and determinations from 2 CFU spots for each replica.

1512

1513   **Fig. 10: Effect of the mutations on colonization and persistence in chicken intestine.**

1514   Two day old chicks were orally gavaged with 10^7 CFU and their caecal content in *C.*

1515   *jejuni* was determined after 5 days by CFU counting of plated caecal homogenate. **Panel**

1516   **A:** Test of wild type and four mutants. **Panel B:** Test of wild type and one additional

1517   mutant. While experiments were performed following the same procedure, a slightly

1518   lower level of colonization and wider distribution of colonization levels were obtained for

1519   wild type in panel B compared with panel A, therefore, the data for the last mutant tested

1520   are displayed separately. For both panels, each data point represents an individual chick.

1521   The numbers at the top of the graphs are the number of chicks from which *C. jejuni* was

1522   isolated over the total number of chicks inoculated for each strain tested. The lower limit

1523   of detection for colonization was ~ 1.5 log CFU/g. Horizontal bars represent the median

1524   of each group. Significant differences (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p <$

1525 0.05) in colonization compared to wild type as determined by the Mann-Whitney test are

1526   indicated. A second experiment was performed with an inoculation dose of 10^6 and

1527   similar data were obtained (data not shown).

1528

A

3,6-O-Me$_2$-D-α-L-*gluco*Hep*p*-(1-3)

MeOPN-4

-2)-β-D-Rib*f*-(1-5)-β-D-Gal*f*NAc-(1-4)-α-D-Glc*p*A-(1-

MeOPN-3   GroN/EtN-6

B
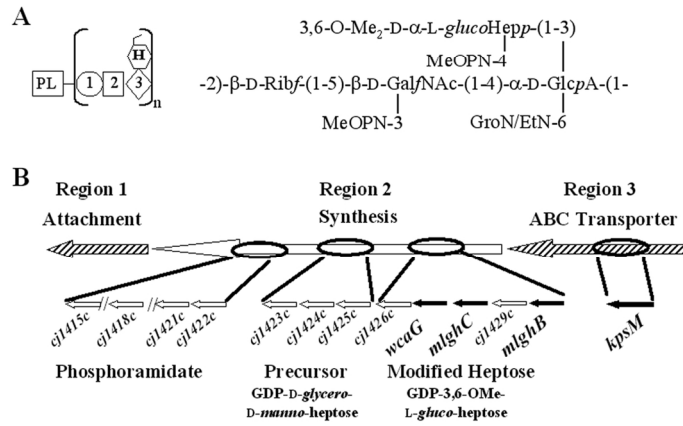
Figure 1: Wong et al.

CPS composition and CPS cluster
149x149mm (300 x 300 DPI)

GDP-D-*glycero*-D-*manno*-heptose

?   C4 oxidase | MlghA ?

GDP-3,6-R-4-keto-D-*lyxo*-heptose

C3, C5 | MlghB   C4 | WcaG
epimerase | (*cj1430c*)   reductase | (*cj1427c*)

C6 methylase ?
(*cj1426c*)

GDP-3,6-R-4-keto-L-*xylo*-heptose   GDP-3,6-R-
D-*manno*-heptose

C4 reductase | MlghC
(*cj1428c*)

?

GDP-3,6-OMe-L-*gluco*-heptose
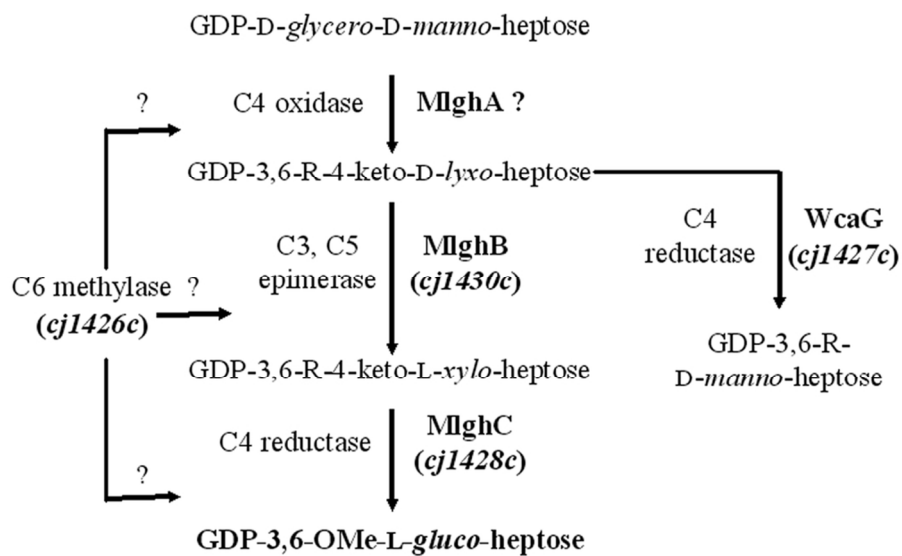
Figure 2: Wong et al.

Heptose modification pathway
99x99mm (300 x 300 DPI)

Wong et al Figure 3
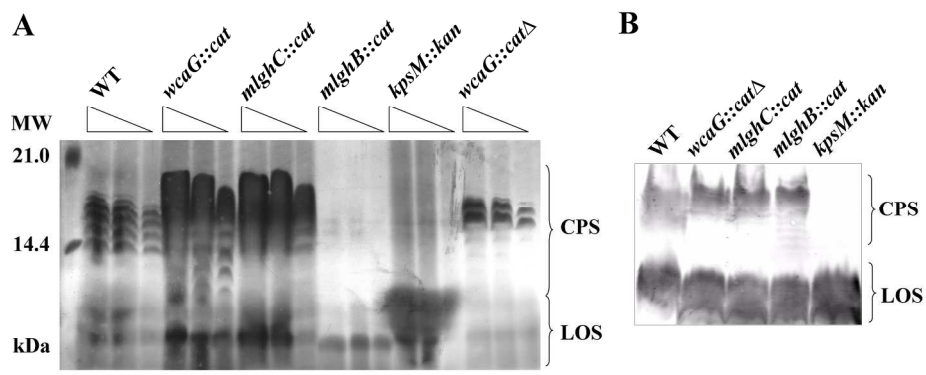
CPS gels
199x199mm (300 x 300 DPI)

WILD-TYPE

$$3,6\text{-}O\text{-}Me_2\text{-}D\text{-}\alpha\text{-}L\text{-}gluco\text{Hep}p\text{-}(1\text{-}3)$$
$$|$$
$$-2)\text{-}\beta\text{-}D\text{-}Rib f\text{-}(1\text{-}5)\text{-}\beta\text{-}D\text{-}Gal f\text{NAc}\text{-}(1\text{-}4)\text{-}\alpha\text{-}D\text{-}Glc p A\text{-}(1\text{-}$$
$$|$$
$$EtN\text{-}6$$

wcaG::*cat, mlghB*::*cat, mlghC*::*cat* (trace)

$$-2)\text{-}\beta\text{-}D\text{-}Rib f\text{-}(1\text{-}5)\text{-}\beta\text{-}D\text{-}Gal f\text{NAc}\text{-}(1\text{-}4)\text{-}\alpha\text{-}D\text{-}Glc p A\text{-}(1\text{-}$$
$$|\qquad\qquad\qquad|$$
$$MeOPN\text{-}3\qquad GroN/EtN\text{-}6$$

wcaG::*catΔ, mlghC*::*cat* (major)

$$-2)\text{-}\beta\text{-}D\text{-}Rib f\text{-}(1\text{-}5)\text{-}\beta\text{-}D\text{-}Gal f\text{NAc}\text{-}(1\text{-}4)\text{-}\alpha\text{-}D\text{-}Glc p A\text{-}(1\text{-}$$
$$|$$
$$GroN/EtN\text{-}6$$
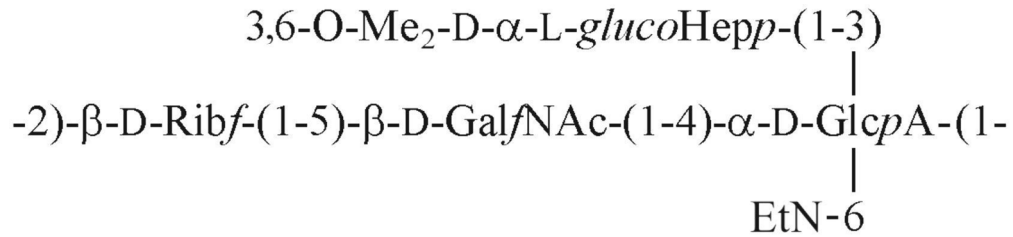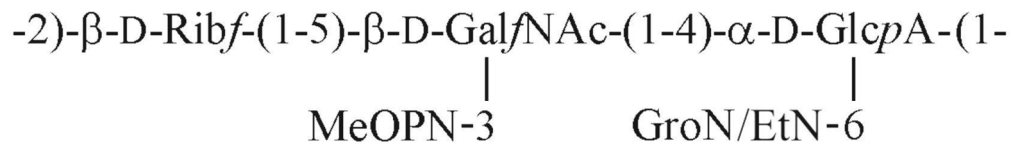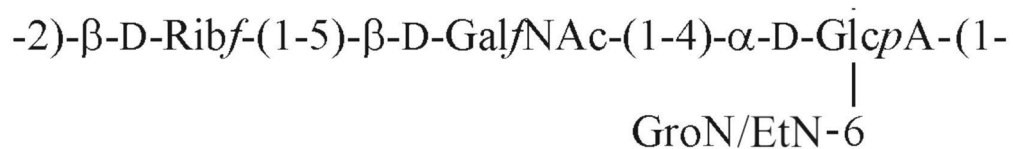
CPS NMR structures
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Molecular Microbiology
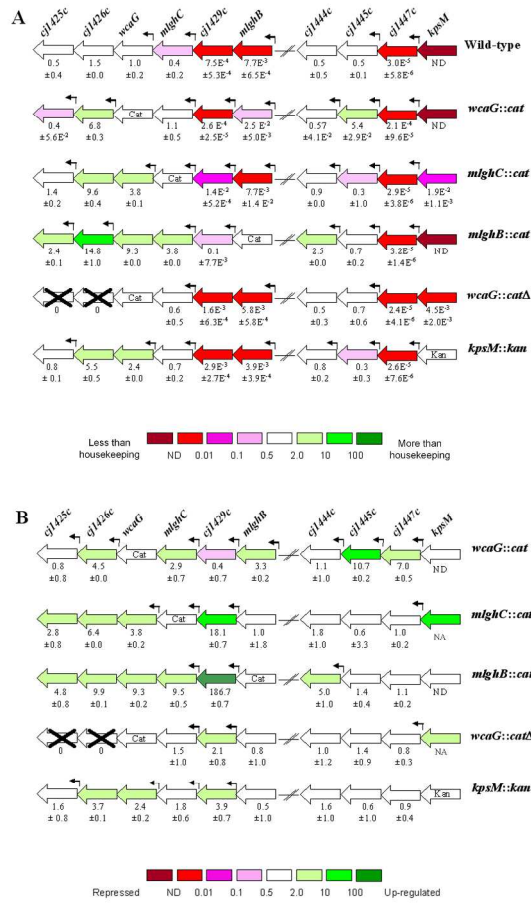
Figure 5 Wong et al

qRT-PCR analysis of CPS cluster
240x384mm (300 x 300 DPI)

Wong et al Figure 6
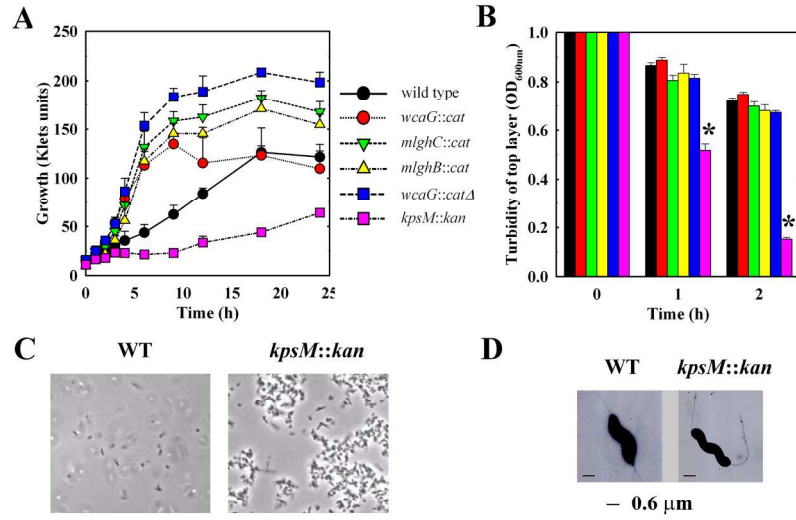
Growth, agglutination and microscopy
199x199mm (300 x 300 DPI)

Figure 7 Wong et al
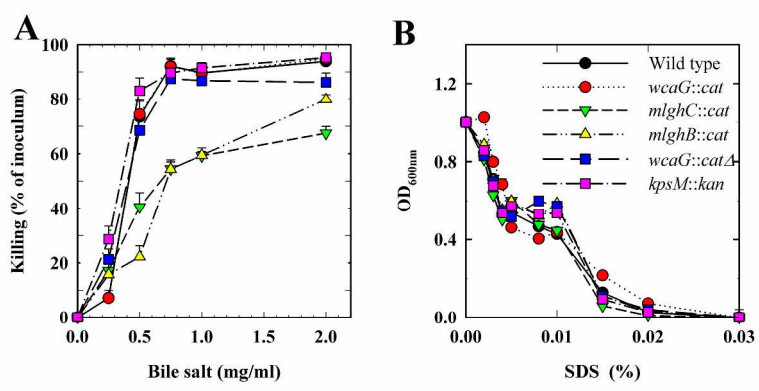
Barrier function towards bile and SDS
279x360mm (300 x 300 DPI)

Figure 8 Wong et al
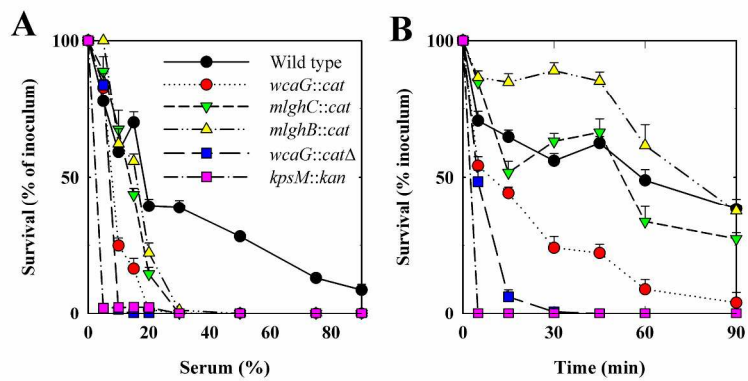
Resistance to serum
278x360mm (300 x 300 DPI)

Figure 9 Wong et al
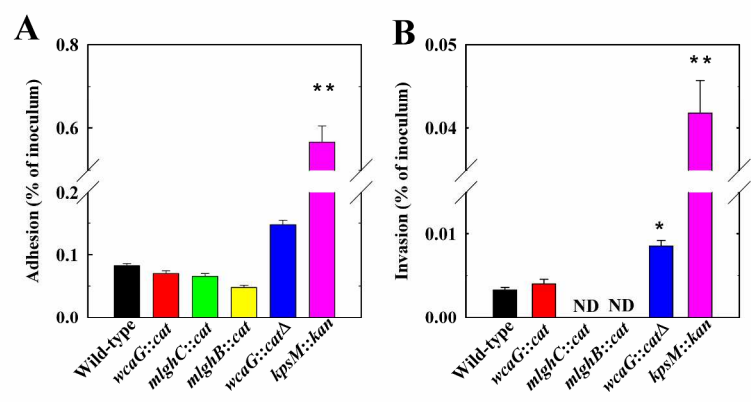
Interactions with epithelial cells
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Wong et al Figure 10
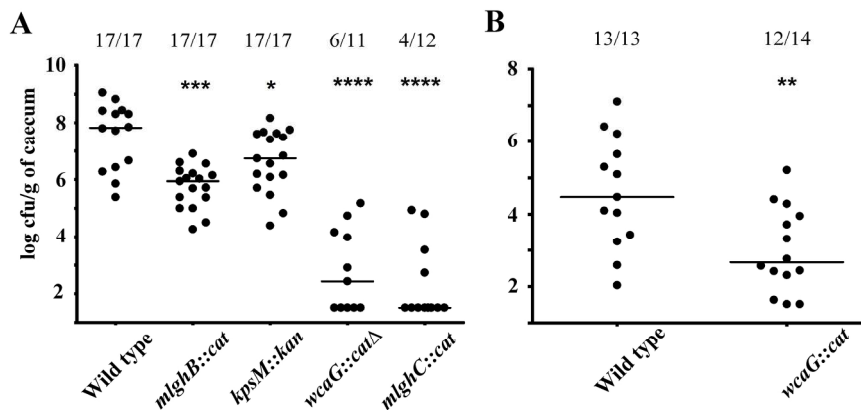
Chicken colonization experiments
199x199mm (300 x 300 DPI)
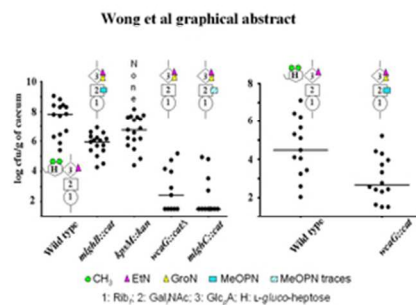
chicken data and CPS structure for graphical abstract
59x39mm (300 x 300 DPI)

Graphical abstract for Wong et al 2015

80 words.

Campylobacter jejuni ATCC 700819 produces a capsule that is important for virulence and contains a modified heptose. Inactivation of any of the genes *wcaG*, *mlghB*, and *mlghC* involved in heptose modification leads to capsule lacking modified heptose and presenting altered MeOPN, EtN and NGro contents. This alters capsule barrier functions and interactions with intestinal cells and decreases colonization and/or persistence in the chicken gut. This demonstrates that these genes contribute to bacterial resistance against host defenses in the gastrointestinal tract.

Legend for figure:

Chicken caecum colonization by heptose modification mutants and schematic representation of their capsule composition.

Supplementary Table 1: List of primers used to construct complementation strains.

When applicable, the restriction sites are indicated in bold letters.

Primer name	Sequence (5' – 3')
1427RevKan	CAAATGGTTCGCTGGGTTTCTTAATTAAAATTTGC AAAGCGATTA
KanFor1427	TAATCGCTTTGCAAATTTTAATTAAGAAACCCAGC GAACCATTTG
1428RevKan	TCAAATGGTTCGCTGGGTTTCTCAATTTTGTGTTTT ATACCATTC
KanFor1428	GAATGGTATAAAACACAAAATTGAGAAACCCAGC GAACCATTTGA
1430RevKan	CAAATGGTTCGCTGGGTTTCTTATCCTTTATTTTTA GTTGCAAG
KanFor1430	CTTGCAACTAAAAATAAAGGATAAGAAACCCAGC GAACCATTTG
OmpE-for	GC**TCTAGA**CTTTAGATGTTTTTATCCTTC
Aph3P3	GC**TCTAGA**GACATCTAAATCTAGGTAC
1427ForOmpE	TAATTTTTGACAAGGAGAATTCTCATGTCAAAAAA AGTTTTAATTACAG
1428ForOmpE	TAATTTTTGACAAGGAGAATTCTCATGCAAACAAA TTCAAAAATATATATA
1430ForOmpE	TAATTTTTGACAAGGAGAATTCTCATGGCAATAGA ATTTGATATACAA
ompERev1427	CTGTAATTAAAAGTTTTTTTGACATGAGAATTCTCC TTGTCAAAAATTA
ompERev1428	TATATATATTTTTGAATTTGTTTGCATGTGAATTCTC CTTGTCAAAAATTA
ompERev1430	TTGTATATCAAATTCTATTGCCATGAGAATTCTCCTT GTCAAAAATTA
16SrRNATop	GG**GGTACC**CTGGAACTCAACTGACGCTAA
23SrRNABottom	ATAAGAAT**GCGGCCGC**TCTTGCACATTGCAGTCCTA

Legends to supplementary figures:

Supplementary Fig. S1: Parts of two-dimensional ^{1}H, ^{31}P HMBC spectra of CPS of the wild type strain and the mutants. One-dimensional ^{1}H and ^{31}P NMR spectra are shown along the horizontal and vertical axes, respectively.

Supplementary Fig. S2: Parts of two-dimensional ^{1}H,^{13}C HSQC spectra of CPS of the wild type strain and the mutants. One-dimensional ^{1}H NMR spectra are shown along the horizontal axis.

Supplementary Fig. S3: Effect of mutations on interactions with RAW 267.4 murine macrophages. Panels A and B: Time course of intracellular bacterial survival performed by CFU counting after elimination of extracellular bacteria by gentamicin treatment and lysis of macrophages by water. The data are expressed as % of original inoculum (panel A) or as a function of the initial bacterial load at time zero after gentamicin (GM) treatment (Panel B). **Panel C:** adherence to macrophages. Incubation was performed at 4°C to inhibit phagocytosis. **Panel D:** Time course of invasion of macrophages before gentamicin treatment. Error bars represent SEM. *: $p<0.05$ between wild type and *kpsM*::*kan* by one way ANOVA. Experiments performed 3 times independently with triplicates within each experiment and data acquisition from 2 CFU spots for each replica. For all panels, the bacteria were centrifuged onto the macrophages to account for motility defects and the legend is the same as in panel A.
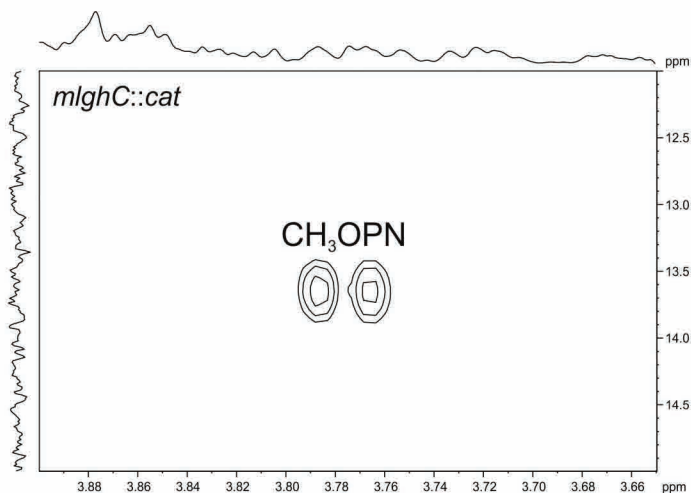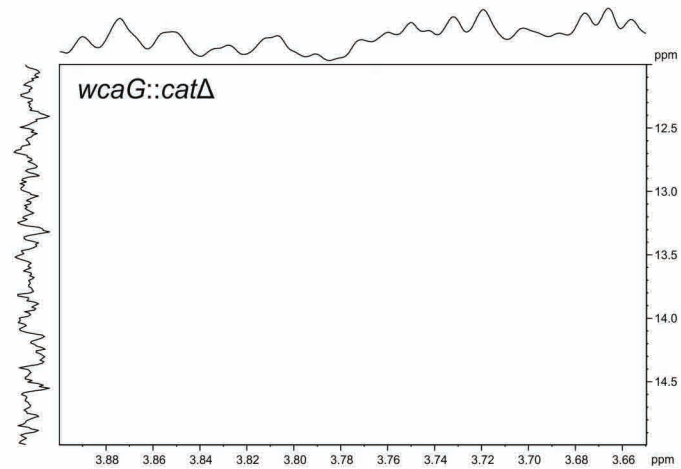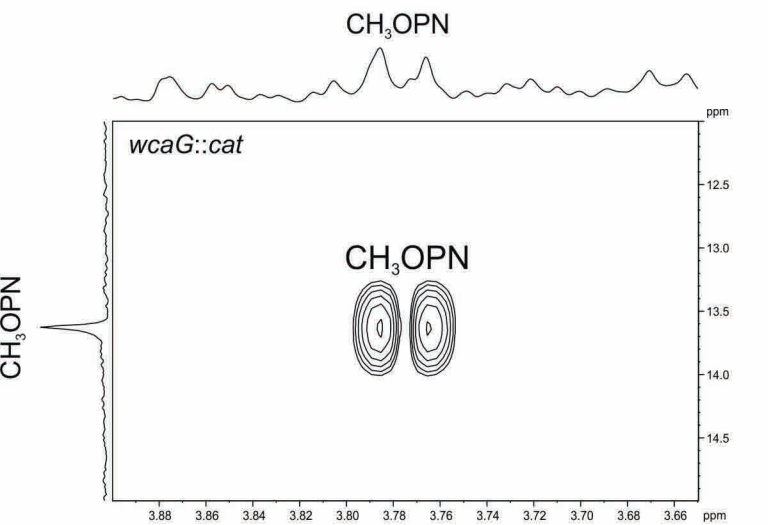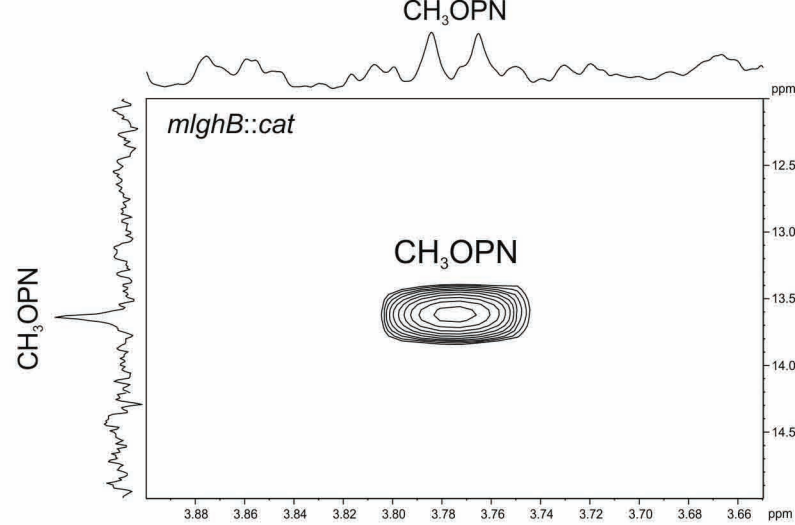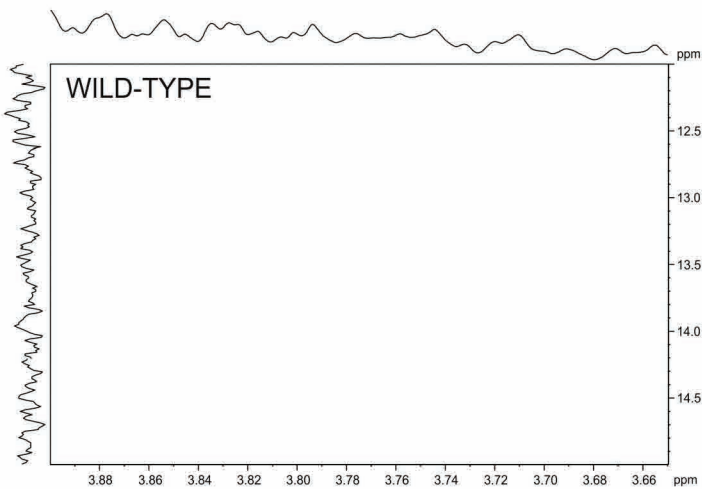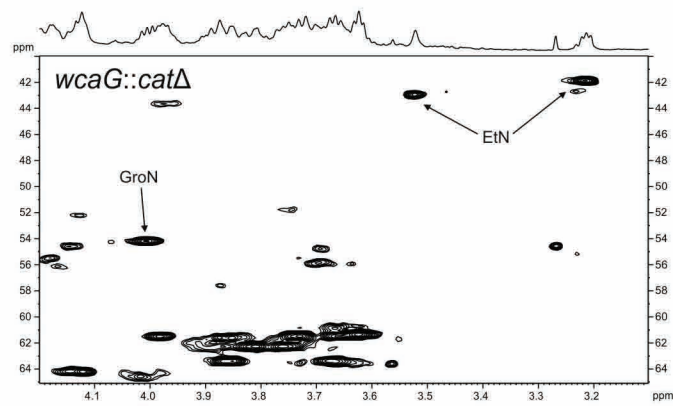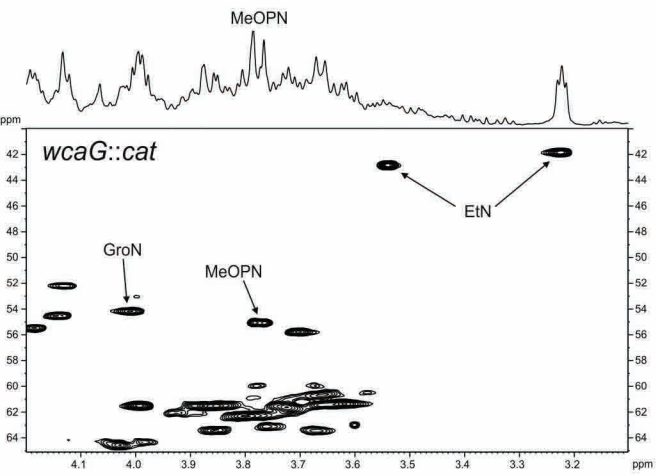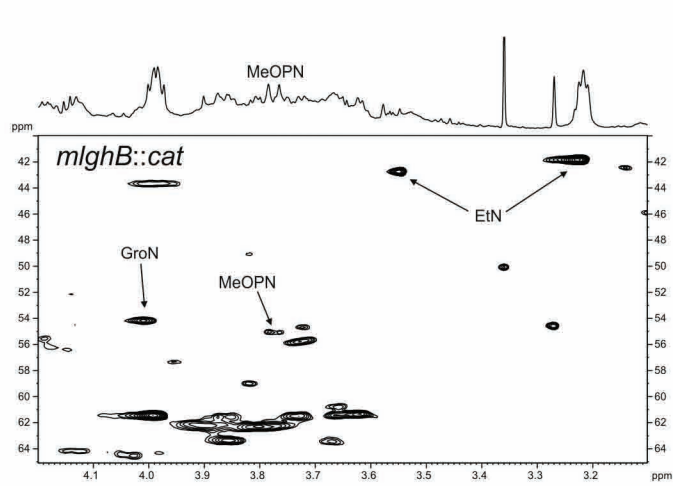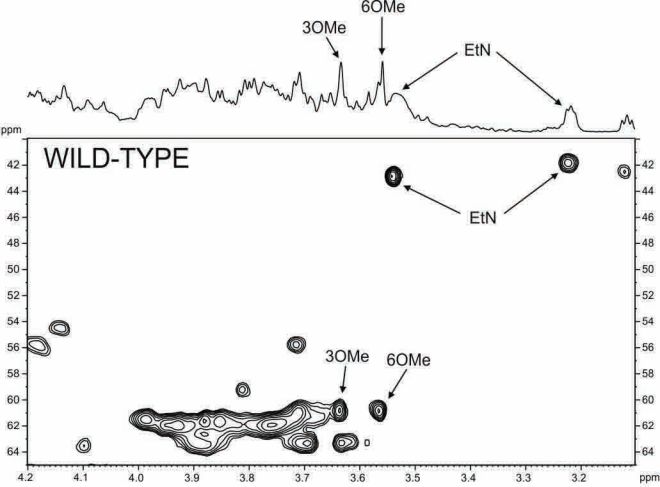
Fig S1
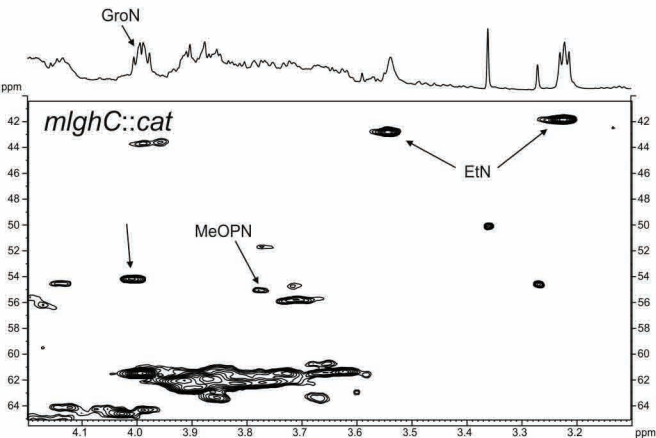
Fig S2

Supplementary Figure S3 Wong et al