

1 Title: Genetics behind the biosynthesis of nonulosonic acid containing lipooligosaccharides in

2 *Campylobacter coli*

3 Running title: *Campylobacter coli* GT-42

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13

14 **ABSTRACT**

15 *Campylobacter jejuni* and *Campylobacter coli* are the most common cause of bacterial
16 gastroenteritis in the world. Ganglioside mimicry by *C. jejuni* lipooligosaccharide (LOS) is the
17 triggering factor of Guillain-Barré syndrome (GBS), an acute polyneuropathy.
18 Sialyltransferases from the glycosyltransferase (GT) family 42 are essential for the expression
19 of ganglioside mimics in *C. jejuni*. Recently, two novel GT-42 genes, *cstIV* and *cstV*, have
20 been identified in *C. coli*. Despite being present in ~11% of currently available *C. coli* genomes,
21 the biological role of *cstIV* and *cstV* is unknown. Here, we show that CstIV and CstV are
22 involved in LOS biosynthesis. Additionally, *cstV* is associated with LOS sialylation, while
23 *cstIV* is linked to the addition of a diacetylated nonulosonic acid residue.

24 INTRODUCTION

25 Nonulosonic acids are a highly diverse family of nine-carbon α -keto acids. The most natural
26 abundant nonulosonic acids are the sialic acids (*N*-acetylneuraminic acid, Neu5Ac) and
27 derivatives¹. Initially thought to be only a deuterostomes feature, sialic acids have been found
28 in virulence associated bacterial cell surface glycoconjugates such as lipopolysaccharides,
29 capsules, pili, and flagella²⁻⁴. Furthermore, these sialylated structures have been shown to
30 influence pathogenesis through immune evasion, adhesion, and invasion^{5,6}. Sialyltransferases,
31 catalyse the transfer of sialic acid from cytidine-5'-monophospho-*N*-acetylneuraminic acid
32 (CMP-Neu5Ac) to an acceptor, are key in the synthesis of sialoglycoconjugates. Known
33 sialyltransferases have been classified into five distinct CAZy (Carbohydrate-active enzymes
34 database) glycosyltransferase (GT) families; GT-29, GT-38, GT-42, GT-52, and GT-80⁷. In
35 *Campylobacter jejuni*, the most common cause of bacterial gastroenteritis, CMP-Neu5Ac
36 biosynthesis (*neuA*, *neuB1*, and *neuC*) and GT-42 genes are present in the lipooligosaccharide
37 (LOS) biosynthesis locus classes A, B, C, M, R, and V⁸⁻¹⁰. *C. jejuni* strains carrying one of
38 these genetic classes synthesize LOS structures generally resembling gangliosides^{9, 11-13}. In
39 some cases, infection with a *C. jejuni* strain expressing ganglioside-like LOS induces
40 production of cross-reactive anti-ganglioside antibodies. This leads to the development of
41 Guillian-Barré syndrome (GBS); an acute autoimmune polyradiculoneuropathy disease with
42 ~5% mortality rate¹⁴.

43 *C. coli*, the second most common cause of campylobacteriosis, has also been isolated from
44 GBS patients¹⁵⁻¹⁸. Nevertheless, the role of *C. coli* in GBS has largely remained unclear due
45 to the seemingly absence of key elements for the synthesis of ganglioside-like LOS (i.e GT-42
46 and *neuABC*). Recently, three newly identified *C. coli* LOS-associated GT-42 genes were
47 reported; *cstIV*, *cstV*, and *cstVI*^{10, 19, 20}. While *cstVI* is generally found as a pseudogene, *cstIV*

48 and *cstV* may potentially be involved in LOS biosynthesis¹⁹. In this manuscript we sought to
49 explore the role of the new GT-42 enzymes CstIV and CstV in LOS biosynthesis.

50 **RESULTS**

51 **CstIV and CstV exhibit no sialyltransferase activity *in vitro*.**

52 To determine whether CstIV and CstV are capable of transferring Neu5Ac, *C. coli* crude
53 protein extracts were tested for sialyltransferase activity using sugar acceptors labelled with
54 either boron-dipyrromethene or BODIPY (BDP) and fluorescein (FCHASE).
55 Monosialyltransferase activity on BDP-Lactose (BDP-Lac) and BDP-*N*-acetyllactosamine
56 (BDP-LacNAc) was detected in *C. coli* 76339 WT, *C. coli* 76339 Δ *cstV*-SF1, and *C. coli* 76339
57 Δ *cstV*-SR4 crude protein extracts (Suppl. Fig. 1). Conversely, no measurable enzymatic
58 activity on any of the tested acceptors was detected in *C. coli* 76339 Δ *cstI*-XR3 and *C. coli*
59 76339 Δ *cstV*-SR Δ *cstI*-XR1 protein extracts. Likewise, no sialyltransferase activity was
60 detected in the crude protein extracts of *C. coli* 65 and 73, *C. coli* 65 Δ *cstIV*-SF5 and *C. coli*
61 73 Δ *cstIV*-SF3. Furthermore, recombinant CstIV and CstV showed no activity with any of the
62 tested acceptors (Suppl. Fig. 2).

63 **CstIV and CstV are involved in LOS biosynthesis.**

64 The LOS of Δ *cstIV* and Δ *cstV* strains showed an increased mobility on silver stained SDS-
65 PAGE gels relative to the WTs (Fig. 1). Thus, deletion of *cstV* in *C. coli* 76339 and *cstIV* in *C.*
66 *coli* 65 and 73 resulted in a truncated LOS. The complemented *cstV* mutant exhibited two LOS
67 bands on SDS-PAGE gels; the upper one corresponding to the WT LOS and the lower
68 molecular weight band to the truncated LOS (Suppl. Fig. 3). This suggests that partial
69 restoration of the phenotype was achieved upon complementation *in cis* of Δ *cstV*-SR4.

70 ***C. coli* 76339 *neuB* is involved in the biosynthesis of CstV substrate.**

71 Since no clear shift in the electrophoretic mobility of *C. coli* 76339 LOS was detected after
72 neuraminidase treatment (Suppl. Fig. 4), *neuBI* was knocked out to determine whether CMP-
73 Neu5Ac was CstV's substrate. The LOS of 76339 Δ *neuB*-SR2 showed a similar profile to those
74 of 76339 Δ *cstV*-SF1 and 76339 Δ *cstV*-SR4 (Suppl. Fig. 5). Thus, deletion of *neuB* results in a
75 seemingly similar LOS truncation to the one observed in Δ *cstV* strains, suggesting the potential
76 involvement of *neuBI* in CstV substrate synthesis.

77 **CstIV and CstV are associated to nonulosonate residues in *C. coli* LOS**

78 Predicted LOS compositions by LC-MS for *C. coli* 76339 WT and mutants are shown on
79 Table 1. *C. coli* 76339 contains a core oligosaccharide linked via two 3-deoxy-D-manno-oct-
80 2-ulosonic acid (Kdo) molecules to a lipid A molecule. The core oligosaccharide of *C. coli*
81 76339 is composed of heptoses (Hep), hexoses (Hex), hexosamines (HexNAc), and NeuAc.
82 The resulting MS/MS spectrum obtained from the O-deacylated LOS of *C. coli* 76339 WT
83 revealed a single ion at m/z 1214.4 corresponding to Hex₃•Hep₂•PEtn₁•KDO₁ (Fig. 2). The
84 fragment ions at m/z 1052.4 and 890.3 correspond to the additional loss of two Hex residues.
85 The spectra also revealed ions that derived from lipid A, m/z 693.5 and m/z 388.3
86 corresponding to HexN₃N₁•P₁•(C14:0 3-OH)₂ and HexN₃N₁•(C14:0 3-OH)₁, respectively.
87 The observation of fragment ions at m/z 292.1 and 274.1 provided evidence for the presence
88 of sialic acid on core region LOS. The MS/MS spectrum of precursor ion m/z 1064.0 from *C.*
89 *coli* 76339 Δ *cstI* is similar to that from *C. coli* 76339 WT, in which the diagnostic ions for
90 sialic acid were detected at m/z 292.1 and 274.2 (Fig. 2b). However, no sialic acid was
91 detected in the MS/MS spectrum *C. coli* 76339 Δ *cstV* (Fig. 2c). Thus, *cstV* is associated to
92 the presence of NeuAc, while *cstI* plays no role in *C. coli* 76339 LOS biosynthesis.

94 A similar lipid A moiety was indicated by the MS/MS spectrum obtained from the *O*-
95 deacylated LOS of *C. coli* strain 73 WT. The spectra also revealed ions that derived from
96 lipid A, m/z 693.5 and m/z 388.3 corresponding to HexN₃N₁•P₁•(C14:0 3-OH)₂ and
97 HexN₃N₁•(C14:0 3-OH)₁, respectively. The observation of fragment ions at m/z 317.2 and
98 299.1 provided evidence for the presence of a residue with a molecular weight of 334.2 Da or
99 316.2 Da for its anhydrous form on core region LOS (Fig. 3a). These masses are consistent
100 with free diNAc-nonulosonate and its conjugated form, respectively. However, these
101 characteristic ions were not detected in the MS/MS spectrum *C. coli* 73 Δ *cstIV*-SF3 (Fig. 3b).
102 Thus, suggesting the role of *cstIV* in the biosynthesis of diNAc-nonulosonate LOS in *C. coli*
103 73.

104 DISCUSSION

105 *C. jejuni*. *C. jejuni* GT-42 were the first glycosyltransferases from this CAZy family to be
106 enzymatically and structurally characterized; CstII is mono/bifunctional exhibiting α 2,3-/ α 2,8-
107 sialyltransferase activity, while CstI and CstIII are monofunctional α 2,3- sialyltransferases ²¹⁻
108 ²⁴. The activity of CstII and CstIII has been shown to be essential for the biosynthesis
109 ganglioside-like LOS structures, which are linked to GBS onset ^{12, 24}. Despite the importance
110 of GT-42 enzymes in virulence and pathogenesis ²⁵⁻²⁸, the activity of these glycosyltransferases
111 has not been explored in other *Campylobacter* species. Approximately 29% of *C. coli* genomes
112 have been found to contain a GT-42 encoding gene within the LOS biosynthesis locus ¹⁹. While
113 *cstVI* was the most common LOS associated GT-42 encoding gene in *C. coli*, in 99% of the
114 analysed genomes it was observed to be present as a pseudo gene. Thus, we focused our
115 attention on the role of *cstIV* and *cstV* in LOS biosynthesis. Until recently, *cstV* had been solely
116 identified in the genome of *C. coli* 76339 ²⁰. However, in a systematic screen of publicly
117 available *C. coli* genomes several *cstV* positive strains were identified ¹⁹. *C. coli* 76339 crude
118 protein extracts were tested for sialyltransferase activity as Neu5Ac had been previously

119 detected in the strain's LOS²⁰. Monosialyltransferase activity was initially detected, but was
120 found to be due to activity of CstI. As in *C. jejuni*, *C. coli* 76339 *cstI* is located outside the LOS
121 biosynthesis locus and encodes a α 2,3- sialyltransferase which has no role in LOS biosynthesis
122^{20,21}. No sialyltransferase activity was detected on the protein extracts of the *cstI* mutant strain.
123 However, transcriptomic analysis showed polycistronic expression of LOS biosynthesis genes
124 indicating the active expression of *cstV*. Similarly, recombinant CstV exhibited no detectable
125 activity on any of the tested acceptors. Deletion of *neuBI* or *cstV* resulted in identical LOS
126 electrophoretic profiles. Additionally, LC-MS analysis showed that deletion of *cstV* resulted in
127 the loss 2 Hex and 1 NeuAc. Thus, it is probable that *cstV* is associated to *C. coli* 76339 LOS
128 sialylation. Yet, further studies are required to identified CstV natural acceptor and corroborate
129 its activity *in vitro*.

130 After *cstVI*, *cstIV* is the most common orthologue being present in ~38% of the genomes
131 positive for a LOS associated GT-42. Previously, no evidence of Neu5Ac had been found in
132 the LOS of strains expressing *cstIV*²⁹. This was to be expected as Neu5Ac biosynthesis genes
133 are rarely present in strains carrying *cstIV*¹⁹. Furthermore, no sialyltransferase activity was
134 detected neither in *C. coli* 65 and 73 protein extracts nor in recombinant CstIV. Thus, we
135 assumed that CstIV was a cryptic, possibly inactive, sialyltransferase with no role in LOS
136 biosynthesis. Nevertheless, deletion of *cstIV* in *C. coli* 65 and 73 resulted in a truncated LOS.
137 Sequence alignment of CstIV with previously characterized GT42 sialyltransferases revealed
138 numerous amino acid substitutions at conserved positions (Suppl. Fig. 6)³⁰. Additionally,
139 superimposition of CstIV on *C. jejuni* CstII structure identified various substitutions at amino
140 acids involved in substrate interactions^{23, 31-33}. Interestingly, most substitutions predicted to
141 impact CstIV were in the amino acids associated with CMP-Neu5Ac, particularly with the
142 Neu5Ac moiety. Moreover, these substitutions were conserved in multiple CstIV orthologues
143^{23, 32, 33}. Altogether, results pointed at the possibility of an alternative sugar donor for CstIV.

144 Detection of a diNAc-nonulosonate residue in *C. coli* 73 WT LOS and its absence in *C. coli* 73
145 Δ *cstIV*-SF3 prompted us to investigate genes potentially linked to the synthesis of this residue.
146 In *C. coli*, *neuB2* (*ptmC*, *legI*) and *neuB3* (*pseI*) are conserved flagella glycosylation genes
147 involved in the synthesis of legionaminic and pseudaminic acid derivatives, respectively³⁴⁻⁴⁰.
148 Deletion of *neuB2* had no impact on *C. coli* 73 LOS electrophoretic mobility (Suppl. Fig. 7),
149 implying that *neuB2* is not involved in the synthesis of CstIV substrate. Despite repetitive
150 attempts, no viable *C. coli* 73 Δ *neuB3* mutants were obtained. Although *neuB3* deletion has
151 been successful in *C. coli* VC167, disruption of flagellin glycosylation and potentially
152 truncation of the LOS might have resulted in a lethal phenotype for *C. coli* 73⁴⁰. In sum, it is
153 tempting to speculate that the diNAc-nonulosonate residue in *C. coli* 73 WT corresponds to
154 pseudaminic acid. However, the nature of this residue cannot be inferred from MS/MS spectra
155 alone, since many diNAc-nonulosonate variants have been identified⁴¹. Thus, this task is still
156 under investigation.

157 **Conclusion**

158 Due to its close relatedness to *C. jejuni* and large horizontal gene transfer between both species,
159 *C. coli* glycobiology has been largely neglected. Similarly to *C. jejuni*, *C. coli* appears to
160 express LOS structures containing nonulosonate acids. The importance of this to *C. coli*
161 ecology and host-pathogen interaction remains to be explore.

162 **METHODS**

163 **Bacterial strains, plasmids, and growth conditions.** Bacterial strains used in this study are
164 listed in Supplemental Table 1. *C. coli* cultivation and DNA isolation were carried out as
165 previously described, unless specified otherwise²⁰.

166 **Construction of Δ *cstIV*, Δ *cstV*, Δ *cstI*, and Δ *neuB* mutants.**

167 Chromosomal mutant strains of *C. coli* 76339 (*cstV*, *cstI*, and *neuB1*)^{19,20}, *C. coli* 73 (*cstIV*
168 and *neuB2*), and *C. coli* (*cstIV*)^{19,29} were generated by homologues recombination with suicide
169 vectors containing genes inactivated by the insertion of an antibiotic resistance cassette (All
170 recombinant plasmids and primers are shown in Supplemental material). The genes *cstV*, *cstIV*,
171 *neuB1*, and *neuB2* were inactivated by the insertion of an erythromycin resistance cassette
172 (EryC)⁴², while *cstI* was disrupted with a chloramphenicol acetyltransferase cassette (CAT)⁴³.
173 Preparation of electrocompetent cells and transformation was done as previously described⁴³.
174 Selection of Δ *cstIV*, Δ *cstV*, Δ *neuB1* and Δ *neuB2* mutants was done on nutrient blood agar
175 (NBA) supplemented with 10 μ g ml⁻¹ of erythromycin, while Δ *cstI* mutants were selected in
176 NBA supplemented with 12.5 μ g ml⁻¹ of chloramphenicol. Homologous recombination of all
177 mutants was verified by PCR.

178 **Complementation of *C. coli* 76339 Δ *cstV*-SR**

179 Complementation of *C. coli* 76339 Δ *cstV*-SR4 was done in *cis* by integration of *cstV* under the
180 active promoter of gamma glutamyltranspeptidase (*ggt*). The *ggt* is an accessory gene in *C.*
181 *coli* and has no role in LOS biosynthesis. Additionally, the *ggt* locus is located far from the
182 LOS locus and its deletion does not induce a loss in bacterial viability. The suicide vector
183 containing an inactivated *ggt* by the insertion of a *cstV* and CAT (pGEM-*ggt*-*cstV*-CAT) was
184 used to transform *C. coli* 76339 Δ *cstV*-SR4 electrocompetent cells as above. Transformants
185 were selected on NBA supplemented with 12.5 μ g ml⁻¹ of chloramphenicol. Homologous
186 recombination of mutants was verified by testing for GGT activity as before⁴⁴.

187 **LOS silver staining.** LOS profiles were assessed by silver staining as described earlier²⁹.
188 Additionally, LOS sensitivity to neuraminidase was assessed. Crude LOS was treated with 2
189 IU/ml of *Clostridium perfringens* neuraminidase (Sigma-Aldrich) overnight at 37 °C.

190 **Mass spectrometry analysis of *C. coli* LOS composition.**

191 Following 1% formaldehyde in PBS (pH 7.4) treatment, *C. coli* cell pellets were washed 3X in
192 PBS and lyophilized. Then, cells were dehydrated by a sequence of 2 washes in each of 70%
193 ethanol (in PBS), 100% ethanol, and 100% acetone. The dehydrated cells were treated with
194 proteinase K, RNase A, and DNase I as previously described ⁴⁵. Digested cells were then
195 treated with hydrazine to cleave *O*-linked fatty acids ⁴⁵. The *O*-deacylated LOS samples were
196 analysed by LC-MS by coupling a Waters Premier Q-TOF with an Agilent 1260 capillary LC
197 system. Mass spectrometry was operated in positive-ion detection mode. Liquid
198 chromatography separation was done on an Agilent Eclipse XDB C8 column (5 μ m, 50 x 1mm).
199 The flow rate was 20 μ l/min. Solvent A: aqueous 0.2% formic acid/0.028% ammonia; solvent
200 B: Isopropanol with 0.2% formic acid/0.028% ammonia. The following gradient was used: 0-
201 2 min. 10% B, 2-16 min linear gradient to 85% B, 16-25 min. 85% B, 25-30 min., and
202 equilibration at 10% B.

203

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340 **CONTRIBUTIONS**

341 A.C and M.R designed and coordinated the study. A.C generated all *C. coli* mutants. A.C,
342 M.G, and W.W participated in enzymatic assays. J.S and J.L performed LC-MS analysis. J.S,
343 J.L, and M.G interpreted LC-MS data. A.C drafted the manuscript. All authors have
344 contributed to data interpretation, have critically reviewed the manuscript, and approved the
345 final version as submitted.

346 **ADDITIONAL INFORMATION**

347 The authors declare that they have no competing interests

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349 **Figure Legends**

350 **Figure 1.** Electrophoresis mobility comparison of *C. coli* LOS of WT and mutant strains. *C.*

351 *jejuni* 81-176 was used as a reference. Samples marked with an asteric were neuraminidase

352 treated.

353 **Figure 2.** MS/MS spectra for the precursor ions of O-deacylated LOS from (a) *C. coli* 76339

354 WT, m/z 1064.0; (b) *C. coli* 76339 Δ cstI, m/z 1064.0; and (c) *C. coli* 76339 Δ cstV, m/z

355 1295.4.

356 **Figure 3.** MS/MS spectra for the precursor ions of O-deacylated LOS from (a) *C. coli* strain

357 73 WT, m/z 1072.4; (b) *C. coli* 73 Δ cstIV-SF3, m/z 1566.0.

358 **Table X.** LC-MS in positive mode data and proposed compositions for *O*-deacylated LOS of *C. coli* 76339 (strain 51) and corresponding *cstI* and *cstV* knock-
 359 out mutants.
 360

Strain	Observed ions				Molecular mass		Proposed compositions		
	$[M+3H]^3_+$	$[M+2H+NH_4]^3_+$	$[M+2H]^2_+$	$[M+H+NH_4]^2_+$	Observed	Calculated ⁱ	Core oligosaccharide	Phosphorylation in lipid A	Acylation in lipid A
wt	1063.43	1069.11			3187.30	3187.35	Kdo ₂ •Hep ₂ •Hex ₄ •HexNAc ₁ •NeuAc ₁	<i>PP</i> Etn	3 <i>N</i> -(C14:0 3-OH)
	1104.43	1110.11			3310.30	3310.36	Kdo ₂ •Hep ₂ •Hex ₄ •HexNAc ₁ •NeuAc ₁ • <i>PE</i> t ₁	<i>PP</i> Etn	3 <i>N</i> -(C14:0 3-OH)
	1138.48	1144.17			3412.46	3412.56	Kdo ₂ •Hep ₂ •Hex ₄ •HexNAc ₁ •NeuAc ₁	<i>PP</i> Etn	4 <i>N</i> -(C14:0 3-OH)
	1179.51	1185.17			3535.51	3535.57	Kdo ₂ •Hep ₂ •Hex ₄ •HexNAc ₁ •NeuAc ₁ • <i>PE</i> t ₁	<i>PP</i> Etn	4 <i>N</i> -(C14:0 3-OH)
Δ <i>cstI</i>	1063.44	1069.11			3187.31	3187.35	Kdo ₂ •Hep ₂ •Hex ₄ •HexNAc ₁ •NeuAc ₁	<i>PP</i> Etn	3 <i>N</i> -(C14:0 3-OH)
	1104.44	1110.12			3310.33	3310.36	Kdo ₂ •Hep ₂ •Hex ₄ •HexNAc ₁ •NeuAc ₁ • <i>PE</i> t ₁	<i>PP</i> Etn	3 <i>N</i> -(C14:0 3-OH)
	1138.51	1144.18			3412.52	3412.56	Kdo ₂ •Hep ₂ •Hex ₄ •HexNAc ₁ •NeuAc ₁	<i>PP</i> Etn	4 <i>N</i> -(C14:0 3-OH)
	1179.51	1185.20			3535.55	3535.57	Kdo ₂ •Hep ₂ •Hex ₄ •HexNAc ₁ •NeuAc ₁ • <i>PE</i> t ₁	<i>PP</i> Etn	4 <i>N</i> -(C14:0 3-OH)
Δ <i>cstV</i>			1225.56	1234.09	2449.14	2449.14	Kdo ₂ •Hep ₂ •Hex ₂ •HexNAc ₁	<i>P</i>	3 <i>N</i> -(C14:0 3-OH)
			1287.08	1295.56	2572.13	2572.15	Kdo ₂ •Hep ₂ •Hex ₂ •HexNAc ₁	<i>PP</i> Etn	3 <i>N</i> -(C14:0 3-OH)
			1348.58		2695.14	2695.16	Kdo ₂ •Hep ₂ •Hex ₂ •HexNAc ₁ • <i>PE</i> t ₁	<i>PP</i> Etn	3 <i>N</i> -(C14:0 3-OH)

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ⁱ Isotope-monoisotopic mass units were used for calculation of molecular mass values based on proposed compositions as follows: HexN, 161.0688; HexN3N, 160.0848; C14:0 3-OH, 226.1933; *PE*t₁, 123.0085; *P*, 79.9663; Kdo, 220.0583; Hep, 192.0634; Hex, 162.0528; HexNAc, 203.0794; NeuAc, 291.0954; H₂O, 18.0106.



