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1 Genetics behind the biosynthesis of nonulosonic acid containing lipooligosaccharides in

2 *Campylobacter coli*

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11 Running Title: Function of *C. coli* GT-42 glycosyltransferases

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15 **ABSTRACT**

16 *Campylobacter jejuni* and *Campylobacter coli* are the most common cause of bacterial
17 gastroenteritis in the world. Ganglioside mimicry by *C. jejuni* lipooligosaccharide (LOS) is
18 the triggering factor of Guillain-Barré syndrome (GBS), an acute polyneuropathy.
19 Sialyltransferases from the glycosyltransferase (GT) family 42 are essential for the
20 expression of ganglioside mimics in *C. jejuni*. Recently, two novel GT-42 genes, *cstIV* and
21 *cstV*, have been identified in *C. coli.* Despite being present in ~11% of currently available *C.*
22 *coli* genomes, the biological role of *cstIV* and *cstV* is unknown. In the present study, mutation
23 studies in two strains expressing either *cstIV* or *cstV* were performed and mass spectrometry
24 was used to investigate differences in the chemical composition of LOS. Attempts were made
25 to identify donor and acceptor molecules using *in vitro* activity tests with recombinant GT-42
26 enzymes. Here, we show that CstIV and CstV are involved in *C. coli* LOS biosynthesis. In
27 particular, *cstV* is associated with LOS sialylation, while *cstIV* is linked to the addition of a
28 diacetylated nonulosonic acid residue.

29 **IMPORTANCE**

30 Despite being a major foodborne pathogen, *Campylobacter coli* glycobiology has been
31 largely neglected. The genetic makeup of the *C. coli* lipooligosaccharide biosynthesis locus
32 was largely unknown until recently. *C. coli* harbour a large set of genes associated to
33 lipooligosaccharide biosynthesis, including several putative glycosyltransferases involved in
34 the synthesis of sialylated lipooligosaccharide in *Campylobacter jejuni*. In the present study,
35 *C. coli* was found to express lipooligosaccharide structures containing sialic acid and other
36 nonulosonate acids. These findings have a strong impact in understanding *C. coli* ecology,
37 host-pathogen interaction, and pathogenesis.

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38 **INTRODUCTION**

39 Nonulosonic acids are a highly diverse family of nine-carbon α-keto acids. The most
40 naturally abundant nonulosonic acids are the sialic acids (*N*-acetylneuraminic acid, Neu5Ac)
41 and derivatives (1). Initially thought to be only a deuterostome feature, sialic acids have been
42 found in virulence associated bacterial cell surface glycoconjugates such as
43 lipopolysaccharides, capsules, pili, and flagella (2-4). Furthermore, these sialylated structures
44 have been shown to influence pathogenesis through immune evasion, adhesion, and invasion
45 (5, 6). Sialyltransferases catalyse the transfer of sialic acid from cytidine-5′-monophospho-*N*-
46 acetylneuraminic acid (CMP-Neu5Ac) to an acceptor and are key in the synthesis of
47 sialoglycoconjugates. Known sialyltransferases have been classified into seven distinct CAZy
48 (Carbohydrate-active enzymes database) glycosyltransferase (GT) families; GT-29, GT-38,
49 GT-42, GT-52, GT-80, GT-97, and GT-100 (7). In *Campylobacter jejuni,* the most common
50 cause of bacterial gastroenteritis, CMP-Neu5Ac biosynthesis (*neuA, neuB,* and *neuC*) and
51 GT-42 genes are present in the lipooligosaccharide (LOS) biosynthesis locus classes A, B, C,
52 M, R, and V (8-10). *C. jejuni* strains carrying one of these genetic classes synthesize LOS
53 structures generally resembling gangliosides (9, 11-13). In some cases, infection with a *C.*
54 *jejuni* strain expressing ganglioside-like LOS induces production of cross-reactive anti-
55 ganglioside antibodies. This leads to the development of Guillian-Barré syndrome (GBS); an
56 acute autoimmune polyradiculoneuropathy disease with ~5% mortality rate (14).

57 *C. coli*, the second most common cause of campylobacteriosis, has also been isolated from
58 GBS patients (15-18). Nevertheless, the role of *C. coli* in GBS has largely remained unclear
59 due to the seemingly absence of key elements for the synthesis of ganglioside-like LOS (i.e
60 GT-42 and *neuABC* genes). Recently, three newly identified *C. coli* LOS-associated GT-42
61 genes were reported in the LOS biosynthesis locus: *cstIV, cstV,* and *cstVI* (10, 19, 20). While
62 *cstVI* is generally found as a pseudogene, *cstIV* and *cstV* may potentially be involved in LOS

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63 biosynthesis (19). In this manuscript we sought to explore the role of the newly identified
64 GT-42 enzymes CstIV and CstV in *C. coli* LOS biosynthesis.

65 **RESULTS**

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

67 The LOS of Δ*cstIV* and Δ*cstV* strains showed an increased mobility on silver stained SDS-
68 PAGE gels relative to the WTs (Fig. 1). Thus, deletion of *cstV* in *C. coli* 76339 and *cstIV* in
69 *C. coli* 73 resulted in a truncated LOS. The complemented *cstV* mutant exhibited two LOS
70 bands on SDS-PAGE gels; the upper one corresponding to the WT LOS and the lower
71 molecular weight band to the truncated LOS (Suppl. Fig. S1). This suggests that partial
72 restoration of the phenotype was achieved upon complementation in *cis* of Δ*cstV*-SR4.
73 Deletion of the second putative sialyltransferase of *C. coli* 76339, *cstI* located in the capsular
74 locus, had no effect on LOS mobility on SDS-PAGE gel (data not shown).

75 Though complementation of Δ*cstIV* strain was infeasible, owing to the absence of a suitable
76 loci, it is unlikely that the mobility shift resulting from *cstIV* disruption was due to a polar
77 effect, as *cstIV* is followed by genes that are transcribed in the opposite direction. While *cstIV*
78 and *cstV* were found to be involved in LOS biosynthesis, neuraminidase treatment had no
79 impact on LOS mobility (Suppl. Fig. S2).

80 ***C. coli* 76339 *neuB1* is involved in the biosynthesis of CstV substrate.**

81 Since no clear shift in the electrophoretic mobility of *C. coli* 76339 LOS was detected after
82 neuraminidase treatment (Suppl. Fig. S2), the putative sialic acid synthase, *neuB1,* located
83 downstream from *cstV* (Fig. 2) was knocked out to determine whether CMP-Neu5Ac was the
84 donor molecule for CstV. The LOS of 76339 Δ*neuB1*-SR2 showed a similar profile to those
85 of 76339 Δ*cstV*-SF1 and 76339 Δ*cstV*-SR4 (Suppl. Fig. S3). Thus, inactivation of *neuB1*

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86 results in a seemingly similar LOS truncation to the one observed in Δ*cstV* strains, suggesting
87 the potential involvement of *neuB1* in the synthesis of the CstV donor.

88 *C. coli* 73 lacks Neu5Ac biosynthesis genes orthologues. However, as other *C. coli* strains, it
89 synthetizes other nonulosonic acids through the activity of *neuB2* and *neuB3* genes. Deletion
90 of *neuB2* had no impact on *C. coli* 73 LOS electrophoretic mobility (data not shown) and,
91 despite repetitive attempts, no viable *C. coli* 73 Δ*neuB3* mutants were obtained.

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

93 Predicted LOS compositions by LC-MS for *C. coli* 76339 WT and mutants are shown on
94 Table 1. *C. coli* 76339 contains a core oligosaccharide linked via two 3-deoxy-D-manno-oct-
95 2-ulosonic acid (Kdo) molecules to a lipid A molecule. The core oligosaccharide of *C. coli*
96 76339 is composed of heptoses (Hep), hexoses (Hex), hexosamines (HexNAc), and NeuAc.
97 The resulting MS/MS spectrum of *m/z* 1064.0 obtained from the *O*-deacylated LOS of *C. coli*
98 76339 WT revealed a single ion at *m/z* 1214.4 corresponding to Hex_3•Hep_2•$PEtn_1$•KDO_1
99 (Fig. 3a). The fragment ions at *m/z* 1052.4 and 890.3 correspond to the additional loss of two
100 Hex residues. The spectra also revealed ions that derived from lipid A, m/z 693.5 and *m/z*
101 388.3 corresponding to $HexN3N_1$•P_1•$(C14:0\ 3\text{-}OH)_2$ and $HexN3N_1$•$(C14:0\ 3\text{-}OH)_1$,
102 respectively. The observation of fragment ions at *m/z* 292.1 and 274.1 provided evidence for
103 the presence of sialic acid on core region LOS. The MS/MS spectrum of precursor ion *m/z*
104 1064.0 from *C. coli* 76339 Δ*cstI* is similar to that from *C. coli* 76339 WT, in which the
105 diagnostic ions for sialic acid were detected at *m/z* 292.1 and 274.1 (Fig. 3b). However, no
106 sialic acid was detected in the MS/MS spectrum *C. coli* 76339 Δ*cstV* (Fig. 3c). Thus, *cstV* is
107 associated to the presence of NeuAc, while *cstI* plays no role in *C. coli* 76339 LOS
108 biosynthesis.

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109 A similar lipid A moiety was indicated by the MS/MS spectrum obtained from the *O*-
110 deacylated LOS of *C. coli* strain 73 WT (Fig. 4a). The spectra also revealed ions that derived
111 from lipid A, *m/z* 693.5 and *m/z* 388.3 corresponding to $HexN3N_1{\bullet}P_1{\bullet}(C14{:}0\ 3\text{-}OH)_2$ and
112 $HexN3N_1{\bullet}(C14{:}0\ 3\text{-}OH)_1$, respectively. The observation of fragment ions at *m/z* 317.2 and
113 299.1 provided evidence for the presence of a residue with a molecular weight of 334.2 Da or
114 316.2 Da for its anhydrous form on core region LOS. These masses are consistent with free
115 diNAc-nonulosonate and its conjugated form, respectively. However, these characteristic ions
116 were not detected in the MS/MS spectrum *C. coli* 73 Δ*cstIV*-SF3 (Fig. 4b). Thus, suggesting
117 the role of *cstIV* in the biosynthesis of diNAc-nonulosonate LOS in *C. coli* 73.

118 **No sialyltransferase activity was detected for CstIV and CstV using *in vitro* assays.**

119 To determine whether CstIV and CstV are capable of transferring Neu5Ac, *C. coli* crude
120 protein extracts were tested for sialyltransferase activity using sugar acceptors labelled with
121 either boron-dipyrromethene (abbreviated as BODIPY or BDP) or fluorescein (FCHASE).
122 No sialyltransferase activity was detected in the crude protein extracts of *C. coli* 73 and *C.*
123 *coli* 73 Δ*cstIV*-SF3. Monospecific α-2,3-sialyltransferase activity was detected in *C. coli*
124 76339 WT, *C. coli* 76339 Δ*cstV*-SF1, and *C. coli* 76339 Δ*cstV*-SR4 crude protein extracts
125 using BDP-Lactose (BDP-Lac) and BDP-*N*-acetyllactosamine (BDP-LacNAc) (Suppl. Fig.
126 S4). Since the genome of *C. coli* 76339 is known to carry the gene encoding the monospecific
127 CstI α-2,3-sialyltransferase, the assays were also performed using *C. coli* 76339Δ*cstI*-XR3
128 and *C. coli* 76339Δ*cstV*-SRΔ*cstI*-XR1 protein extracts (data not shown). No measurable
129 enzymatic activity was detected with any of the tested acceptors in these Δ*cstI* strains which
130 demonstrated that the activity detected in *C. coli* 76339 WT was due to CstI. Furthermore, all
131 tested recombinant CstIV and CstV showed no activity with any of the tested acceptors,
132 suggesting that either none of the tested glycans was a suitable acceptor or that another
133 nonulosonate is the actual donor for these enzymes (data not shown).

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134 **DISCUSSION**

135 *C. jejuni* GT-42 were the first glycosyltransferases from this CAZy family to be
136 enzymatically and structurally characterized; CstII variants can be either monofunctional
137 α2,3- sialyltransferases or bifunctional α2,3-/α2,8-sialyltransferase, while CstI and CstIII are
138 monofunctional α2,3- sialyltransferases (21-24). CstII and CstIII activity has been shown to
139 be essential for the biosynthesis of ganglioside-like LOS structures, which are linked to GBS
140 onset (12, 24). Despite the importance of GT-42 enzymes in virulence and pathogenesis (25-
141 28), the activity of these glycosyltransferases has not been explored in other *Campylobacter*
142 species. Approximately 29% of *C. coli* genomes have been found to contain a GT-42
143 encoding gene within the LOS biosynthesis locus (19). While *cstVI* was the most common
144 LOS associated GT-42 encoding gene in *C. coli*, in 99% of the analysed genomes it was
145 observed to be present as a pseudo gene (19). Thus, we focused our attention on the role of
146 *cstIV* and *cstV* in LOS biosynthesis. Until recently, *cstV* had been solely identified in the
147 genome of *C. coli* 76339 (20). However, in a systematic screen of publicly available *C. coli*
148 genomes several *cstV* positive strains were identified (19). Since *in vitro* assays have been
149 previously used to determine the activity of *C. jejuni* GT-42 enzymes (9, 21), a similar
150 approach was attempted to define CstIV and CstV activity. *C. coli* 76339 crude protein
151 extracts were tested for sialyltransferase activity, as Neu5Ac had been previously detected in
152 the strain's LOS (20). Monofunctional sialyltransferase activity was initially observed but
153 was found to be due to CstI activity. As in *C. jejuni*, *C. coli* 76339 *cstI* is located outside the
154 LOS biosynthesis locus, and encodes an α2,3-sialyltransferase which has no role in LOS
155 biosynthesis (20, 21). Although transcriptomic analysis showed polycistronic expression of
156 LOS biosynthesis genes, indicating the active expression of *cstV* (data not shown), no
157 sialyltransferase activity was detected on the protein extracts of the *cstI* mutant strain.
158 Inactivation of *neuB1* or *cstV* resulted in identical LOS electrophoretic profiles. Additionally,

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159 LC-MS analysis showed that the inactivation of *cstV* resulted in the loss 2 Hex and 1 NeuAc
160 residues. Nevertheless, recombinant CstV exhibited no detectable activity with any of the
161 tested acceptors. Thus, it is very likely that *cstV* is associated to *C. coli* 76339 LOS
162 sialylation. Yet, further studies are required to identified CstV natural acceptor and
163 corroborate its activity *in vitro.*

164 After *cstVI, cstIV* is the most common orthologue; being present in ~38% of the genomes
165 positive for a LOS associated GT-42. Previously, no evidence of Neu5Ac had been found in
166 the LOS of strains containing a *cstIV* orthologue (29). This was to be expected as Neu5Ac
167 biosynthesis genes are rarely present in strains carrying *cstIV* (19). Furthermore, no
168 sialyltransferase activity was detected neither in *C. coli* 73 protein extracts nor in
169 recombinant CstIV. Nevertheless, deletion of *cstIV* in *C. coli* 73 resulted in a truncated LOS.
170 Thus, suggesting a link between *cstIV* and LOS biosynthesis. Sequence alignment of CstIV
171 with previously characterized GT-42 sialyltransferases revealed numerous amino acid
172 substitutions at conserved positions (Suppl. Fig. S5) (30). Additionally, superimposition of
173 CstIV on *C. jejuni* CstII structure identified various substitutions at amino acids involved in
174 substrate interactions (23, 31-33). Interestingly, most substitutions predicted to impact CstIV
175 activity were in the amino acids associated with CMP-Neu5Ac, particularly with the Neu5Ac
176 moiety. Moreover, these substitutions were conserved in multiple CstIV orthologues (23, 32,
177 33). Altogether, results pointed at the possibility of an alternative sugar donor for CstIV.
178 Detection of a diNAc-nonulosonate residue in *C. coli* 73 WT LOS and its absence in *C. coli*
179 73 Δ*cstIV*-SF3 prompted an investigation on genes potentially linked to the synthesis of this
180 residue. In *C. coli, neuB2* (*ptmC, legI*) and *neuB3* (*pseI*) are conserved flagella glycosylation
181 genes involved in the synthesis of legionaminic and pseudaminic acid derivatives,
182 respectively (34-40). Deletion of *neuB2* had no impact on *C. coli* 73 LOS electrophoretic
183 mobility, implying that *neuB2* is not involved in the synthesis of CstIV donor. Despite

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184 repetitive attempts, no viable *C. coli* 73 Δ*neuB3* mutants were obtained. Although *neuB3*
185 deletion has been successful in *C. coli* VC167, disruption of flagellin glycosylation and the
186 potential truncation of the LOS might have resulted in a lethal phenotype for *C. coli* 73 (40).
187 In sum, it is tempting to speculate that the diNAc-nonulosonate residue in *C. coli* 73 WT
188 corresponds to pseudaminic acid. However, the nature of this residue cannot be inferred from
189 MS/MS spectra alone since many diNAc-nonulosonate variants have been identified (41).

190 In conclusion, although we could not determine the complete structures of the LOS outer
191 cores of *C. coli* 73 and *C. coli* 76339, we have established that they both contain nonulosonic
192 acid. We have also unequivocally demonstrated that CstIV and CstV are involved in the
193 synthesis of LOS in their respective stains and, more specifically, they are responsible of the
194 transfer of a nonulosonic acid residue to the outer core.

195 METHODS

196 Bacterial strains, plasmids, and growth conditions.

197 Bacterial strains used in this study are listed in Table 2. Two *C. coli* strains, expressing either
198 *cstIV* or *cstV*, were selected. Figure 2 shows a schematic representation of the LOS locus and
199 the position of the insertion of the antibiotic resistance cassette for the mutational studies. *C.*
200 *coli* 73 possesses LOS locus class II, which contains a copy of *cstIV* and lacks sialic acid
201 biosynthesis genes *neuABC* or other copies of putative sialyltransferases. However, strain 73
202 possesses the conserved biosynthesis pathways for both legionaminic (including legionaminic
203 acid synthetase *neuB2*) and pseudaminic acid (including pseudaminic acid synthetase *neuB3*)
204 in the flagella glycosylation region (29). *C. coli* 76339 harbours two copies of putative
205 sialyltransferases: *cstV* as part of LOS locus class IX (Fig. 2) and a *C. jejuni cstI* orthologue
206 located in the capsule region (19, 20). In addition to the conserved legionaminic and
207 pseudaminic acid biosynthesis pathways, as in *C. coli* 73, *C. coli* 76339 possesses all the

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208 genes for the biosynthesis of sialic acid (*neuAB1C*) within the LOS locus class IX (Fig. 2). *C.*
209 *coli* cultivation and DNA isolation were carried out as previously described, unless specified
210 otherwise (20).

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

212 Chromosomal mutant strains of *C. coli* 76339 (19, 20) and *C. coli* 73 (19, 29) were generated
213 by homologous recombination with suicide vectors containing genes inactivated by the
214 insertion of an antibiotic resistance cassette. All recombinant plasmids and primers are shown
215 in Supplemental material (Suppl. Fig. S6-S11). The genes *cstIV, cstV, neuB1*, *neuB2,* and
216 *neuB3* were inactivated by the insertion of an erythromycin resistance cassette (EryC) (42),
217 while *cstI* was disrupted with a chloramphenicol acetyltransferase cassette (CAT) (43). The
218 inactivation of *cstV* in *C. coli* 76339 was performed by inserting *eryC* cassette either in the
219 direction of the gene (SR) or in the opposite direction (SF). Preparation of electrocompetent
220 cells and transformation was done as previously described (43). Selection of the mutants was
221 done on nutrient blood agar (NBA) supplemented containing either 10 μg ml^{-1} of
222 erythromycin or 12.5 μg ml^{-1} of chloramphenicol. Homologous recombination of all mutants
223 was verified by PCR. Figure 2 shows a summary of the mutations performed in the LOS
224 locus of *C. coli* strains 76339 and 73.

225 **Complementation studies**

226 Complementation of *C. coli* 76339 Δ*cstV*-SR4 was done in *cis* by integration of *cstV* under
227 the active promoter of gamma glutamyltranspeptidase (*ggt*). The *ggt* is an accessory gene in
228 *C. coli* and has no role in LOS biosynthesis. Additionally, the *ggt* locus is located far from the
229 LOS locus and its deletion does not induce a loss in bacterial viability. The suicide vector
230 containing an inactivated *ggt* by the insertion of a *cstV* and CAT (pGEM-ggt-cstV-CAT)
231 (Suppl. Fig. S12) was used to transform *C. coli* 76339 Δ*cstV*-SR4 electrocompetent cells as

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232 above. Transformants were selected on NBA supplemented with 12.5 μg ml^{-1} of
233 chloramphenicol. Homologous recombination of mutants was verified by testing for GGT
234 activity as before (44). Complementation of *cstIV* was not possible due to the absence of a
235 suitable locus.

236 **LOS silver staining.**

237 LOS profiles were assessed by silver staining as described earlier (29). Additionally, LOS
238 sensitivity to neuraminidase was assessed by treating crude LOS with 2 IU/ml of *Clostridium*
239 *perfringens* neuraminidase (Sigma-Aldrich) overnight at 37 ºC.

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

241 Following 1% formaldehyde in PBS (pH 7.4) treatment, *C. coli* cell pellets were washed 3X
242 in PBS and lyophilized. Then, cells were dehydrated by a sequence of 2 washes in each of
243 70% ethanol (in PBS), 100% ethanol, and 100% acetone. The dehydrated cells were treated
244 with proteinase K, RNAse A, and DNAse I as previously described (45). Digested cells were
245 then treated with hydrazine to cleave *O*-linked fatty acids (45). The *O*-deacylated LOS
246 samples were analysed by LC-MS by coupling a Waters Premier Q-TOF with an Agilent
247 1260 capillary LC system. Mass spectrometry was operated in positive-ion detection mode.
248 Liquid chromatography separation was done on an Agilent Eclipse XDB C8 column (5 µm,
249 50 x 1 mm). The flow rate was 20 µL/min. Solvent A: aqueous 0.2% formic acid/0.028%
250 ammonia; solvent B: Isopropanol with 0.2% formic acid/0.028% ammonia. The following
251 gradient was used: 0-2 min. 10% B, 2-16 min linear gradient to 85% B, 16-25 min. 85% B,
252 25-30 min., and equilibration at 10% B.

253 **Sialyltransferase activity test in *C. coli* protein extracts**

254 To test for sialyltransferase activity, *C. coli* 76339 and 73 were grown for 16 h in nutrient
255 broth 2 (Oxoid) (100 rpm, microaerobic atmosphere, and 37 °C). Cells were harvested by

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256 centrifugation (10,000 × *g* for 15 min at 4°C) and resuspended in 50 mM HEPES pH 7.5
257 containing a protease inhibitor cocktail (Sigma). Cells were then lysed by mechanical
258 disruption and debris was removed by centrifugation (10,000 × *g* for 15 min at 4°C).
259 Sialyltransferase activity of protein extracts was tested on boron-dipyrromethene or BODIPY
260 (BDP) labelled Lac, LacNAc, and 3'Sialyllactose or fluorescein (FCHASE) labelled α-
261 GalNAc, β-GalNAc, GM3, α-Gal, β-GlcNAc, α-Glc, β-Glc, Hep-Hep-Glc, as acceptors.
262 Reactions were performed at 37°C in 10 µl volumes containing 50 mM HEPES pH 7.5, 10
263 mM $MgCl_2$, 1 mM CMP-NeuAc, 0.5 mM labelled acceptor, and 6 µl of extract. To stop
264 enzymatic reactions an equal volume of 80% acetonitrile was added. Enzymatic activity was
265 assessed by thin-layer chromatography on silica using a solvent system of ethyl
266 acetate/methanol/water/acetic acid 4:2:1:0.1.

267 **Expression and activity of recombinant *C. coli* GT-42 enzymes**

268 Gene *cstIV* from *C. coli* 73 and *cstV* from *C. coli* 76339 were amplified and ligated to pCW
269 and pCW-MalET plasmids (46). Ligation products were then electroporated in *E. coli* 10β for
270 plasmid amplification. After sequence confirmation, plasmids were electroporated into *E. coli*
271 AD202 or BL21 for protein expression. Cells containing the protein expression vectors were
272 grown in 200 mL of 2YT medium supplemented with 150 µg/mL ampicillin and 0.2% of
273 glucose at 25°C with 250 rpm shaking. After reaching an A_{600nm} of ~0.6, protein over-
274 expression was induced with 0.5 mM of isopropyl-β-D thiogalactopyranoside (IPTG) and
275 cultures were further incubated for 16 h. Cells were harvested by centrifugation (10,000 × *g*
276 for 15 min at 4°C) and crude protein extracts were run in 12% SDS-PAGE gels and stained
277 with Coomassie blue to verify overexpression. In addition, *cstIV* and *cstV* genes were
278 synthesized with a T7 promoter and a ribosome binding site upstream of the coding sequence
279 and a T7 terminator downstream from the stop codon (Thermo Scientific). Synthesized
280 products were inserted into pMA-T vector backbone and proteins were synthesized using the

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281 cell-free PURExpress *in vitro* Protein Synthesis Kit (New England Biolabs Inc.).

282 Recombinant proteins were screened for sialyltransferase activity as described above.

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473 **CONTRIBUTIONS**

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

479 **ADDITIONAL INFORMATION**

480 The authors declare that they have no competing interests.

481 DISCLAIMER

482 M. R is currently employed with the European Food Safety Authority (EFSA) in its BIOCONTAM
483 Unit that provides scientific and administrative support to EFSA's scientific activities in the area of
484 Microbial Risk Assessment. The positions and opinions presented in this article are those of the
485 authors alone and are not intended to represent the views or scientific works of EFSA.

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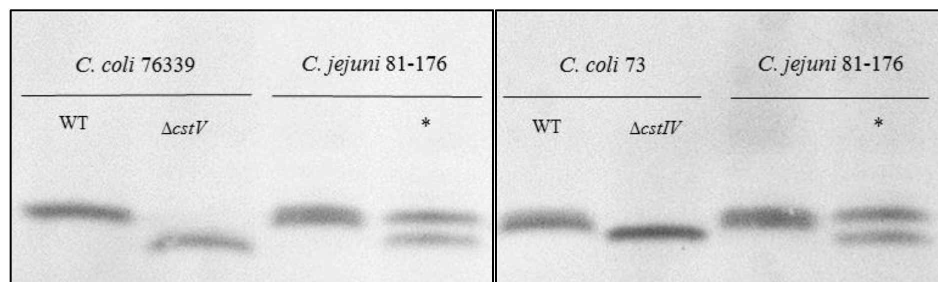

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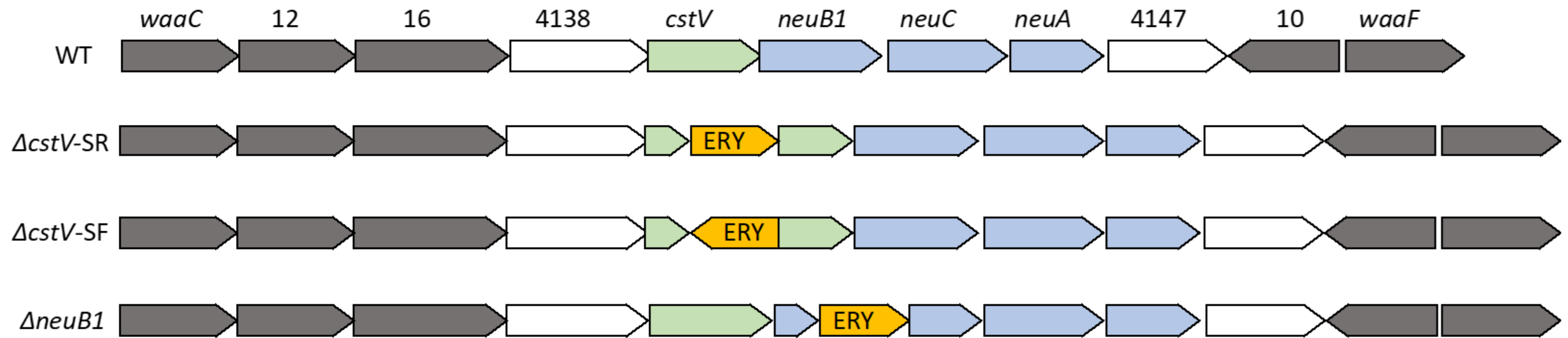
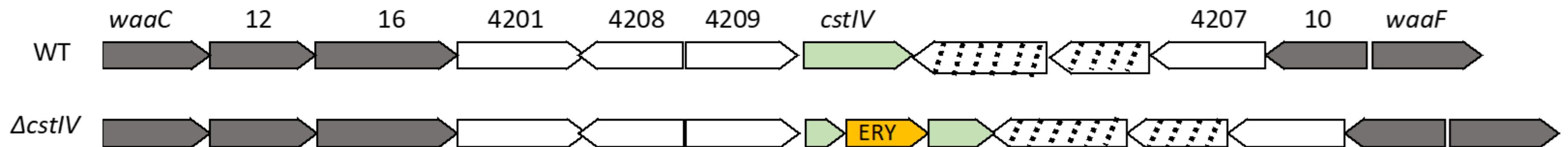
491 **Figure 1.** Electrophoresis mobility comparison of (A) *C. coli* 76339 LOS of WT and mutant strains and (B) *C. coli* 73 LOS of WT and mutant strains. *C. jejuni*
492 81-176 was used as a reference. Lanes marked with an asterisk show *C. jejuni* 81-176 LOS samples treated with neuraminidase to indicate the expected
493 mobility when a Neu5Ac residue is removed.

494





495

496 **Figure 2.** Schematic representation of (A) *C. coli* 76339 LOS locus class IX and (B) *C. coli* 73 LOS locus class II wild types and corresponding mutants. Grey
497 arrows are conserved genes across LOS locus classes as described in (10). Light green arrows represent the GT-42 encoding genes. Light blue arrows represent
498 the genes involved in CMP-Neu5Ac biosynthesis. White arrows show accessory genes with unknown functions. Striped arrows represent pseudogenes in LOS
499 locus class II. Orange arrow indicated with ERY corresponds to the antibiotic resistance cassette used for producing the mutants. Numbers correspond to the
500 gene clusters as described in (19).

501

502 **Table 1.** LC-MS in positive mode data and proposed compositions for *O*-deacylated LOS of *C. coli* 76339 and corresponding *cstI* and *cstV* knock-out mutants.
503

Strain	Observed ions (*m/z*)				Molecular mass (Da)		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	$Kdo_2•Hep_2•Hex_4•HexNAc_1•NeuAc_1$	*PP*Etn	3 *N*-(C14:0 3-OH)
	1104.43	1110.11			3310.30	3310.36	$Kdo_2•Hep_2•Hex_4•HexNAc_1•NeuAc_1•PEtn_1$	*PP*Etn	3 *N*-(C14:0 3-OH)
	1138.48	1144.17			3412.46	3412.56	$Kdo_2•Hep_2•Hex_4•HexNAc_1•NeuAc_1$	*PP*Etn	4 *N*-(C14:0 3-OH)
	1179.51	1185.17			3535.51	3535.57	$Kdo_2•Hep_2•Hex_4•HexNAc_1•NeuAc_1•PEtn_1$	*PP*Etn	4 *N*-(C14:0 3-OH)
Δ*cstI*	1063.44	1069.11			3187.31	3187.35	$Kdo_2•Hep_2•Hex_4•HexNAc_1•NeuAc_1$	*PP*Etn	3 *N*-(C14:0 3-OH)
	1104.44	1110.12			3310.33	3310.36	$Kdo_2•Hep_2•Hex_4•HexNAc_1•NeuAc_1•PEtn_1$	*PP*Etn	3 *N*-(C14:0 3-OH)
	1138.51	1144.18			3412.52	3412.56	$Kdo_2•Hep_2•Hex_4•HexNAc_1•NeuAc_1$	*PP*Etn	4 *N*-(C14:0 3-OH)
	1179.51	1185.20			3535.55	3535.57	$Kdo_2•Hep_2•Hex_4•HexNAc_1•NeuAc_1•PEtn_1$	*PP*Etn	4 *N*-(C14:0 3-OH)
Δ*cstV*			1225.56	1234.09	2449.14	2449.14	$Kdo_2•Hep_2•Hex_2•HexNAc_1$	*P*	3 *N*-(C14:0 3-OH)
			1287.08	1295.56	2572.13	2572.15	$Kdo_2•Hep_2•Hex_2•HexNAc_1$	*PP*Etn	3 *N*-(C14:0 3-OH)
			1348.58		2695.14	2695.16	$Kdo_2•Hep_2•Hex_2•HexNAc_1•PEtn_1$	*PP*Etn	3 *N*-(C14:0 3-OH)

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505 [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;
506 C14:0 3-OH, 226.1933; *P*Etn, 123.0085; *P*, 79.9663; Kdo, 220.0583; Hep, 192.0634; Hex, 162.0528; HexNAc, 203.0794; NeuAc, 291.0954; H_2O, 18.0106.
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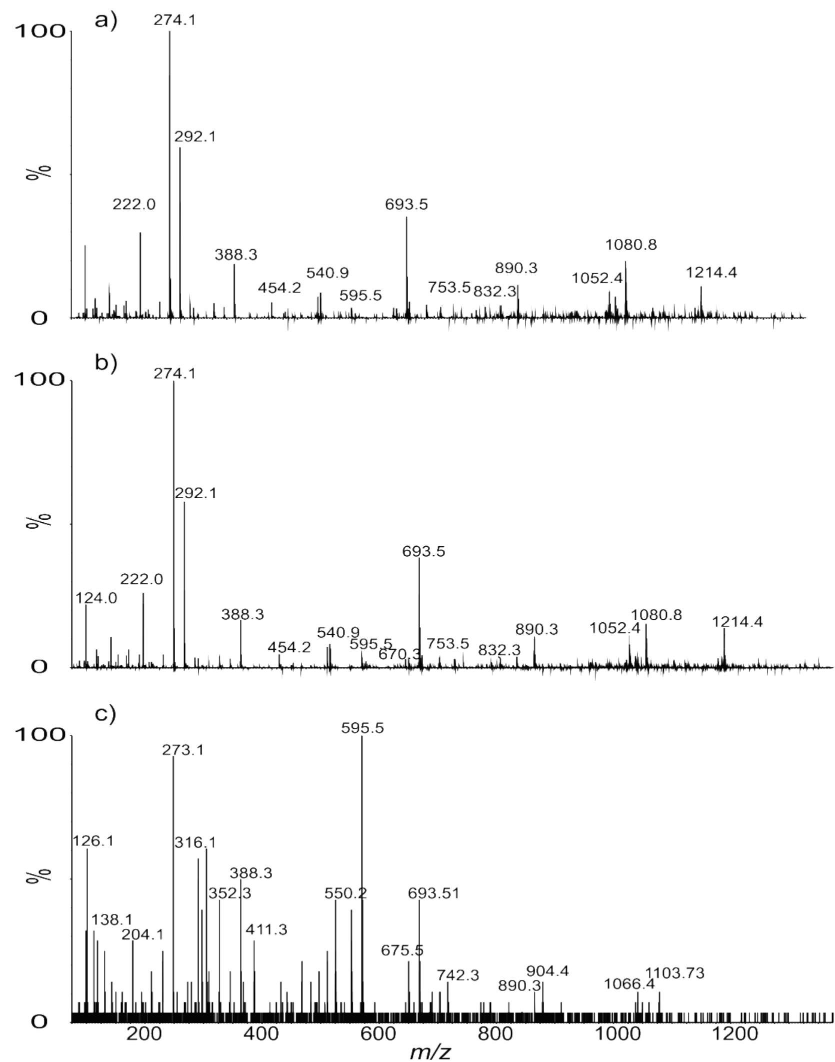

511 **Figure 3.** MS/MS spectra for the precursor ions of *O*-deacylated LOS from (a) *C. coli* 76339 WT, *m/z* 1064.0; (b) *C. coli* 76339 Δ*cstI*, *m/z* 1064.0; and (c) *C.*

512 *coli* 76339 Δ*cstV*, *m/z* 1295.4.

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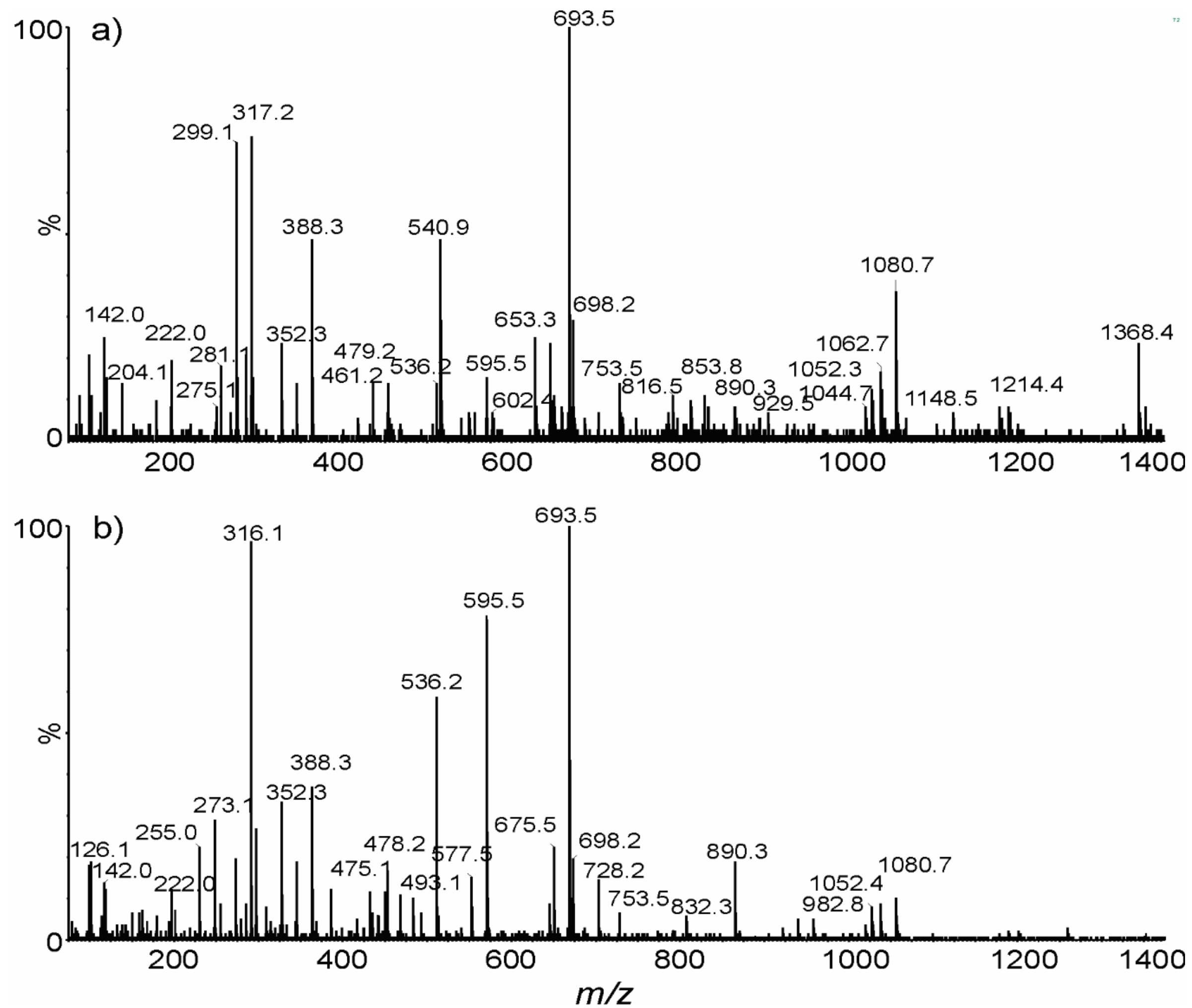

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515 **Figure 4.** MS/MS spectra for the precursor ions of *O*-deacylated LOS from (a) *C. coli* strain 73 WT, *m/z* 1072.4; (b) *C. coli* 73 Δ*cstIV*-SF3, *m/z* 1566.0.

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518 **Table 2.** Bacterial strains

Strain or plasmid	**Genotype and/or phenotype**	**Reference or source**
C. coli 76339	*cstI, cstV, neuB*	(20)
C. coli 76339 Δ*cstV*-SF1	*cstI,* Δ*cstV*: Ery*, neuB*	This study
C. coli 76339 Δ*cstV*-SR4	*cstI,* Δ*cstV*: Ery*, neuB*	This study
C. coli 76339 Δ*neuB*-SR1	*cstI,* Δ*neuB*: Ery*, cstV*	This study
C. coli 76339 Δ*cstI*-XR3	Δ*cstI*: CAT*, cstV, neuB*	This study
C. coli 76339 Δ*cstV*-SR4 Δ*cstI*-XR1	Δ*cstI*: CAT*,* Δ*cstV*: Ery*, neuB*	This study
C. coli 76339 Δ*neuB*-SR2	*cstI,* Δ*neuB*: Ery*, cstV*	This study
C. coli 76339 Δ*cstV*-SR4 Δ*ggt:cstV*-2	*cstI,* Δ*cstV*: Ery*, neuB,* Δggt:*cstV:*CAT	This study
C. coli 73	*cstIV*	(29)
C. coli 73 Δ*cstIV*-SF3	Δ*cstIV*:Ery	This study
C. coli 73 Δ*neuB2*	Δ*neuB2*:Ery	This study
C. jejuni 81-176		
E. coli AD202		(46)

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