Title: Genetics behind the biosynthesis of nonulosonic acid containing lipooligosaccharides in *Campylobacter coli*

Running title: *Campylobacter coli* GT-42

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ABSTRACT

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

INTRODUCTION

Nonulosonic acids are a highly diverse family of nine-carbon α-keto acids. The most natural abundant nonulosonic acids are the sialic acids (*N*-acetylneuraminic acid, Neu5Ac) and derivatives [1]. Initially thought to be only a deuterostomes feature, sialic acids have been found in virulence associated bacterial cell surface glycoconjugates such as lipopolysaccharides, capsules, pili, and flagella [2-4]. Furthermore, these sialylated structures have been shown to influence pathogenesis through immune evasion, adhesion, and invasion [5, 6]. Sialyltransferases, catalyse the transfer of sialic acid from cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) to an acceptor, are key in the synthesis of sialoglycoconjugates. Known sialyltransferases have been classified into five distinct CAZy (Carbohydrate-active enzymes database) glycosyltransferase (GT) families; GT-29, GT-38, GT-42, GT-52, and GT-80 [7]. In *Campylobacter jejuni*, the most common cause of bacterial gastroenteritis, CMP-Neu5Ac biosynthesis (*neuA*, *neuB1*, and *neuC*) and GT-42 genes are present in the lipooligosaccharide (LOS) biosynthesis locus classes A, B, C, M, R, and V [8-10]. *C. jejuni* strains carrying one of these genetic classes synthesize LOS structures generally resembling gangliosides [9, 11-13]. In some cases, infection with a *C. jejuni* strain expressing ganglioside-like LOS induces production of cross-reactive anti-ganglioside antibodies. This leads to the development of Guillian-Barré syndrome (GBS); an acute autoimmune polyradiculoneuropathy disease with ~5% mortality rate [14].

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

and *cstV* may potentially be involved in LOS biosynthesis [19]. In this manuscript we sought to explore the role of the new GT-42 enzymes CstIV and CstV in LOS biosynthesis.

RESULTS

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

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

CstIV and CstV are involved in LOS biosynthesis.

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

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

Since no clear shift in the electrophoretic mobility of *C. coli* 76339 LOS was detected after neuraminidase treatment (Suppl. Fig. 4), *neuB1* was knocked out to determine whether CMP-Neu5Ac was CstV's substrate. The LOS of 76339 Δ*neuB*-SR2 showed a similar profile to those of 76339 Δ*cstV*-SF1 and 76339 Δ*cstV*-SR4 (Suppl. Fig. 5). Thus, deletion of *neuB* results in a seemingly similar LOS truncation to the one observed in Δ*cstV* strains, suggesting the potential involvement of *neuB1* in CstV substrate synthesis.

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

Predicted LOS compositions by LC-MS for *C. coli* 76339 WT and mutants are shown on Table 1. *C. coli* 76339 contains a core oligosaccharide linked via two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) molecules to a lipid A molecule. The core oligosaccharide of *C. coli* 76339 is composed of heptoses (Hep), hexoses (Hex), hexosamines (HexNAc), and NeuAc. The resulting MS/MS spectrum obtained from the O-deacylated LOS of *C. coli* 76339 WT revealed a single ion at *m/z* 1214.4 corresponding to Hex_3•Hep_2•$PEtn_1$•KDO_1 (Fig. 2). The fragment ions at m/z 1052.4 and 890.3 correspond to the additional loss of two Hex residues. The spectra also revealed ions that derived from lipid A, m/z 693.5 and m/z 388.3 corresponding to $HexN3N_1$•P_1•$(C14{:}0\ 3\text{-}OH)_2$ and $HexN3N_1$•$(C14{:}0\ 3\text{-}OH)_1$, respectively. The observation of fragment ions at *m/z* 292.1 and 274.1 provided evidence for the presence of sialic acid on core region LOS. The MS/MS spectrum of precursor ion *m/z* 1064.0 from *C. coli* 76339 Δ*cstI* is similar to that from *C. coli* 76339 WT, in which the diagnostic ions for sialic acid were detected at *m/z* 292.1 and 274.2 (Fig. 2b). However, no sialic acid was detected in the MS/MS spectrum *C. coli* 76339 Δ*cstV* (Fig. 2c). Thus, *cstV* is associated to the presence of NeuAc, while *cstI* plays no role in *C. coli* 76339 LOS biosynthesis.

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

DISCUSSION

C. jejuni. *C. jejuni* GT-42 were the first glycosyltransferases from this CAZy family to be enzymatically and structurally characterized; CstII is mono/bifunctional exhibiting α2,3-/α2,8-sialyltransferase activity, while CstI and CstIII are monofunctional α2,3- sialyltransferases [21-24]. The activity of CstII and CstIII has been shown to be essential for the biosynthesis ganglioside-like LOS structures, which are linked to GBS onset [12, 24]. Despite the importance of GT-42 enzymes in virulence and pathogenesis [25-28], the activity of these glycosyltransferases has not been explored in other *Campylobacter* species. Approximately 29% of *C. coli* genomes have been found to contain a GT-42 encoding gene within the LOS biosynthesis locus [19]. While *cstVI* was the most common LOS associated GT-42 encoding gene in *C. coli*, in 99% of the analysed genomes it was observed to be present as a pseudo gene. Thus, we focused our attention on the role of *cstIV* and *cstV* in LOS biosynthesis. Until recently, *cstV* had been solely identified in the genome of *C. coli* 76339 [20]. However, in a systematic screen of publicly available *C. coli* genomes several *cstV* positive strains were identified [19]. *C. coli* 76339 crude protein extracts were tested for sialyltransferase activity as Neu5Ac had been previously

detected in the strain's LOS [20]. Monosialyltransferase activity was initially detected, but was found to be due to activity of CstI. As in *C. jejuni*, *C. coli* 76339 *cstI* is located outside the LOS biosynthesis locus and encodes a α2,3- sialyltransferase which has no role in LOS biosynthesis [20, 21]. No sialyltransferase activity was detected on the protein extracts of the *cstI* mutant strain. However, transcriptomic analysis showed polycistronic expression of LOS biosynthesis genes indicating the active expression of *cstV*. Similarly, recombinant CstV exhibited no detectable activity on any of the tested acceptors. Deletion of *neuB1* or *cstV* resulted in identical LOS electrophoretic profiles. Additionally, LC-MS analysis showed that deletion of *cstV* resulted in the loss 2 Hex and 1 NeuAc. Thus, it is probable that *cstV* is associated to *C. coli* 76339 LOS sialylation. Yet, further studies are required to identified CstV natural acceptor and corroborate its activity *in vitro.*

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

Detection of a diNAc-nonulosonate residue in *C. coli* 73 WT LOS and its absence in *C. coli* 73 Δ*cstIV*-SF3 prompted us to investigate genes potentially linked to the synthesis of this residue. In *C. coli*, *neuB2* (*ptmC, legI*) and *neuB3* (*pseI*) are conserved flagella glycosylation genes involved in the synthesis of legionaminic and pseudaminic acid derivatives, respectively [34-40]. Deletion of *neuB2* had no impact on *C. coli* 73 LOS electrophoretic mobility (Suppl. Fig. 7), implying that *neuB2* is not involved in the synthesis of CstIV substrate. Despite repetitive attempts, no viable *C. coli* 73 Δ*neuB3* mutants were obtained. Although *neuB3* deletion has been successful in *C. coli* VC167, disruption of flagellin glycosylation and potentially truncation of the LOS might have resulted in a lethal phenotype for *C. coli* 73 [40]. In sum, it is tempting to speculate that the diNAc-nonulosonate residue in *C. coli* 73 WT corresponds to pseudaminic acid. However, the nature of this residue cannot be inferred from MS/MS spectra alone, since many diNAc-nonulosonate variants have been identified [41]. Thus, this task is still under investigation.

Conclusion

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

METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are listed in Supplemental Table 1. *C. coli* cultivation and DNA isolation were carried out as previously described, unless specified otherwise [20].

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

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

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

Complementation of *C. coli* 76339 Δ*cstV*-SR4 was done in *cis* by integration of *cstV* under the active promoter of gamma glutalmyltranspeptidase (*ggt*). The *ggt* is an accessory gene in *C. coli* and has no role in LOS biosynthesis. Additionally, the *ggt* locus is located far from the LOS locus and its deletion does not induce a loss in bacterial viability. The suicide vector containing an inactivated *ggt* by the insertion of a *cstV* and CAT (pGEM-ggt-cstV-CAT) was used to transform *C. coli* 76339 Δ*cstV*-SR4 electrocompetent cells as above. Transformants were selected on NBA supplemented with 12.5 µg ml^{-1} of chloramphenicol. Homologous recombination of mutants was verified by testing for GGT activity as before [44].

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

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

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

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CONTRIBUTIONS

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

ADDITIONAL INFORMATION

The authors declare that they have no competing interests

Figure Legends

Figure 1. Electrophoresis mobility comparison of *C. coli* LOS of WT and mutant strains. *C. jejuni* 81-176 was used as a reference. Samples marked with an asteric were neuraminidase treated.

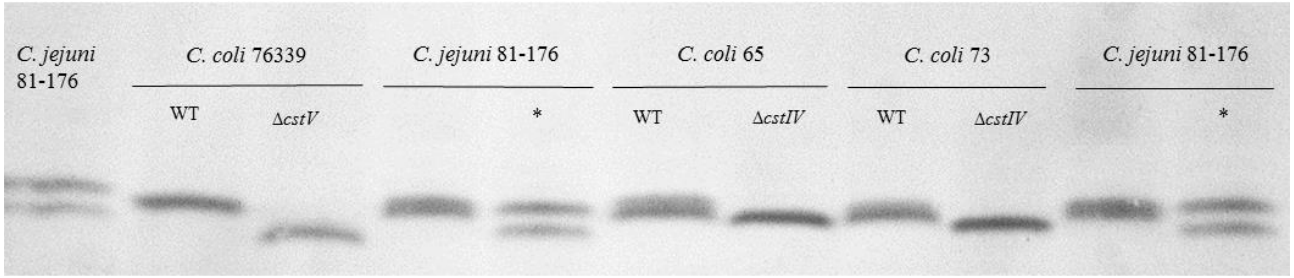
Figure 2. MS/MS spectra for the precursor ions of O-deacylated LOS from *(a) C. coli* 76339 WT, *m/z* 1064.0; (b) *C. coli* 76339 ΔcstI, *m/z* 1064.0; and (c) *C. coli* 76339 ΔcstV, *m/z* 1295.4.

Figure 3. 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.

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-out mutants.

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•$HexNAc_1$•$NeuAc_1$•*P*Etn_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•$HexNAc_1$•$NeuAc_1$•*P*Etn_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•$HexNAc_1$•$NeuAc_1$•*P*Etn_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•$HexNAc_1$•$NeuAc_1$•*P*Etn_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•$HexNAc_1$•*P*Etn_1	*PP*Etn	3 *N*-(C14:0 3-OH)

[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; *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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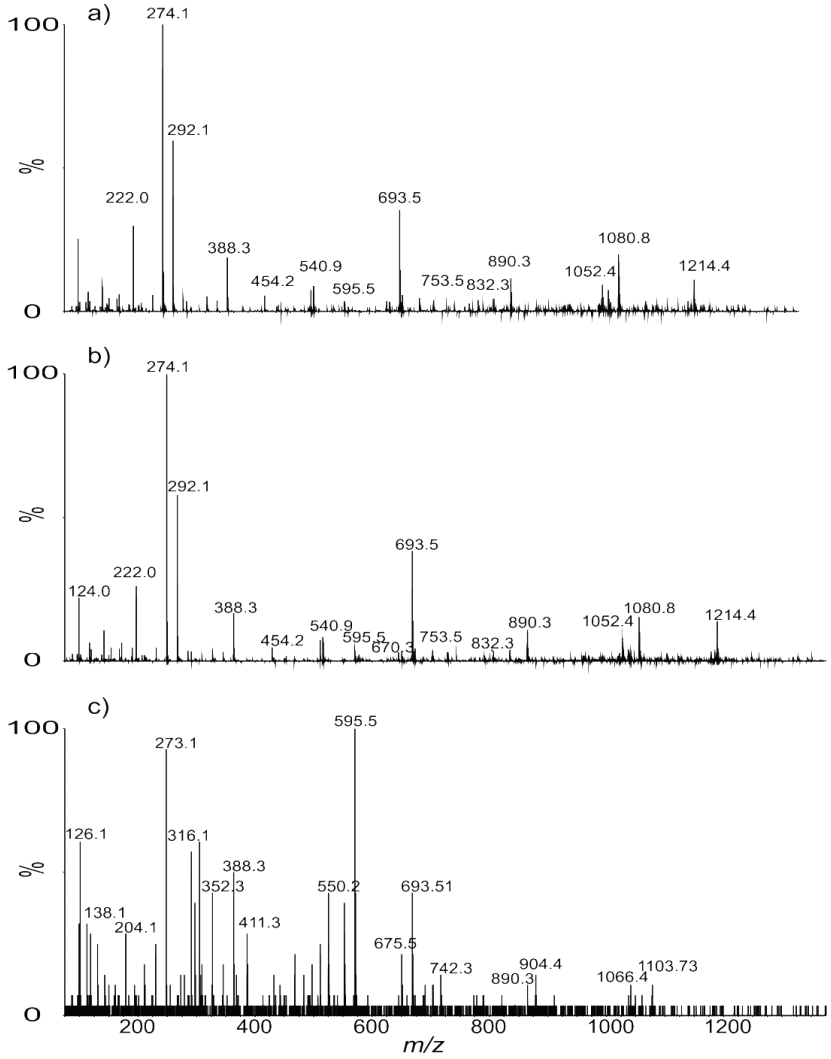
C. coli 76339
WT
ΔcstV
C. jejuni 81-176
*
C. coli 65
WT
ΔcstIV
C. coli 73
WT
ΔcstIV
C. jejuni 81-176
*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454.2
595.5
753.5
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b)
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1214.4
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670.3
753.5
832.3
c)
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550.2
693.51
138.1
204.1
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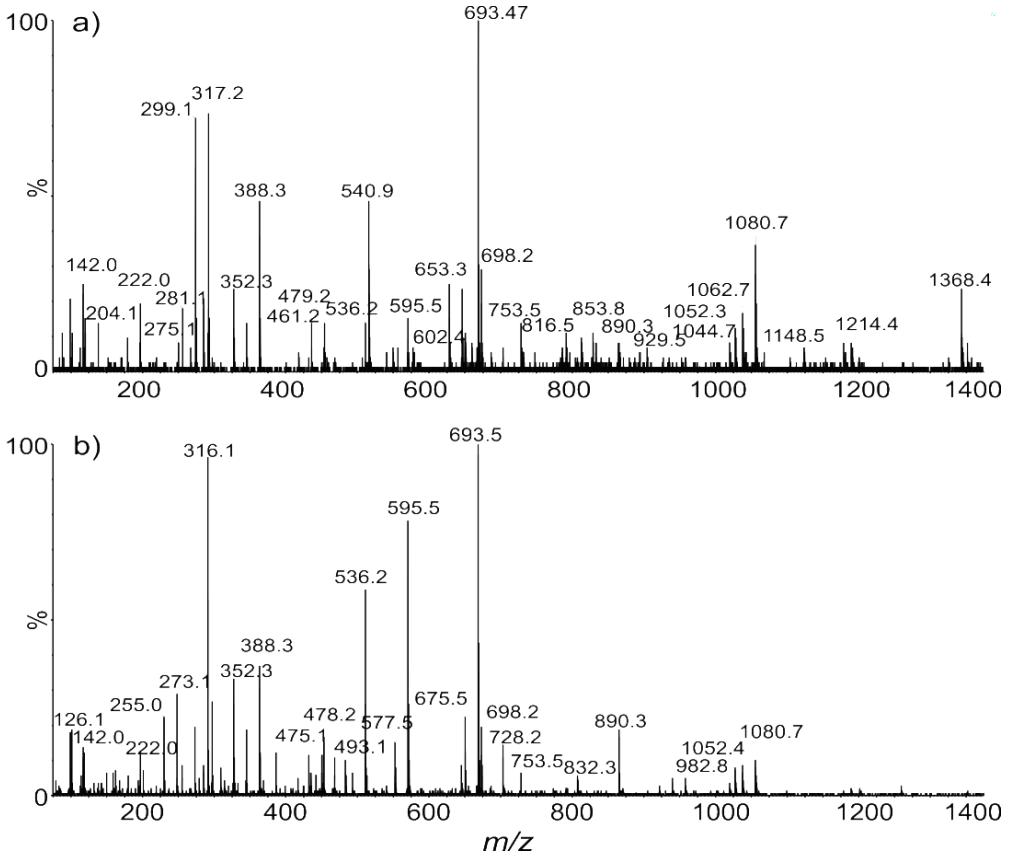
m/z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816.5
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602.4
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1200
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m/z