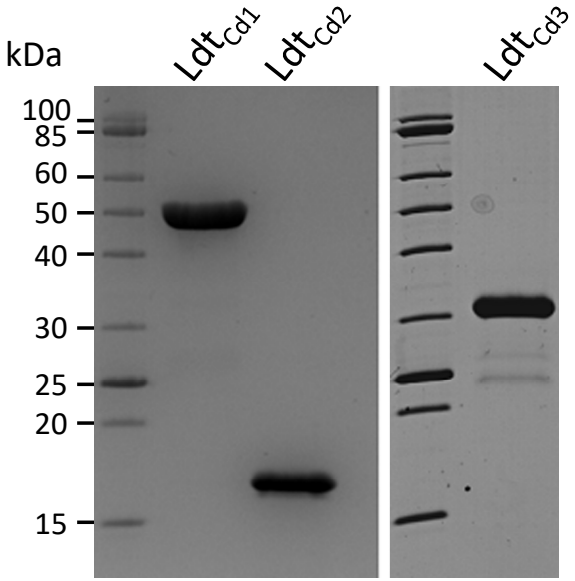


Figure S1. SDS-PAGE analysis of Ldt_{Cd1}, Ldt_{Cd2} and Ldt_{Cd3} purified recombinant proteins used for *in vitro* assays.

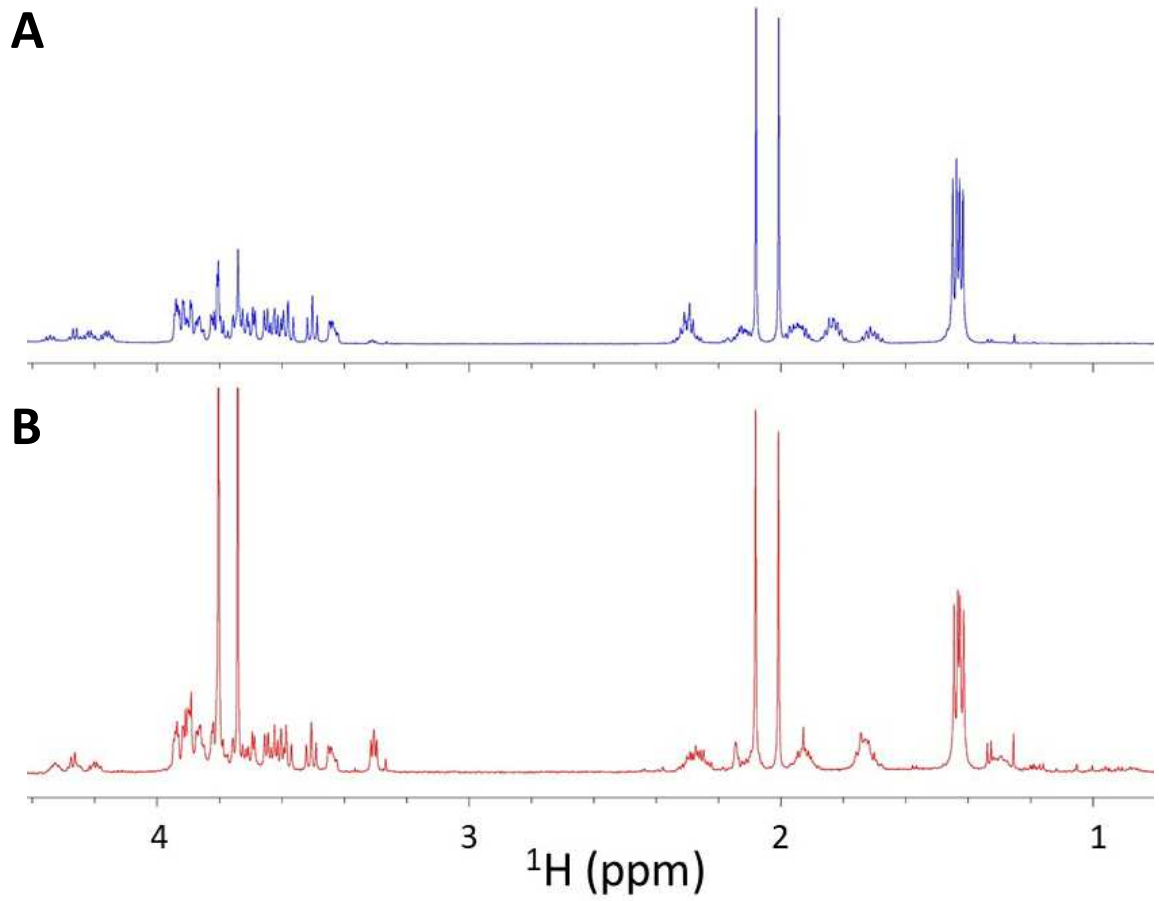
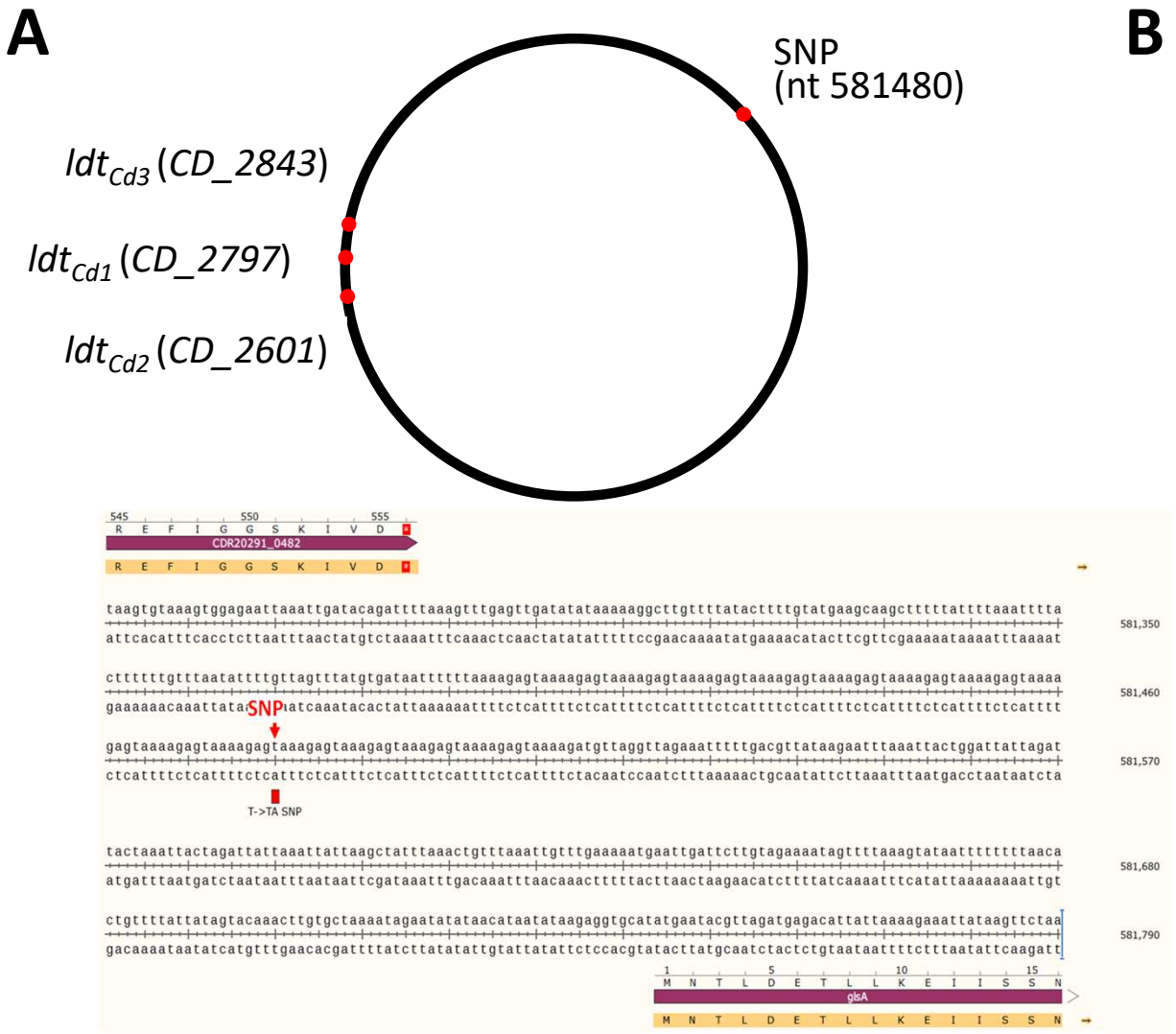


Figure S2. One-dimensional ^{1}H NMR spectra of (**A**) gm-AEJ monomer (peak 1 in Figure 1) and (**B**) gm-AEJ=gm-AEJ (-H2O) dimer (peak a in Figure 1). The two singlet *N*-acetyl methyl signals are at around 2 ppm, and the two methyl doublets are at around 1.4 ppm.



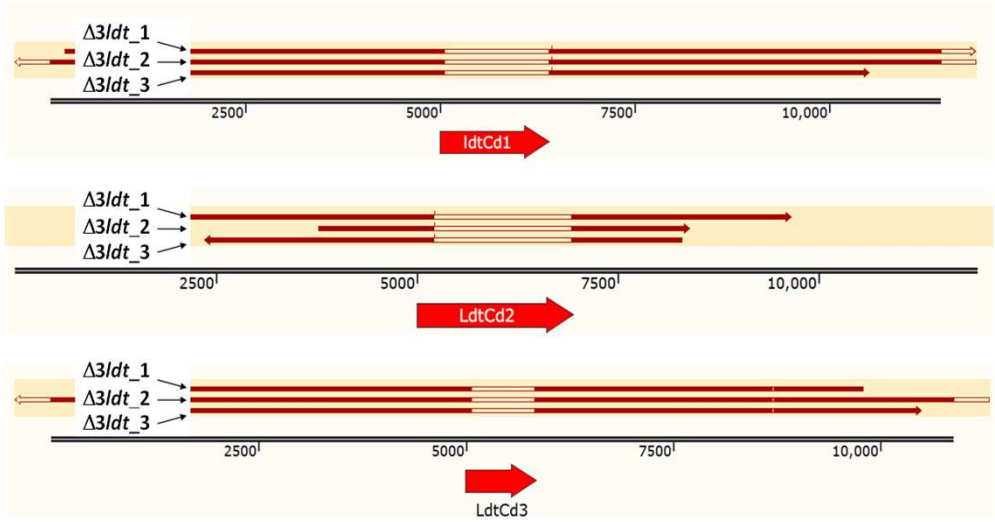


Figure S3. Genome analysis of *C. difficile* WT and Δ3*ldt* derivative. **A**, schematic representation of the R20291 chromosome depicting the localisation of the 3 *ldt*$_{Cd}$ genes and the SNP detected in the triple mutant in position 581480 (T→TA). The sequence in figure A shows the position of the mutation between the two adjacent genes. **B**, sequence alignments of the contigs assembled from the 3 independent genomes sequenced. Each DNA was purified from the culture that was used for peptidoglycan analysis.

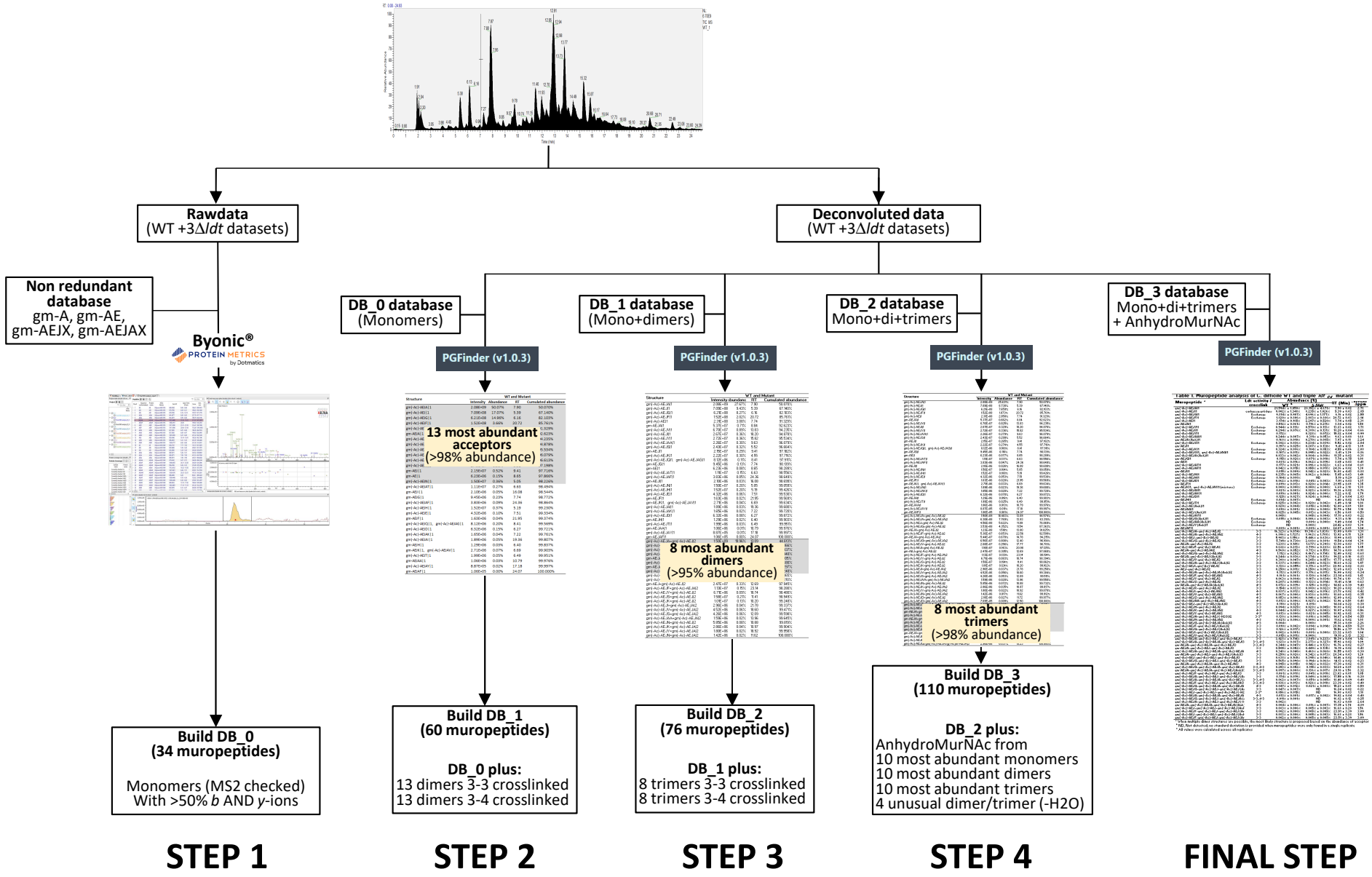
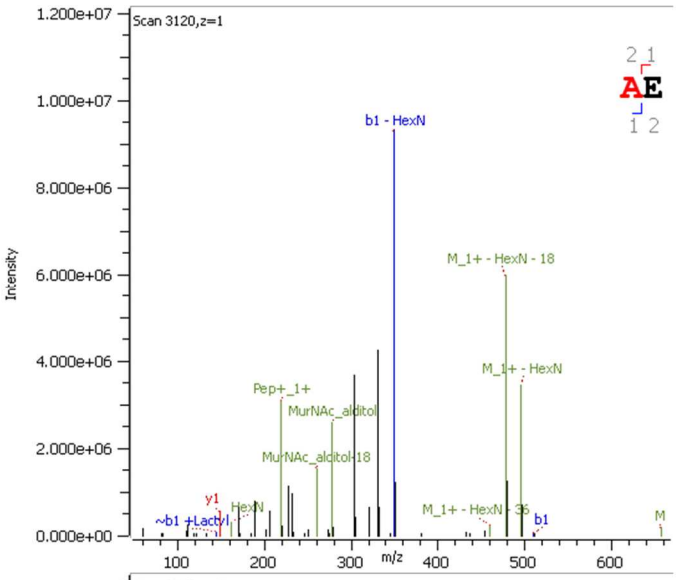
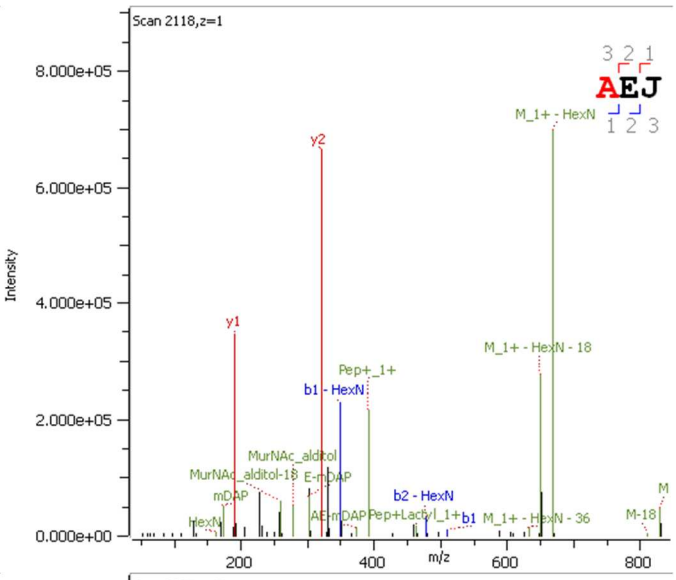


Figure S4. Strategy for peptidoglycan structural analysis. Sequential searches were performed using the Byonic™ and PGFinder software. The monomer database DB_0 was built based on the MS/MS analysis carried out with Byonic™. The identification of the most abundant monomers was used to build the following databases containing dimers (DB_1), trimers (DB_2) and anhydroMurNAc groups (DB_3). DB_3 was used for a final « one off » search.


Scan 3120,z=1
AE
b1 - HexN
M_1+ - HexN - 18
M_1+ - HexN
Pep+_1+
MurNAc_alditol
MurNAc_alditol-18
y1
HexN
~b1 +Lactyl
M_1+ - HexN - 36
b1
M
Intensity
m/z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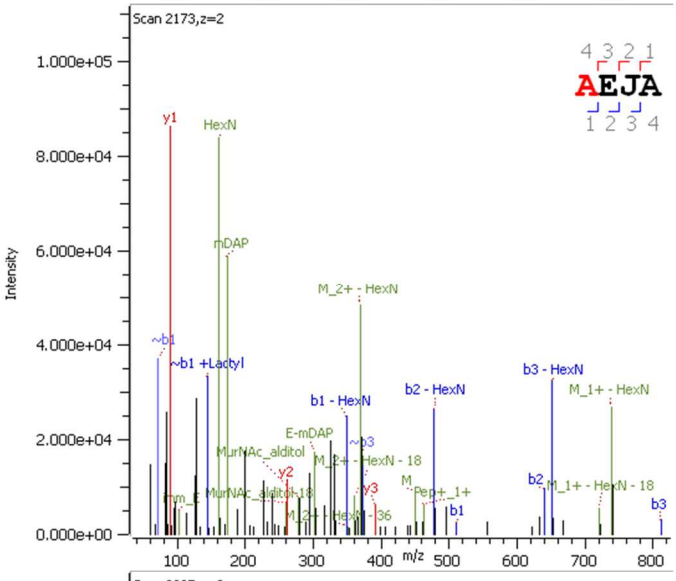
M_1+ - HexN
y2
y1
M_1+ - HexN - 18
Pep+_1+
b1 - HexN
MurNAc_alditol
MurNAc_alditol-18
E-mDAP
mDAP
b2 - HexN
HexN
AE-mDAP
Pep+Lactyl_1+
b1
M_1+ - HexN - 36
M-18
M
Intensity
m/z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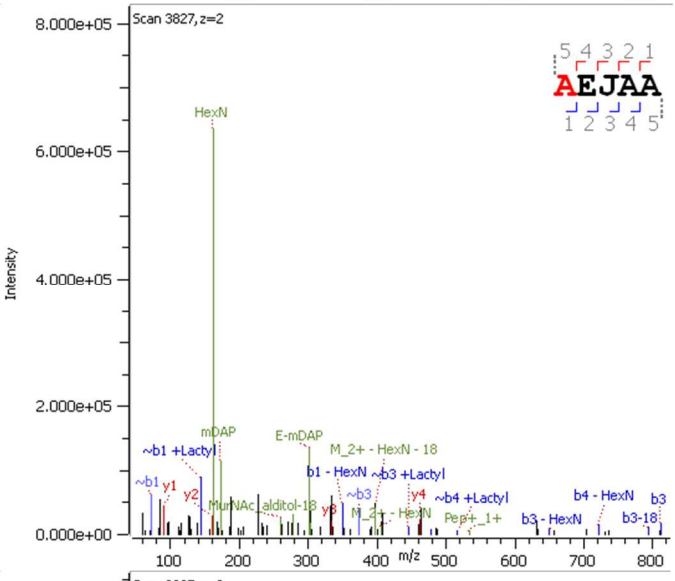
y1
HexN
mDAP
M_2+ - HexN
~b1
~b1 +Lactyl
b3 - HexN
b1 - HexN
b2 - HexN
M_1+ - HexN
E-mDAP
~b3
MurNAc_alditol
M_2+ - HexN - 18
y2
y3
M
Pep+_1+
b2
M_1+ - HexN - 18
b1
b3
imm_F
MurNAc_alditol-18
M_2+ - HexN - 36
Intensity
m/z


Scan 3827,z=2
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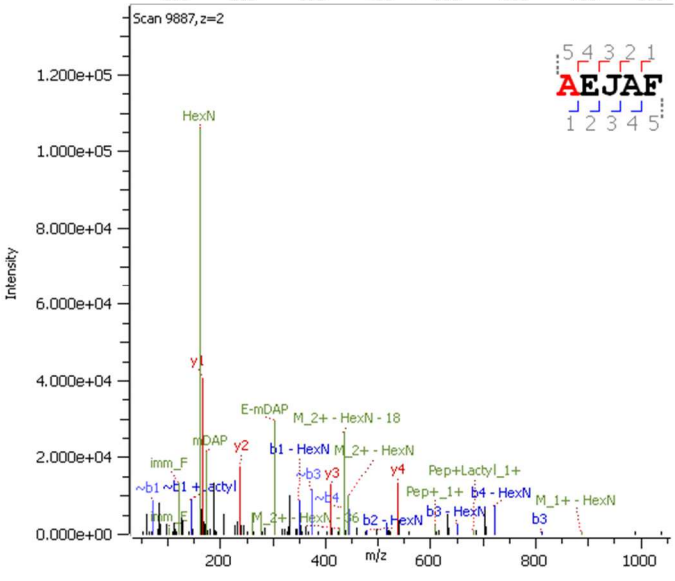
HexN
mDAP
E-mDAP
~b1 +Lactyl
M_2+ - HexN - 18
b1 - HexN
~b3 +Lactyl
~b1
y1
y2
~b3
y4
~b4 +Lactyl
b4 - HexN
b3
MurNAc_alditol-18
y3
M_2+ - HexN
Pep+_1+
b3 - HexN
b3-18
Intensity
m/z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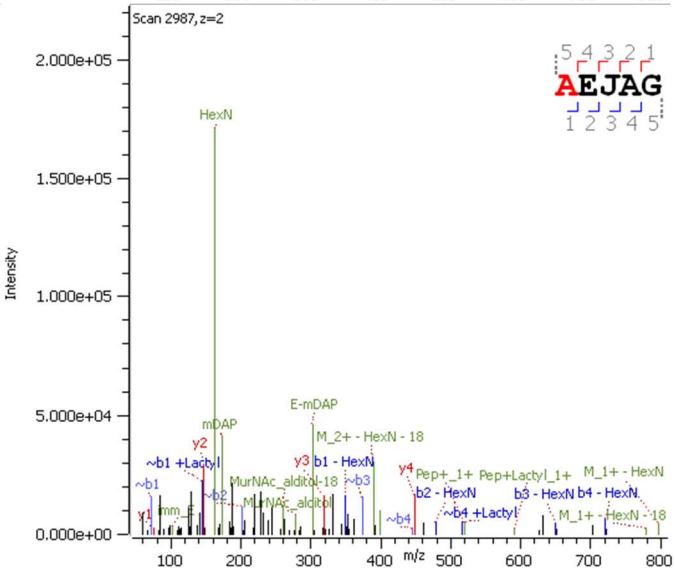
y1
E-mDAP
M_2+ - HexN - 18
mDAP
y2
b1 - HexN
M_2+ - HexN
imm_F
~b3
y3
y4
Pep+Lactyl_1+
~b1
~b1 +Lactyl
~b4
Pep+_1+
b4 - HexN
M_1+ - HexN
b3 - HexN
b3
M_2+ - HexN - 36
b2 - HexN
Intensity
m/z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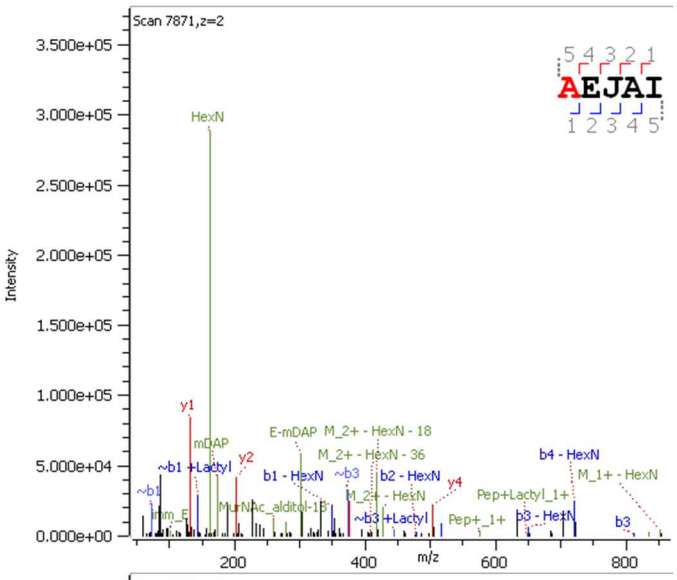
HexN
E-mDAP
mDAP
M_2+ - HexN - 18
y2
~b1 +Lactyl
y3
b1 - HexN
y4
Pep+_1+
Pep+Lactyl_1+
M_1+ - HexN
~b1
~b2
b2 - HexN
b3 - HexN
b4 - HexN
MurNAc_alditol-18
~b3
MurNAc_alditol
~b4
~b4 +Lactyl
M_1+ - HexN - 18
y1
imm_F
Intensity
m/z


Scan 7871,z=2
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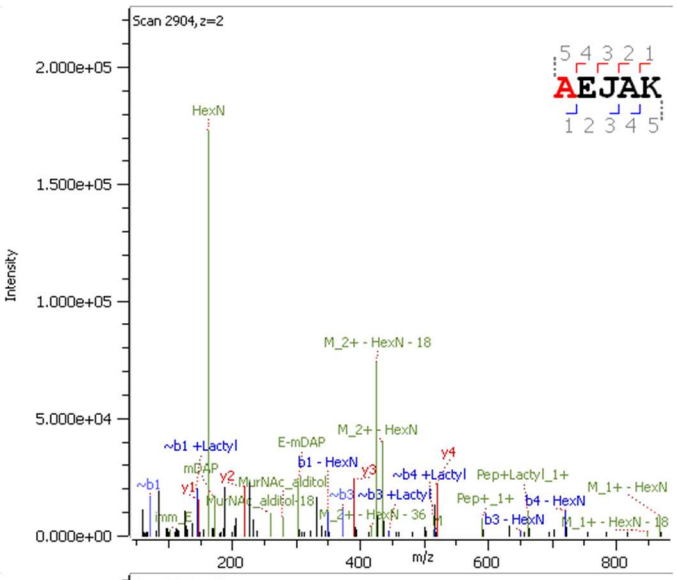
Intensity
m/z
HexN
mDAP
y1
y2
E-mDAP
M_2+ - HexN - 18
M_2+ - HexN - 36
b4 - HexN
M_1+ - HexN
b1 - HexN
b2 - HexN
~b1 +Lactyl
~b3
~b1
imm_E
MurNAc_alditol-18
M_2+ - HexN
y4
~b3 +Lactyl
Pep+Lactyl_1+
Pep+_1+
b3 - HexN
b3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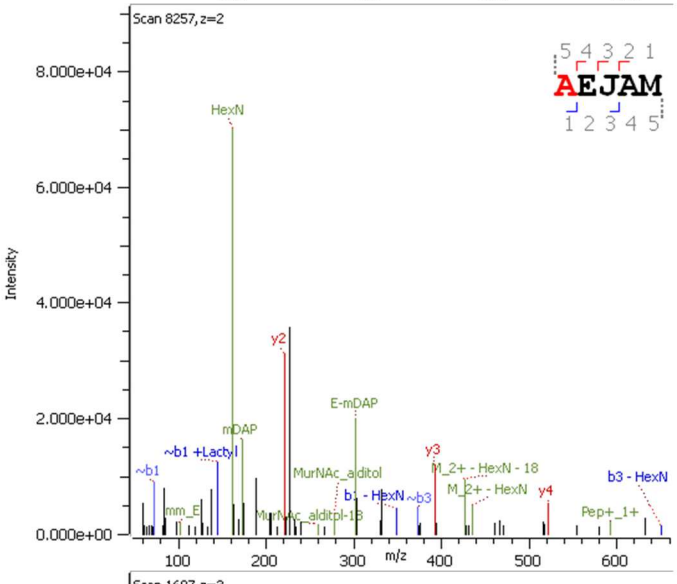
Intensity
m/z
HexN
M_2+ - HexN - 18
~b1 +Lactyl
E-mDAP
M_2+ - HexN
mDAP
b1 - HexN
y3
y4
~b4 +Lactyl
Pep+Lactyl_1+
~b1
y1
y2
MurNAc_alditol
MurNAc_alditol-18
~b3
~b3 +Lactyl
Pep+_1+
b4 - HexN
M_1+ - HexN
imm_E
M_2+ - HexN - 36
b3 - HexN
M_1+ - HexN - 18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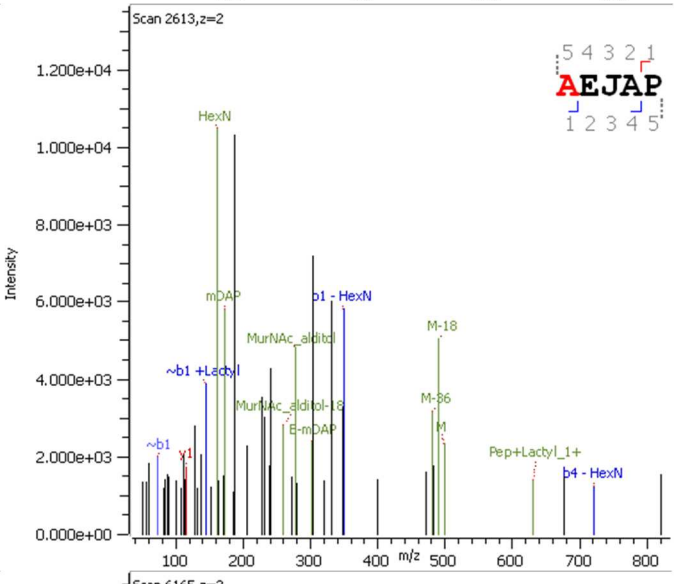
Intensity
m/z
HexN
y2
E-mDAP
mDAP
y3
~b1 +Lactyl
~b1
M_2+ - HexN - 18
MurNAc_alditol
b3 - HexN
b1 - HexN
~b3
M_2+ - HexN
y4
imm_E
MurNAc_alditol-18
Pep+_1+


Scan 2613,z=2
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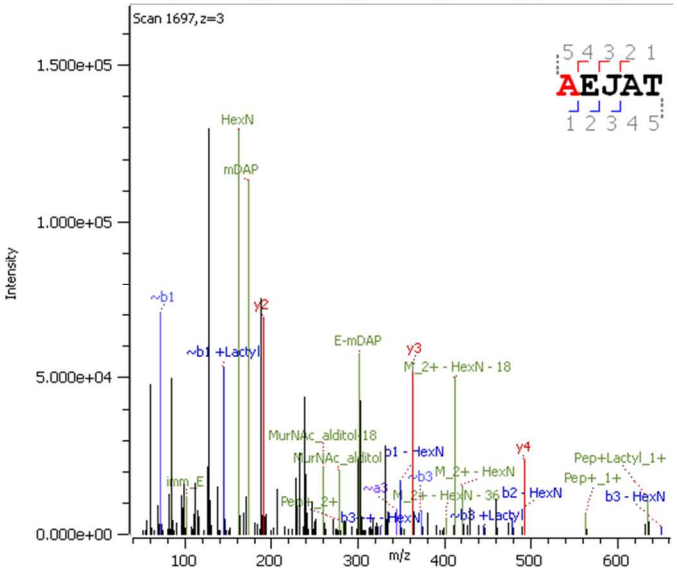
Intensity
m/z
HexN
mDAP
b1 - HexN
M-18
MurNAc_alditol
~b1 +Lactyl
MurNAc_alditol-18
M-36
E-mDAP
M
~b1
y1
Pep+Lactyl_1+
b4 - HexN


Scan 1697,z=3
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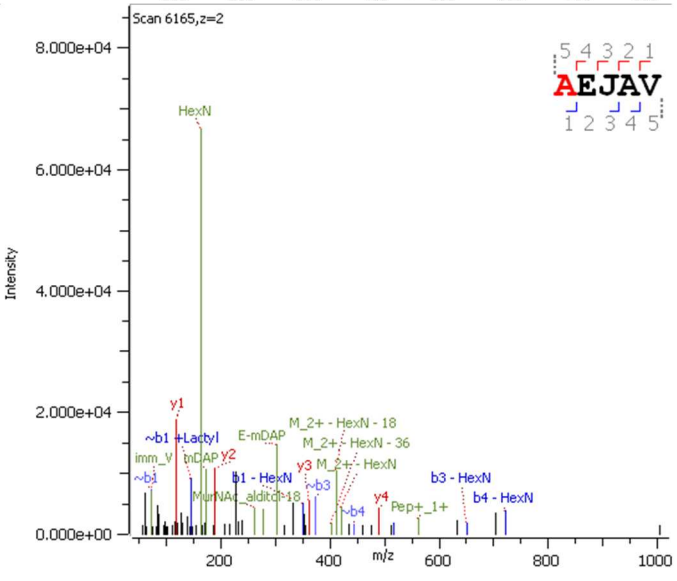
Intensity
m/z
HexN
mDAP
~b1
y2
~b1 +Lactyl
E-mDAP
y3
M_2+ - HexN - 18
MurNAc_alditol-18
MurNAc_alditol
b1 - HexN
y4
Pep+Lactyl_1+
imm_E
~b3
M_2+ - HexN
Pep+_1+
~a3
M_2+ - HexN - 36
b2 - HexN
b3 - HexN
~b3 +Lactyl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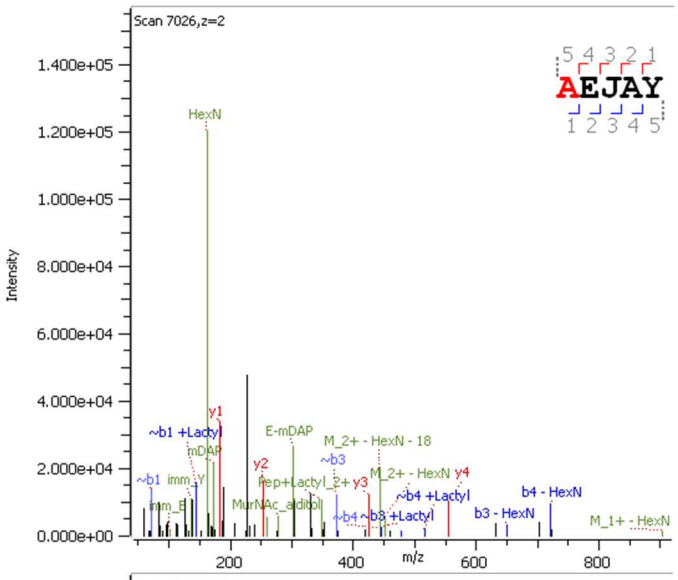
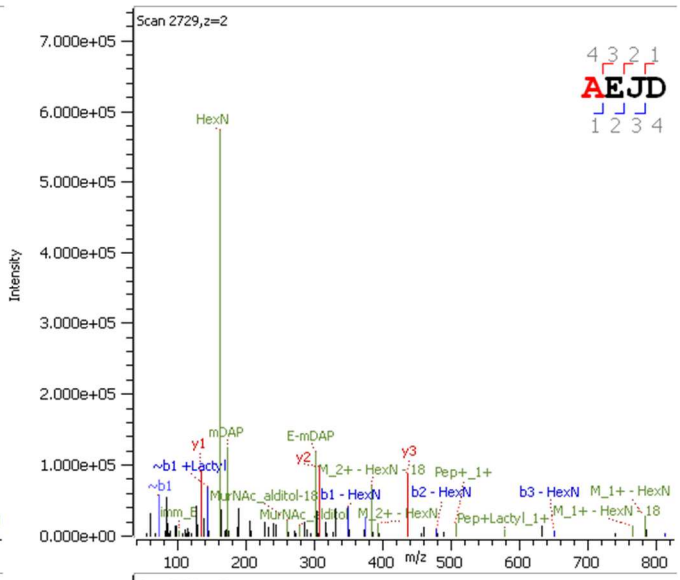
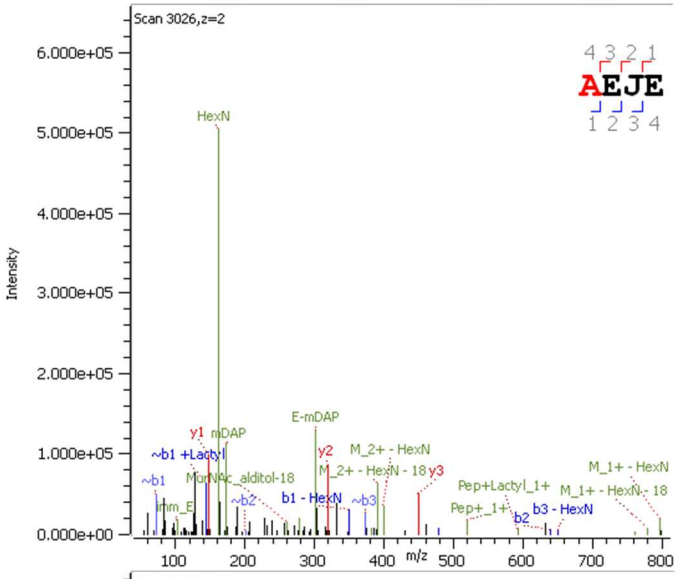
Intensity
m/z
HexN
y1
~b1 +Lactyl
E-mDAP
M_2+ - HexN - 18
M_2+ - HexN - 36
imm_V
mDAP
y2
y3
M_2+ - HexN
~b1
b1 - HexN
~b3
b3 - HexN
MurNAc_alditol-18
y4
~b4
Pep+_1+
b4 - HexN


Scan 7026,z=2
5 4 3 2 1
AEJAY
1 2 3 4 5
HexN
y1
~b1 +Lactyl
E-mDAP
M_2+ - HexN - 18
mDAP
~b3
y2
Pep+Lactyl_2+
y3
M_2+ - HexN
y4
~b1
imm_Y
~b4 +Lactyl
b4 - HexN
imm_E
MurNAc_alditol
~b4
~b3 +Lactyl
b3 - HexN
M_1+ - HexN
Intensity
m/z


Scan 2729,z=2
4 3 2 1
AEJD
1 2 3 4
HexN
mDAP
E-mDAP
y1
y2
y3
~b1 +Lactyl
M_2+ - HexN - 18
Pep+_1+
~b1
MurNAc_alditol-18
b1 - HexN
b2 - HexN
b3 - HexN
M_1+ - HexN
imm_E
MurNAc_alditol
M_2+ - HexN
Pep+Lactyl_1+
M_1+ - HexN - 18
Intensity
m/z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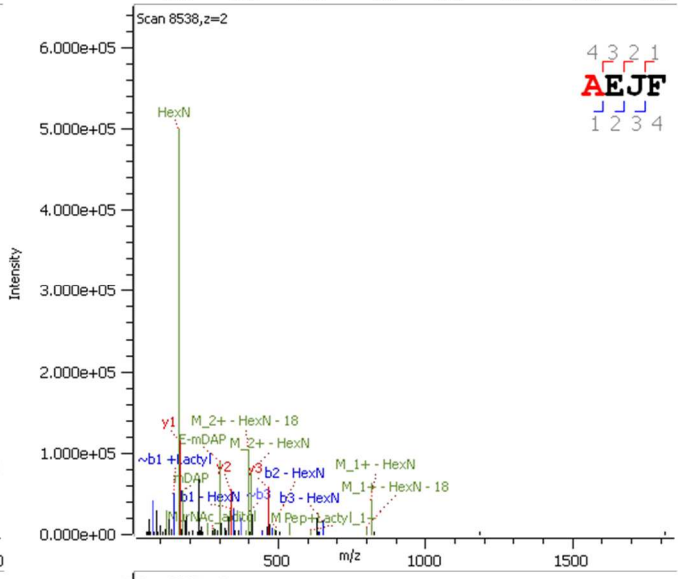
AEJE
1 2 3 4
HexN
E-mDAP
y1
mDAP
y2
M_2+ - HexN
~b1 +Lactyl
M_2+ - HexN - 18
y3
M_1+ - HexN
~b1
MurNAc_alditol-18
Pep+Lactyl_1+
M_1+ - HexN - 18
imm_E
~b2
b1 - HexN
~b3
Pep+_1+
b3 - HexN
b2
Intensity
m/z


Scan 8538,z=2
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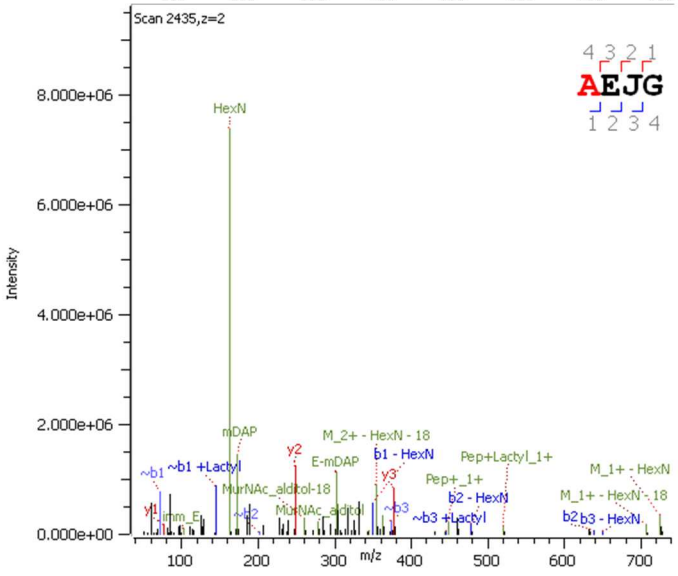
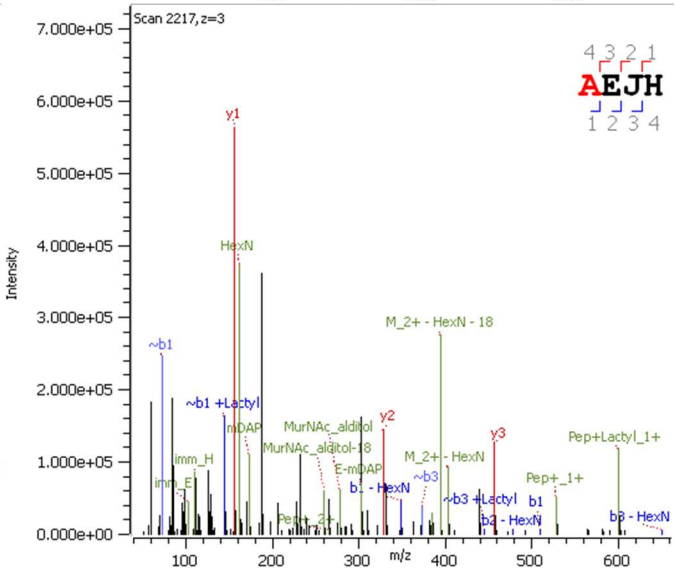
AEJF
1 2 3 4
HexN
y1
M_2+ - HexN - 18
E-mDAP
M_2+ - HexN
~b1 +Lactyl
M_1+ - HexN
mDAP
y2
y3
b2 - HexN
M_1+ - HexN - 18
b1 - HexN
~b3
b3 - HexN
MurNAc_alditol
M
Pep+Lactyl_1+
Intensity
m/z


Scan 2435,z=2
4 3 2 1
AEJG
1 2 3 4
HexN
mDAP
M_2+ - HexN - 18
y2
b1 - HexN
E-mDAP
Pep+Lactyl_1+
~b1
~b1 +Lactyl
y3
Pep+_1+
M_1+ - HexN
MurNAc_alditol-18
b2 - HexN
M_1+ - HexN - 18
y1
imm_E
~b2
MurNAc_alditol
~b3
~b3 +Lactyl
b2
b3 - HexN
Intensity
m/z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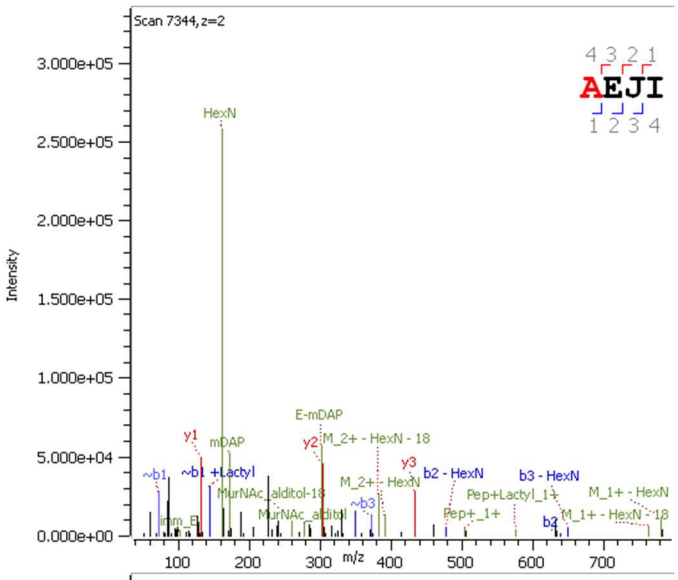
4 3 2 1
AEJH
1 2 3 4
y1
HexN
~b1
M_2+ - HexN - 18
~b1 +Lactyl
mDAP
MurNAc_alditol
y2
MurNAc_alditol-18
y3
Pep+Lactyl_1+
imm_H
E-mDAP
M_2+ - HexN
imm_E
b1 - HexN
~b3
Pep+_1+
~b3 +Lactyl
b1
b2 - HexN
b3 - HexN
Intensity
m/z


Scan 7344, z=2
AEJI
Intensity
m/z
HexN
mDAP
E-mDAP
y1
y2
y3
~b1
~b1 +Lactyl
MurNAc_alditol-18
MurNAc_alditol
~b3
M_2+ - HexN - 18
M_2+ - HexN
b2 - HexN
b3 - HexN
Pep+Lactyl_1+
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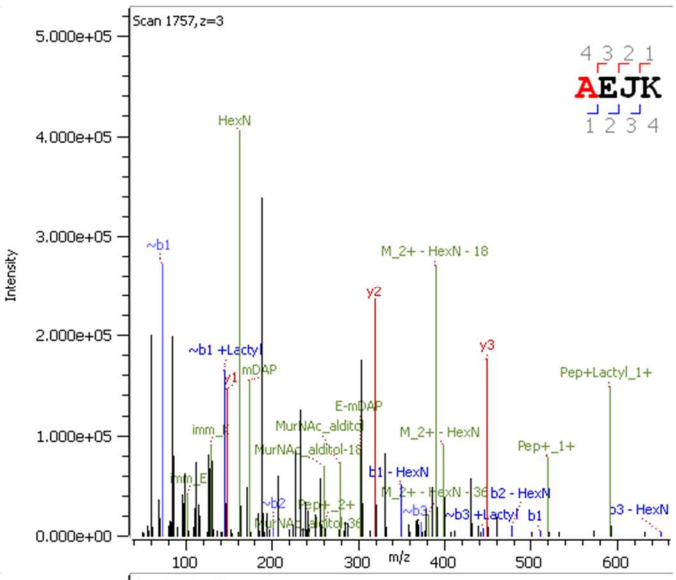
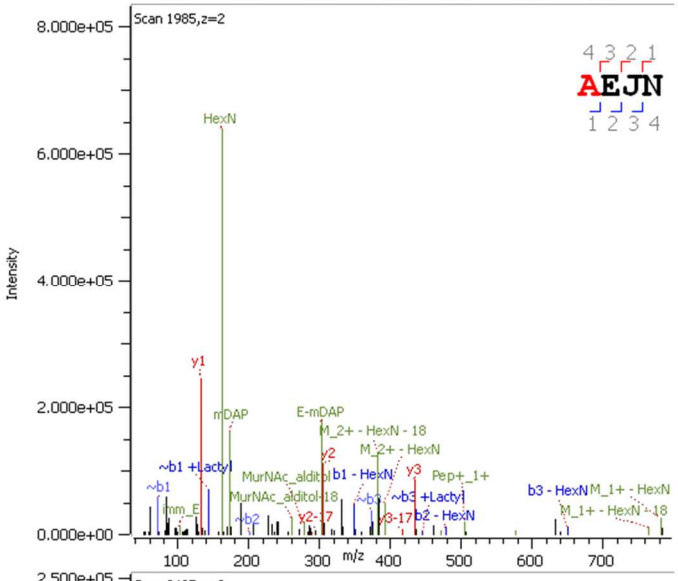
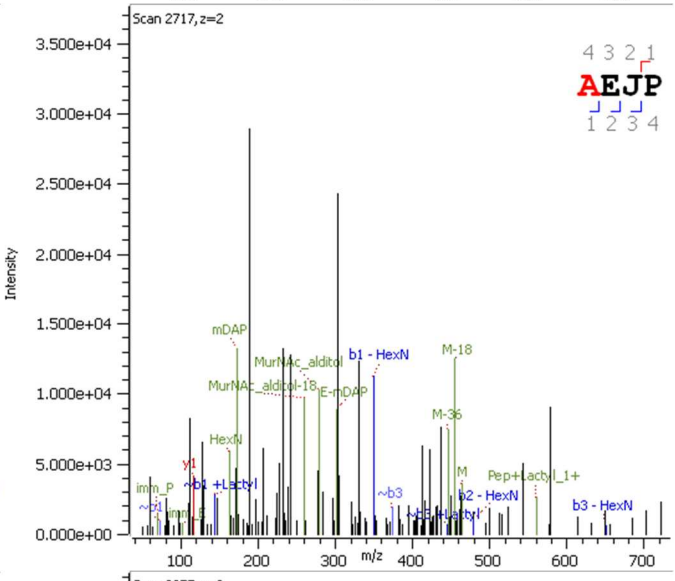
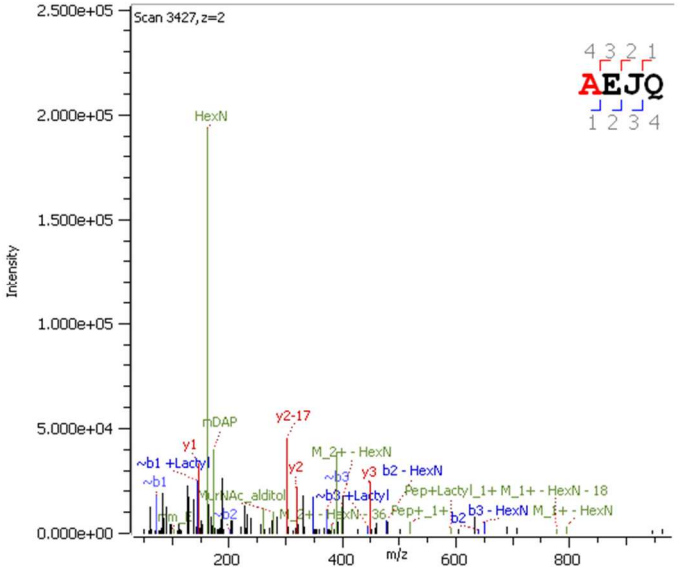
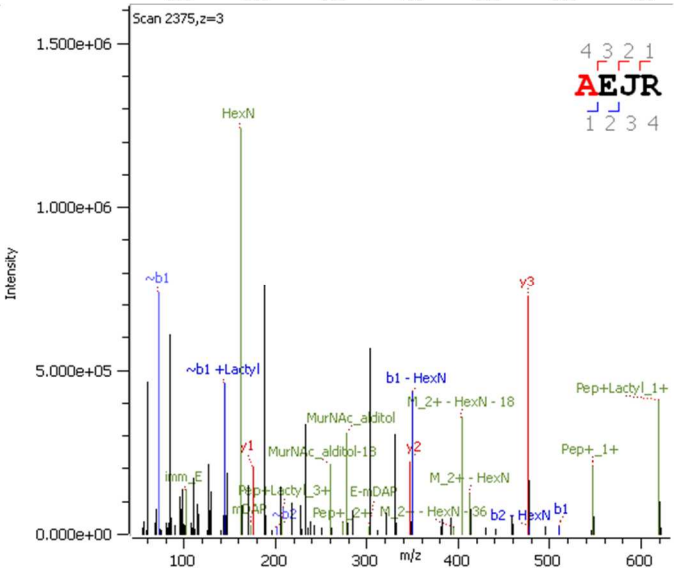
M_1+ - HexN
M_1+ - HexN - 18
imm_E
b2


Scan 1757, z=3
AEJK
Intensity
m/z
HexN
~b1
M_2+ - HexN - 18
y2
y3
~b1 +Lactyl
mDAP
y1
Pep+Lactyl_1+
E-mDAP
MurNAc_alditol
MurNAc_alditol-18
M_2+ - HexN
Pep+_1+
imm_E
b1 - HexN
M_2+ - HexN - 36
b2 - HexN
~b3
~b3 +Lactyl
b1
b3 - HexN


Scan 1985, z=2
AEJN
Intensity
m/z
HexN
y1
mDAP
E-mDAP
M_2+ - HexN - 18
M_2+ - HexN
y2
~b1 +Lactyl
~b1
MurNAc_alditol
b1 - HexN
y3
Pep+_1+
MurNAc_alditol-18
~b3
~b3 +Lactyl
b3 - HexN
M_1+ - HexN
imm_E
~b2
y2-17
y3-17
b2 - HexN
M_1+ - HexN - 18


Scan 2717, z=2
AEJP
Intensity
m/z
mDAP
MurNAc_alditol
b1 - HexN
M-18
MurNAc_alditol-18
E-mDAP
M-36
HexN
y1
imm_P
~b1
~b1 +Lactyl
imm_E
~b3
~b3 +Lactyl
M
Pep+Lactyl_1+
b2 - HexN
b3 - HexN


Scan 3427, z=2
AEJQ
Intensity
m/z
HexN
mDAP
y2-17
y1
~b1 +Lactyl
M_2+ - HexN
y2
~b3
y3
b2 - HexN
~b1
MurNAc_alditol
~b3 +Lactyl
Pep+Lactyl_1+
M_1+ - HexN - 18
imm_E
~b2
M_2+ - HexN - 36
Pep+_1+
b3 - HexN
M_1+ - HexN
b2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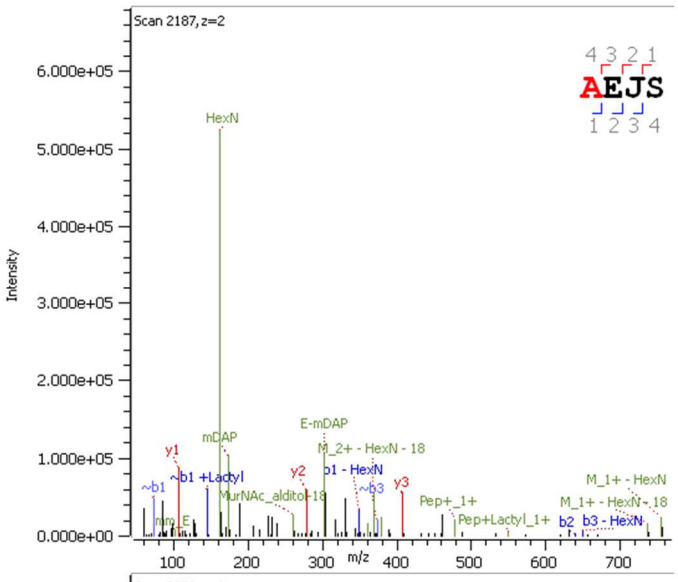
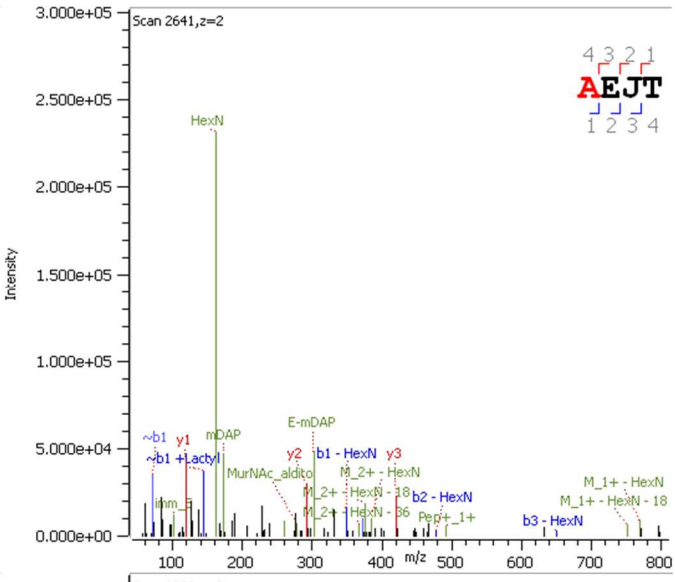
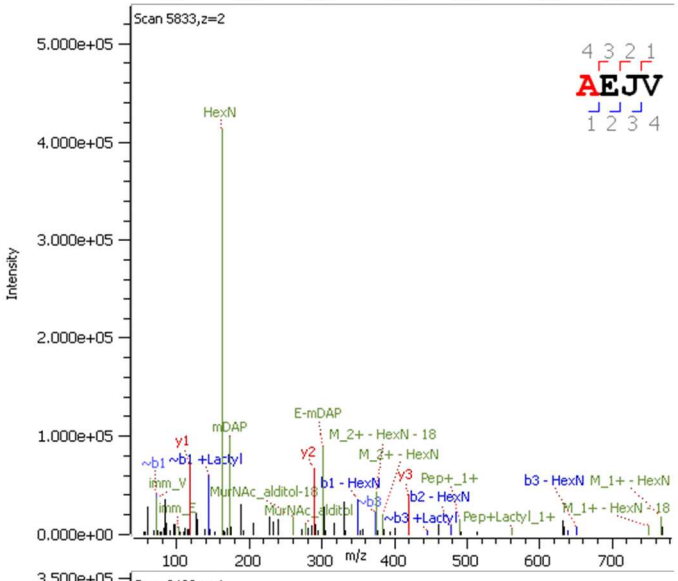
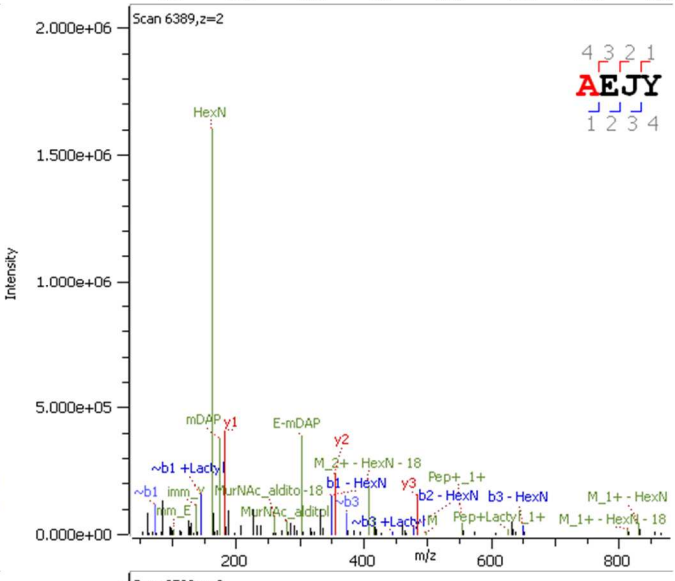
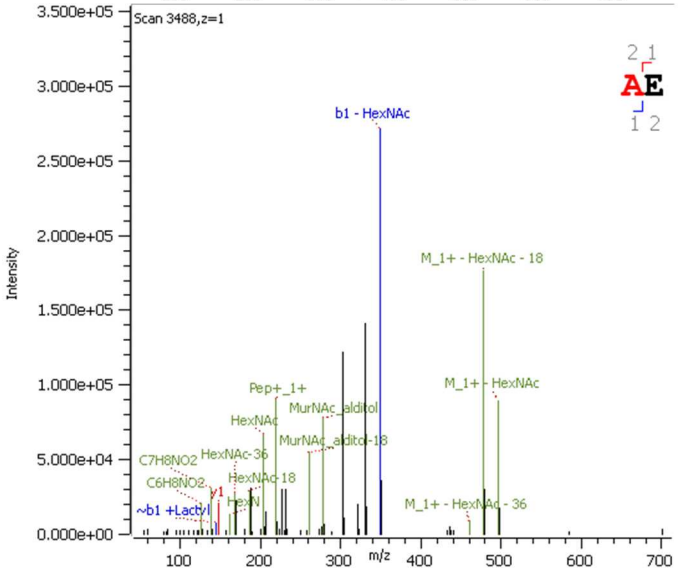
AEJR
Intensity
m/z
HexN
~b1
y3
~b1 +Lactyl
b1 - HexN
M_2+ - HexN - 18
Pep+Lactyl_1+
MurNAc_alditol
MurNAc_alditol-18
y1
y2
Pep+_1+
imm_E
M_2+ - HexN
Pep+Lactyl_3+
E-mDAP
mDAP
~b2
Pep+_2+
M_2+ - HexN - 36
b2 - HexN
b1

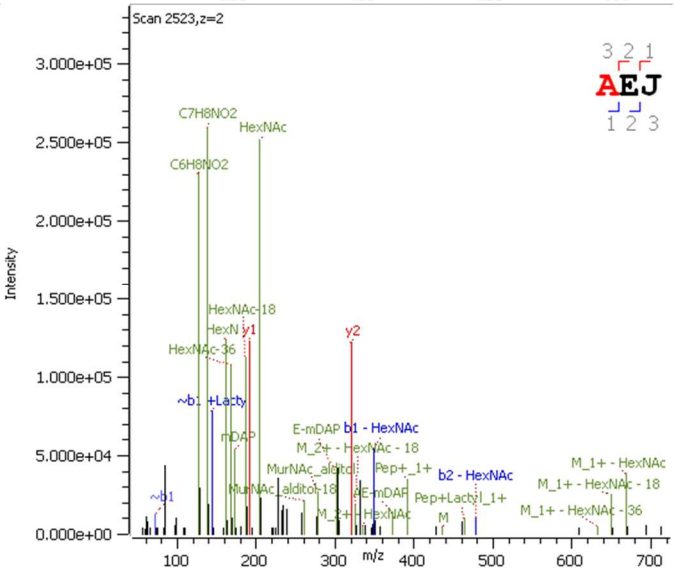

Scan 2187,z=2
4 3 2 1
AEJS
1 2 3 4
HexN
mDAP
E-mDAP
M_2+ - HexN - 18
y1
~b1 +Lactyl
b1 - HexN
y2
~b3
y3
~b1
MurNAc_alditol-18
Pep+_1+
M_1+ - HexN
M_1+ - HexN - 18
Pep+Lactyl_1+
b2
b3 - HexN
imm_E
Intensity
m/z

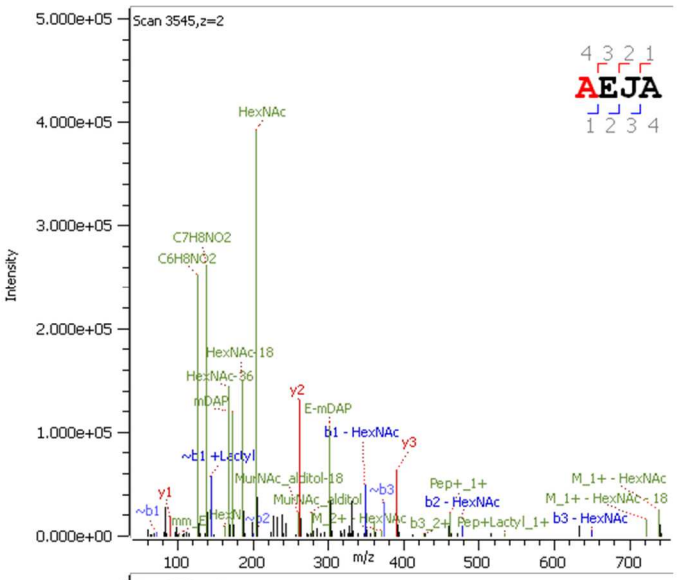

Scan 2641,z=2
4 3 2 1
AEJT
1 2 3 4
HexN
~b1
y1
mDAP
E-mDAP
~b1 +Lactyl
y2
b1 - HexN
y3
MurNAc_alditol
M_2+ - HexN
M_2+ - HexN - 18
M_2+ - HexN - 36
b2 - HexN
Pep+_1+
b3 - HexN
M_1+ - HexN
M_1+ - HexN - 18
imm_E
Intensity
m/z

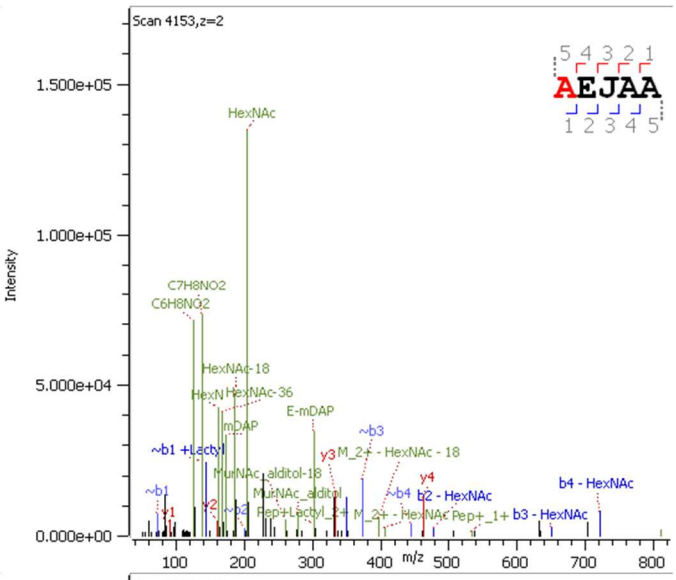

Scan 5833,z=2
4 3 2 1
AEJV
1 2 3 4
HexN
mDAP
E-mDAP
M_2+ - HexN - 18
M_2+ - HexN
y1
~b1
~b1 +Lactyl
y2
b1 - HexN
y3
Pep+_1+
b3 - HexN
M_1+ - HexN
imm_V
MurNAc_alditol-18
b2 - HexN
imm_E
MurNAc_alditol
~b3
~b3 +Lactyl
Pep+Lactyl_1+
M_1+ - HexN - 18
Intensity
m/z

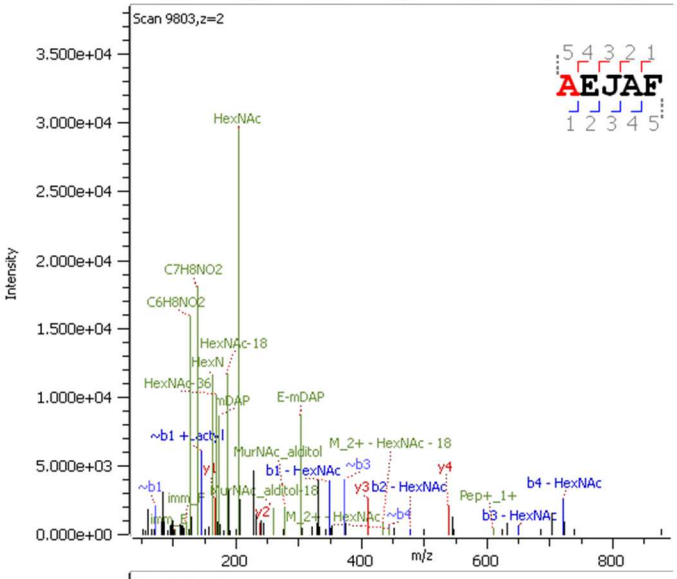

Scan 6389,z=2
4 3 2 1
AEJY
1 2 3 4
HexN
mDAP
y1
E-mDAP
y2
~b1 +Lacty
M_2+ - HexN - 18
~b1
imm_V
MurNAc_alditol-18
b1 - HexN
y3
Pep+_1+
imm_E
MurNAc_alditol
~b3
b2 - HexN
b3 - HexN
M_1+ - HexN
~b3 +Lactyl
M
Pep+Lactyl_1+
M_1+ - HexN - 18
Intensity
m/z

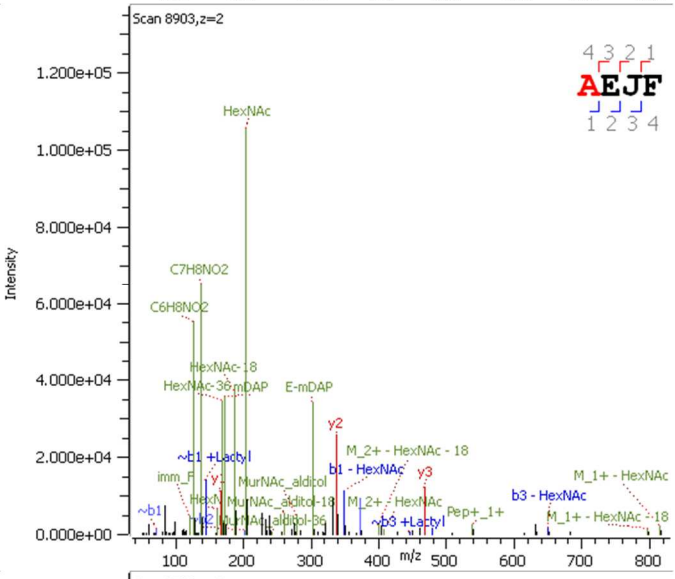

Scan 3488,z=1
2 1
AE
1 2
b1 - HexNAc
M_1+ - HexNAc - 18
M_1+ - HexNAc
Pep+_1+
MurNAc_alditol
HexNAc
MurNAc_alditol-18
C7H8NO2
HexNAc-36
C6H8NO2
HexNAc-18
HexN
y1
~b1 +Lactyl
M_1+ - HexNAc - 36
Intensity
m/z

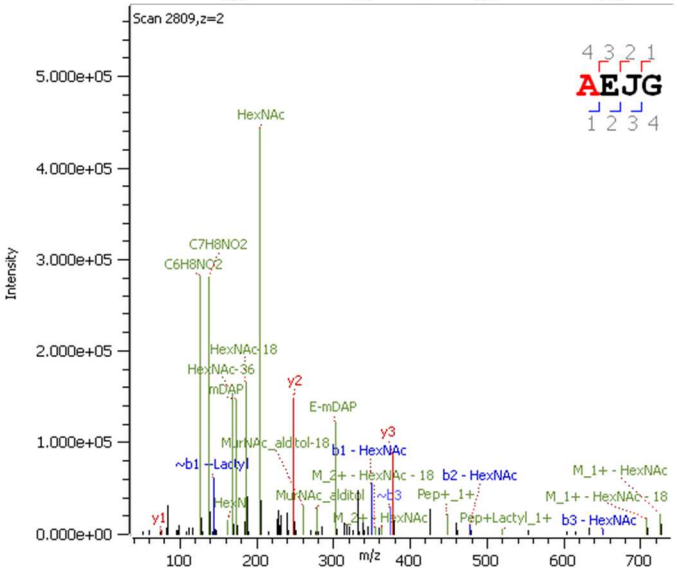

Scan 2523,z=2
3 2 1
AEJ
1 2 3
C7H8NO2
HexNAc
C6H8NO2
HexNAc-18
HexN
y1
y2
HexNAc-36
~b1 +Lacty
mDAP
E-mDAP
b1 - HexNAc
M_2+ - HexNAc - 18
MurNAc_alditol
Pep+_1+
b2 - HexNAc
MurNAc_alditol-18
~b1
AE-mDAP
Pep+Lactyl_1+
M_1+ - HexNAc
M_1+ - HexNAc
M_1+ - HexNAc - 18
M_1+ - HexNAc - 36
Intensity
m/z

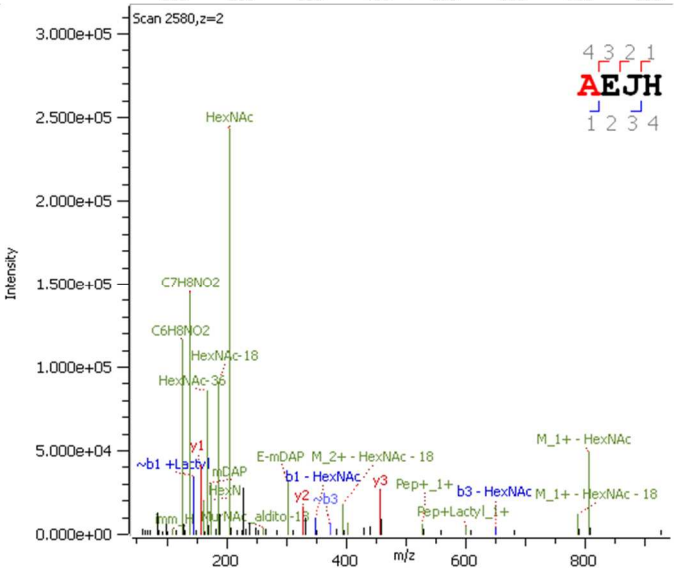

Scan 3545,z=2
4 3 2 1
AEJA
1 2 3 4
Intensity
m/z
HexNAc
C7H8NO2
C6H8NO2
HexNAc-18
HexNAc-36
mDAP
E-mDAP
y2
b1 - HexNAc
y3
~b1 +Lactyl
MurNAc_alditol-18
MurNAc_alditol
~b3
Pep+_1+
b2 - HexNAc
M_1+ - HexNAc
M_1+ - HexNAc - 18
y1
~b1
Imm_F
HexN
~b2
M_2+ - HexNAc
b3_2+
Pep+Lactyl_1+
b3 - HexNAc


Scan 4153,z=2
5 4 3 2 1
AEJAA
1 2 3 4 5
Intensity
m/z
HexNAc
C7H8NO2
C6H8NO2
HexNAc-18
HexN
HexNAc-36
E-mDAP
mDAP
~b3
~b1 +Lactyl
y3
M_2+ - HexNAc - 18
MurNAc_alditol-18
~b1
MurNAc_alditol
~b4
b2 - HexNAc
y4
b4 - HexNAc
y1
y2
~b2
Pep+Lactyl_1+
M_2+ - HexNAc
Pep+_1+
b3 - HexNAc


Scan 9803,z=2
5 4 3 2 1
AEJAF
1 2 3 4 5
Intensity
m/z
HexNAc
C7H8NO2
C6H8NO2
HexNAc-18
HexN
HexNAc-36
mDAP
E-mDAP
~b1 +Lactyl
MurNAc_alditol
M_2+ - HexNAc - 18
b1 - HexNAc
~b3
y4
~b1
y1
MurNAc_alditol-18
y3
b2 - HexNAc
b4 - HexNAc
Pep+_1+
Imm_F
y2
M_2+ - HexNAc
~b4
b3 - HexNAc


Scan 8903,z=2
4 3 2 1
AEJF
1 2 3 4
Intensity
m/z
HexNAc
C7H8NO2
C6H8NO2
HexNAc-18
HexNAc-36
mDAP
E-mDAP
y2
M_2+ - HexNAc - 18
~b1 +Lactyl
b1 - HexNAc
y3
M_1+ - HexNAc
Imm_F
y1
MurNAc_alditol
b3 - HexNAc
~b1
MurNAc_alditol-18
M_2+ - HexNAc
Pep+_1+
M_1+ - HexNAc - 18
~b3 +Lactyl


Scan 2809,z=2
4 3 2 1
AEJG
1 2 3 4
Intensity
m/z
HexNAc
C7H8NO2
C6H8NO2
HexNAc-18
HexNAc-36
mDAP
y2
E-mDAP
y3
MurNAc_alditol-18
b1 - HexNAc
~b1 +Lactyl
M_2+ - HexNAc - 18
b2 - HexNAc
M_1+ - HexNAc
HexN
MurNAc_alditol
~b3
Pep+_1+
M_1+ - HexNAc - 18
y1
M_2+ - HexNAc
Pep+Lactyl_1+
b3 - HexNAc


Scan 2580,z=2
4 3 2 1
AEJH
1 2 3 4
Intensity
m/z
HexNAc
C7H8NO2
C6H8NO2
HexNAc-18
HexNAc-36
y1
~b1 +Lactyl
mDAP
E-mDAP
M_2+ - HexNAc - 18
b1 - HexNAc
y3
M_1+ - HexNAc
HexN
y2
~b3
Pep+_1+
b3 - HexNAc
M_1+ - HexNAc - 18
Imm_F
MurNAc_alditol-18
Pep+Lactyl_1+

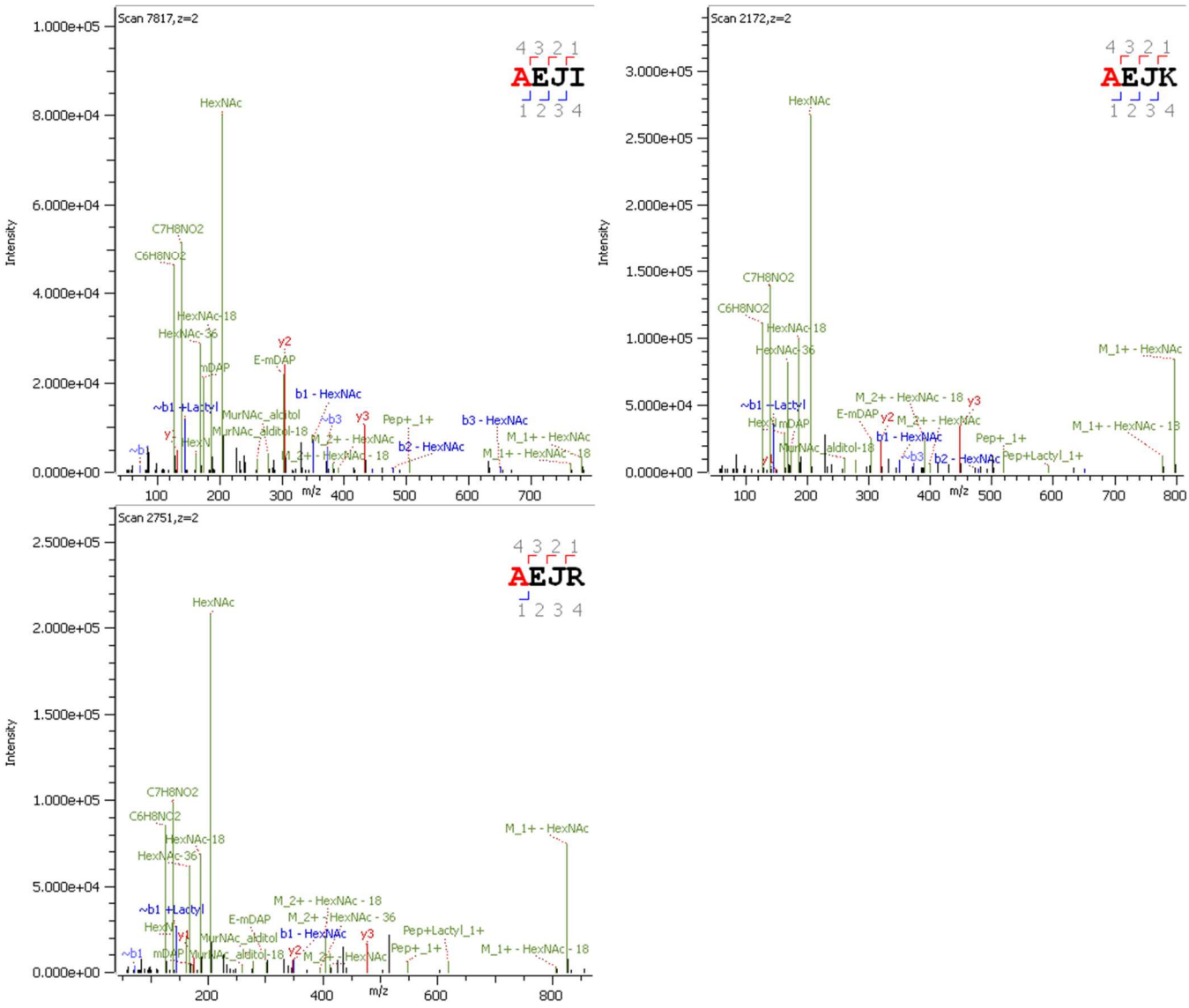


Figure S5. Tandem mass spectrometry analysis of *C. difficile* monomers using the Byonic™ module.

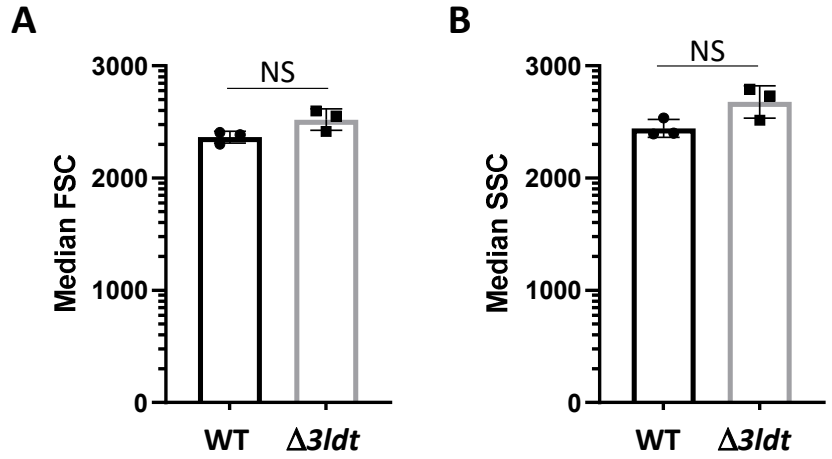


Figure S6. Comparative analysis of WT and Δ*3ldt* cell size by flow cytometry. Comparison of median forward scattered (FSC) (**A**) and side scattered (SSC) light values (**B**) corresponding to WT and Δ3*ldt* mutant; NS, $P>0.05$; n = 3 (Student unpaired *t*-test with Welch's correction).

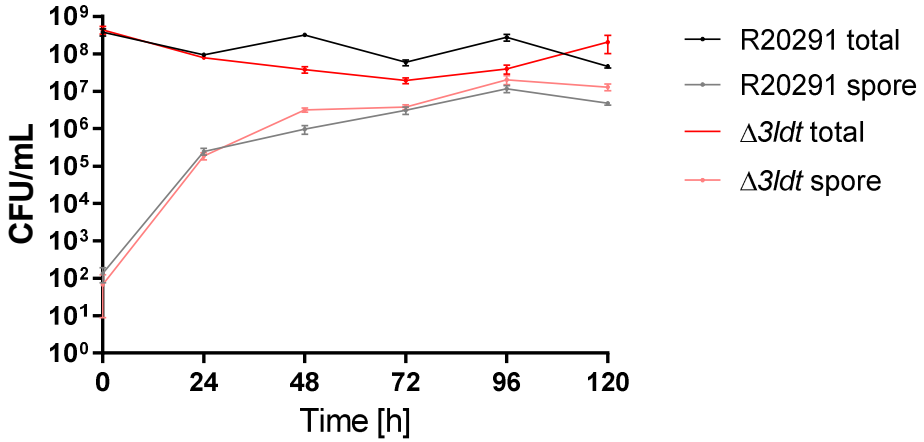


Figure S7. Comparative analysis of WT and Δ3*ldt* sporulation efficiency. Stationary-phase cultures were incubated anaerobically for 5 days, and samples were taken at 24-h intervals for analysis. Total cell numbers were determined by counting the number of CFU on BHIS agar containing the germinant taurocholate. Spore numbers were determined by the same method following incubation at 70°C for 30 min. Experiments were carried out in triplicate on biological duplicate samples. The means ± standard deviations (error bars) are shown.

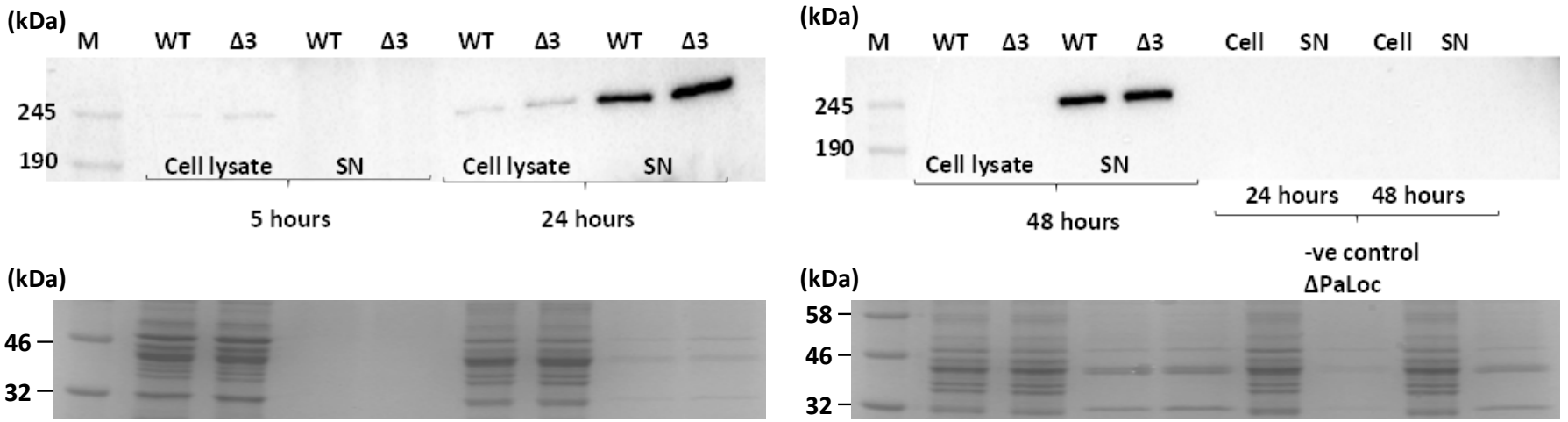


Figure S8. **Analysis of toxin release by *C. difficile* R20291 and the Δ3*ldt* mutant.** Cells were grown overnight and inoculated in TY broth at a 1/100 dilution. Cultures were harvested at different time points to prepare cell lysates and concentrated culture supernatants (SN). Toxin B was detected by Western blot (chemiluminescence) using a mouse monoclonal Anti toxin B antibody (MA1-7413) and a secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase. A strain with a deletion in the gene encoding toxin B (ΔPaLoc) was used as a negative control. M, Molecular weight marker.