Supplemental Materials

Methods

Evaluation of D-amino acid incorporation into peptidoglycan

Three mL LB was inoculated with a single colony of *dtd*$^{+}$ *B. subtilis* and incubated in a roller drum at 37˚C. After three hours, these cultures were diluted 1:1000 into MSgg and 48.5 mL of culture were transferred into a glass dish containing a total of 1.5 mL treatment (D-tryptophan) or water (untreated). These biofilm cultures were incubated statically at 25˚C for 48 hours, at which time cells were harvested by centrifugation at 4000 rpm for 25 minutes.

Pelleted cells were resuspended in 2 mL cold distilled H_2O, added drop-wise to boiling 50 mL solutions of 5% SDS, and boiled for 15 minutes. After cooling to room temperature, the samples were pelleted at 4000 rpm for 25 minutes at room temperature. SDS was removed from the pellets by several rounds of washing in warm distilled H_2O and re-pelleting. Pellets were resuspended in a solution of 100 mM Tris-HCl, 20 mM $MgSO_4$, pH 7.5. 20 µg/mL DNase I, 40 µg/mL RNAse, and 80 µg/mL α-amylase (final concentrations from 10 mg/ml stocks in 50% glycerol) were added and samples were incubated shaking at 37˚C for 2 hours. 80 µg/mL trypsin (10 mg/ml stock) was added as well as $CaCl_2$ to a final concentration of 10 mM, and the samples were incubated shaking at 37˚C overnight. The following day, SDS was added to achieve a final SDS concentration of 1%, and samples were boiled for 2 hours. After washing out the SDS with warm water as described above, the sample was pelleted and resuspended in 0.02% NaN_3.

To digest the cell wall for LC-MS analysis, 50 µL of the cell wall sample was moved to a fresh eppendorf tube, mixed with 10 µL 500 mM sodium phosphate buffer (pH 6.0), 2.5 µL 4000 U/mL mutanolysin, and 37.5 µL dH_2O, and incubated shaking overnight at 37˚C. Another aliquot of mutanolysin was added the next morning, and the samples were incubated shaking at 37˚C for at least three hours. The samples were centrifuged at 16000 rpm for 10 min at 4˚C, and the supernatant was transferred to fresh 1.5 mL eppendorf tubes. One volume of freshly dissolved 10 mg/mL $NaBH_4$ (in dH_2O) was added for every two volumes of supernatant. The solution was mixed gently every 10 minutes for 30 minutes, and the reaction was quenched with 20% H_3PO_4 (1.2 µL of acid was added for every 10 µL of base). After centrifuging until all bubbles disappeared, the samples were frozen in liquid nitrogen, lyophilized, and resuspended in 25 µL dH_2O.

LC-MS chromatograms were obtained on an Agilent Technologies 1100 series LC-MSD instrument using electrospray ionization (ESI) in positive mode and a Waters Symmetry Shield RP18 column (5 µm, 3.9X150 mm) with matching column guard. The fragments were separated by water for 5 minutes followed by a gradient of 0% acetonitrile in water to 20% acetonitrile in water for 40 minutes, always at a constant flow rate of 0.5 mL/min. Both the water and acetonitrile contained 0.1% formic acid to aid in ionization. (M+2H)/2 ions were extracted from the chromatograms for selected disaccharide fragments.

To confirm the structure of the D-Trp incorporation peaks, high-resolution LC-MS/MS was performed on the peptidoglycan isolated from *dtd*$^+$ cells treated with 1 mM D-Trp.

Chromatograms were obtained in positive mode (ES) on a Bruker maXis impact Q-Tof with Agilent 1290 HPLC using the same column and method as the nominal mass LC-MS above. Differences in retention times between the nominal and high-resolution chromatograms can be attributed to dead volume differences between instruments.

Table S1. Bacterial strains and primers used in this study.

Strain name	**Relevant genotype or property**	**Reference**
Turbo *E. coli*	*recA*$^{+}$, *endA1,* Δ*(hsdS-mcrB)5*	NEB
NCIB3610	Wild biofilm-forming strain	Laboratory stock
SLH31	*dtd*^{T2A} in NCIB3610	This study
ALM89	*sacA::P*$_{epsA}$*-lux* in NCIB3610, Cm^{R}	Chen et al, 2011
SLH32	*sacA::P*$_{epsA}$*-lux* in SLH31, Cm^{R}	This study
ALM91	*sacA::P*$_{tapA}$*-lux* in NCIB3610, Cm^{R}	Chen et al, 2011
SLH33	*sacA::P*$_{tapA}$*-lux* in SLH31, Cm^{R}	This study

Primer Number	Primer Sequence
7	CCAGCTTTTGTTCCCTTTAGTGAGGATCGAAAGCGGATTAGAGAAATAC
8	AATGAAGAGGATATGTACGCGGC
51	CGTTGTAAAACGACGGCCAGTGAATTCGTCTGGAAACGGCTCTCTAAACAA
52	GAAATGAGATTAGTTGTTCAGCGAG
53	CTAATCTCATTTCTAACCCCTTTAGTTC
54	AACAGCTATGACCATGATTACGCCAAGCTTTCTGTTCCGATTGACTTTTCTTACCG

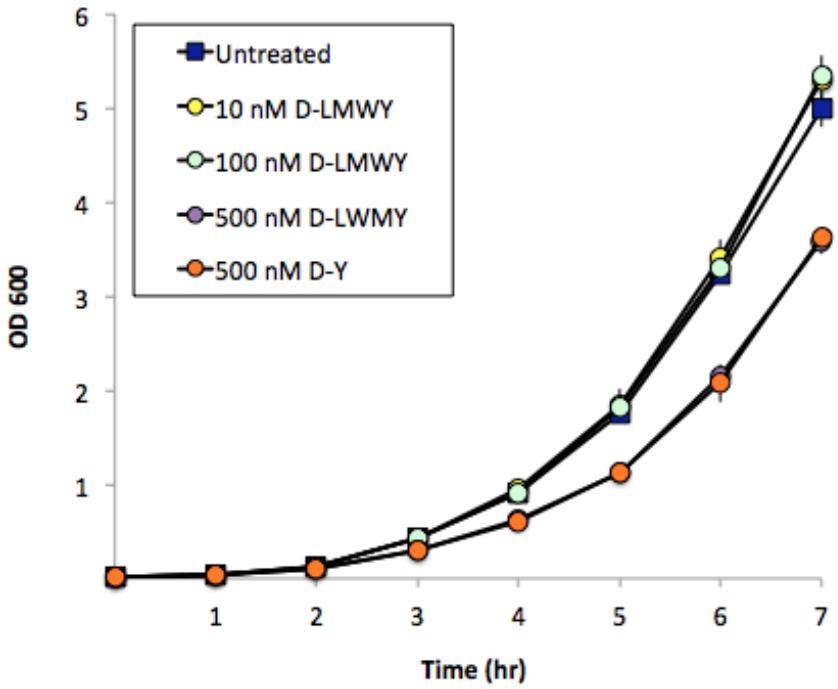


Figure S1. Biofilm-inhibitory concentrations of D-amino acids also inhibit growth. The optical density of untreated cells or cells treated with D-Y or with equimolar mixtures of D-LMWY was measured every hour. Concentrations were chosen to reflect conditions that do not inhibit pellicle formation (10 nM, 100 nM) and those that mildly weaken pellicle formation (500 nM). Cultures were grown in shaking unmodified MSgg at 37˚C. Results represent the average of duplicate experiments, and error bars show the standard deviation.

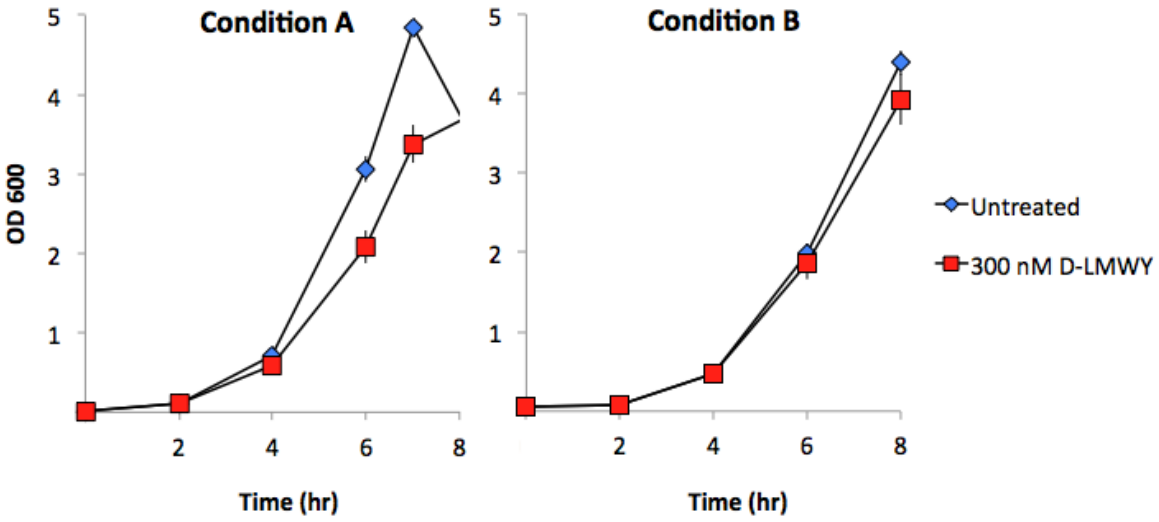


Figure S2. D-LMWY at 300 nM does not inhibit growth under specific growth conditions. *B. subtilis* 3610 was grown as described in Materials and Methods without modifications (Condition A) or with the following modifications (Condition B). For Condition B, cells were inoculated to OD_{600} 0.05 in a final volume of 3 mL and were incubated in a roller drum at 37˚C. Experiments were performed in quadruplet.

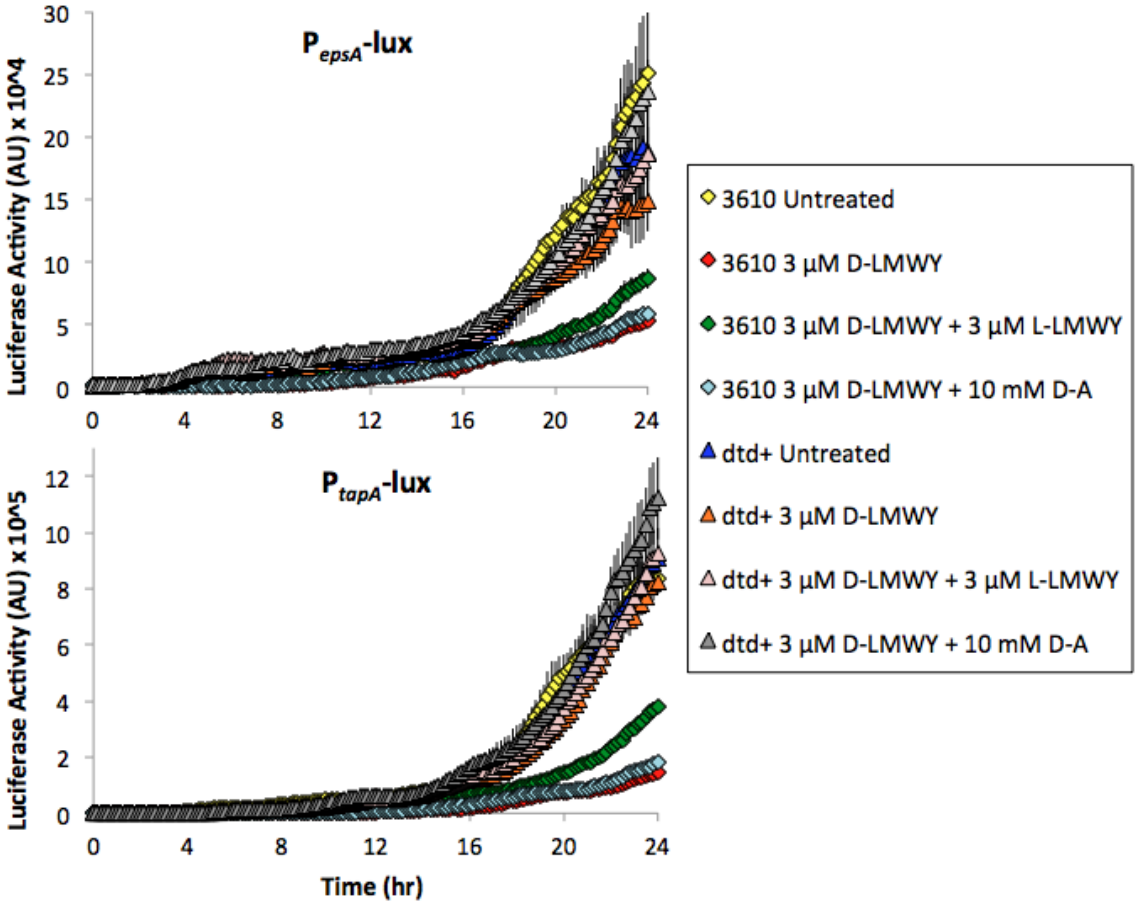
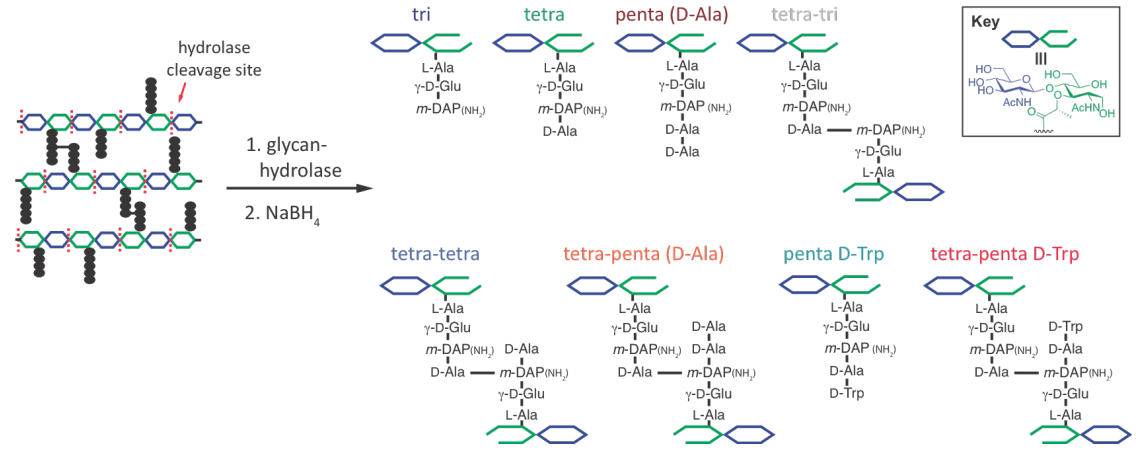


Figure S3. Expression of biofilm matrix genes is sensitive to D-LMWY in 3610 cells, but not in cells repaired for D-aminoacyl-tRNA deacylase activity. 3610 or *dtd*$^{+}$ cells harboring a luciferase reporter for *epsA* or *tapA* expression were grown in MSgg. Luciferase luminescence was normalized to optical density. Results represent the average of triplicate experiments, and the error bars show the standard deviation.

A

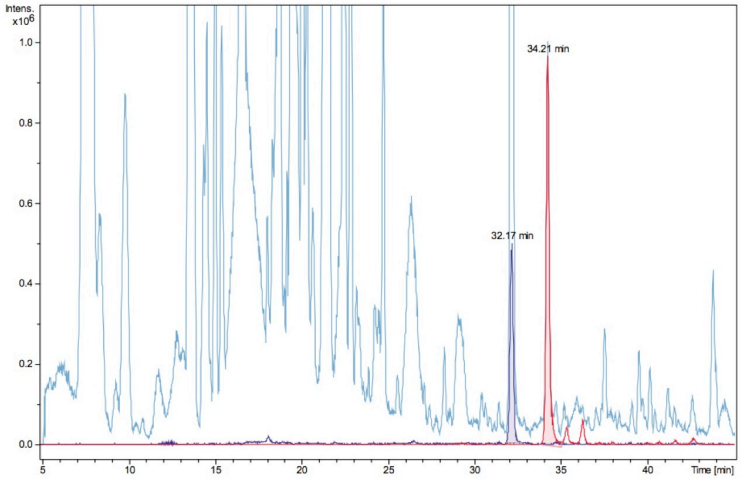


B

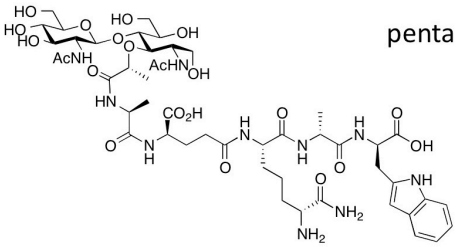
Fragment	Extracted (M+2H)/2 Mass
tri	435.7
tetra	471.2
penta (D-Ala)	506.7
tetra-tri	896.9
tetra-tetra	932.4
tetra-penta (D-Ala)	967.9
penta D-Trp	564.3
tetra-penta D-Trp	1025.5

Figure S4. Peptidoglycan fragment analysis by LC-MS. A) *B. subtilis* cell wall was harvested, digested with a glycan hydrolase, and reduced with sodium borohydride to yield disaccharide fragments for separation and detection by LCMS. Note: the structures of these fragments are proposed based on the work of Atrih et al (1). B) Masses of the (M+2H)/2 ions extracted in Figure 7.

A



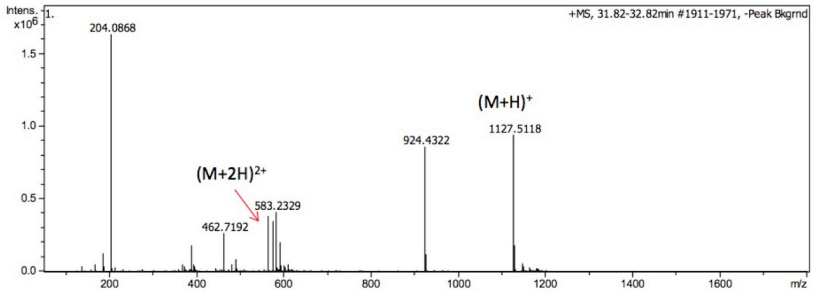
B



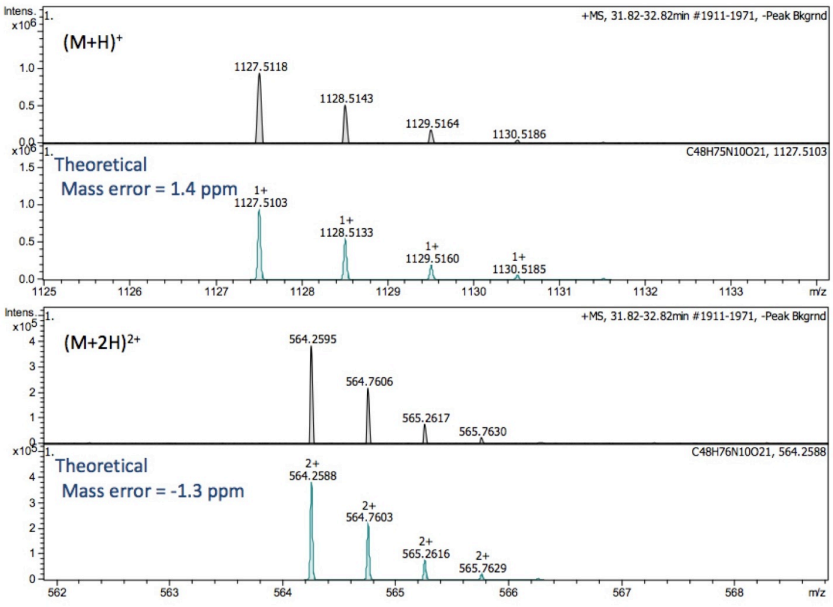
penta D-Trp fragment
(32 min.):

$C_{48}H_{74}N_{10}O_{21}$
$(M+H)^+$: 1127.5103
$(M+2H)^{2+}$: 564.2588

C



D



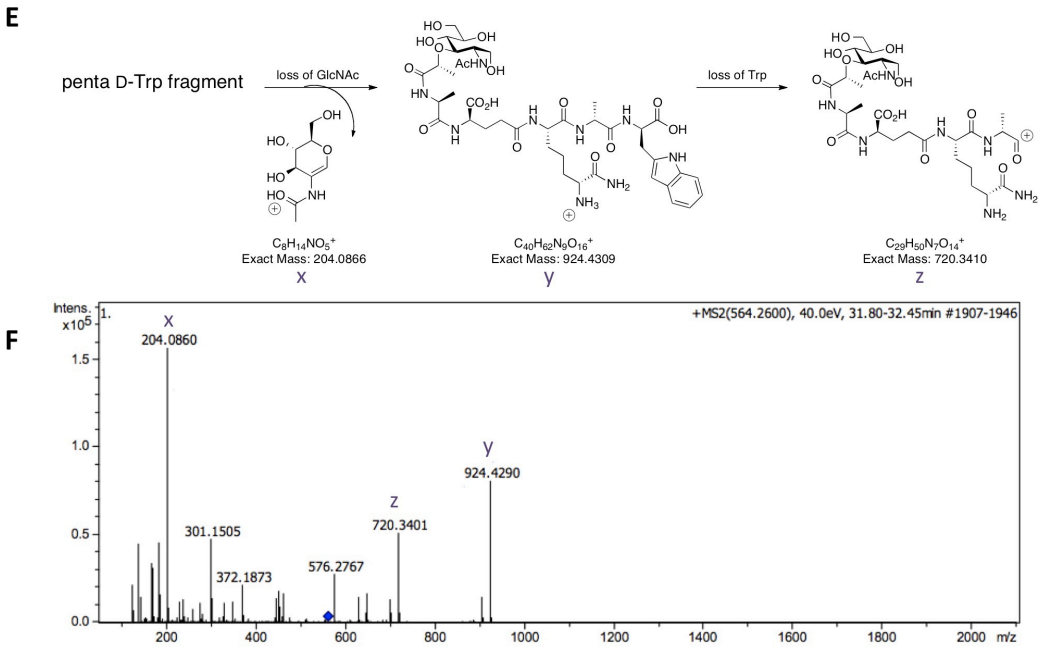


Figure S5. High-resolution LC-MS/MS fragmentation of the *dtd*$^+$ 1 mM D-Trp sample supports the proposed penta D-Trp structure. A) Representative total ion chromatogram of the *dtd*$^+$ 1 mM D-Trp sample overlaid with extracted ion chromatograms for the proposed penta D-Trp (32 minutes) and tetra-penta D-Trp (34 minutes) fragments. B) Proposed structure, molecular formula, and m/z values for the penta D-Trp fragment eluting at 32 minutes. C) Full scan mass spectrum for the peak at 32 minutes. D) Expanded views of the mass spectrum for the peak at 32 minutes confirm the proposed elemental composition of the penta D-Trp fragment within 5 ppm. E and F) The product ion scan mass spectrum of the peak at 32 minutes supports the proposed structure of the penta D-Trp fragment. As depicted in E, loss of the glucosamine derivative (x) generates fragment y, which loses tryptophan to yield fragment z.

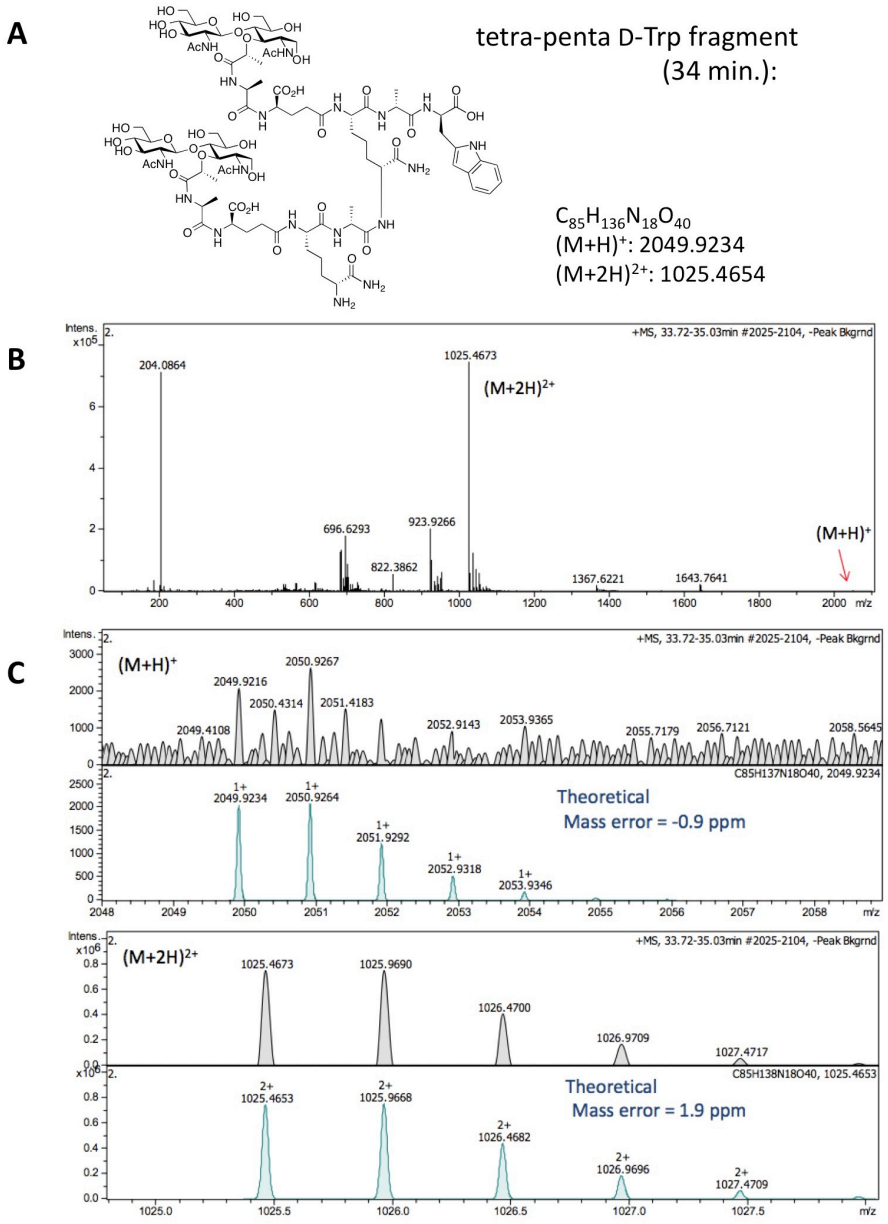


Figure S6. High-resolution LC-MS supports the proposed tetra-penta D-Trp elemental composition. A) Proposed structure, molecular formula, and m/z values for the tetra-penta D-Trp fragment eluting at 34 minutes in panel A of Figure S5. B) Full scan mass spectrum for the peak at 34 minutes. C) Expanded views of the mass spectrum for the peak at 34 minutes confirm the proposed elemental composition of the tetra-penta D-Trp fragment within 5 ppm.

References

1. **Atrih A, Bacher G, Allmaier G, Williamson MP, Foster SJ.** 1999. Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. Journal of bacteriology **181**:3956-3966.