Supplementary Information for:

Identification of a new family of peptidoglycan transpeptidases reveals atypical crosslinking is essential for viability in *Clostridioides difficile*

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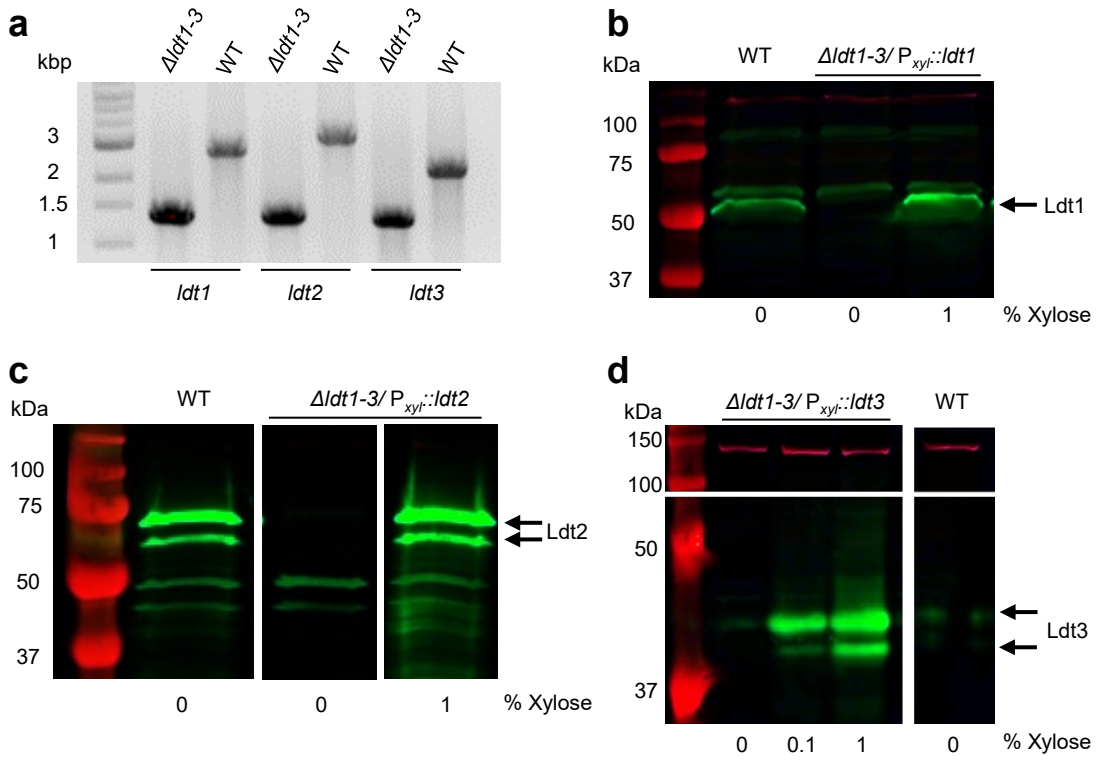
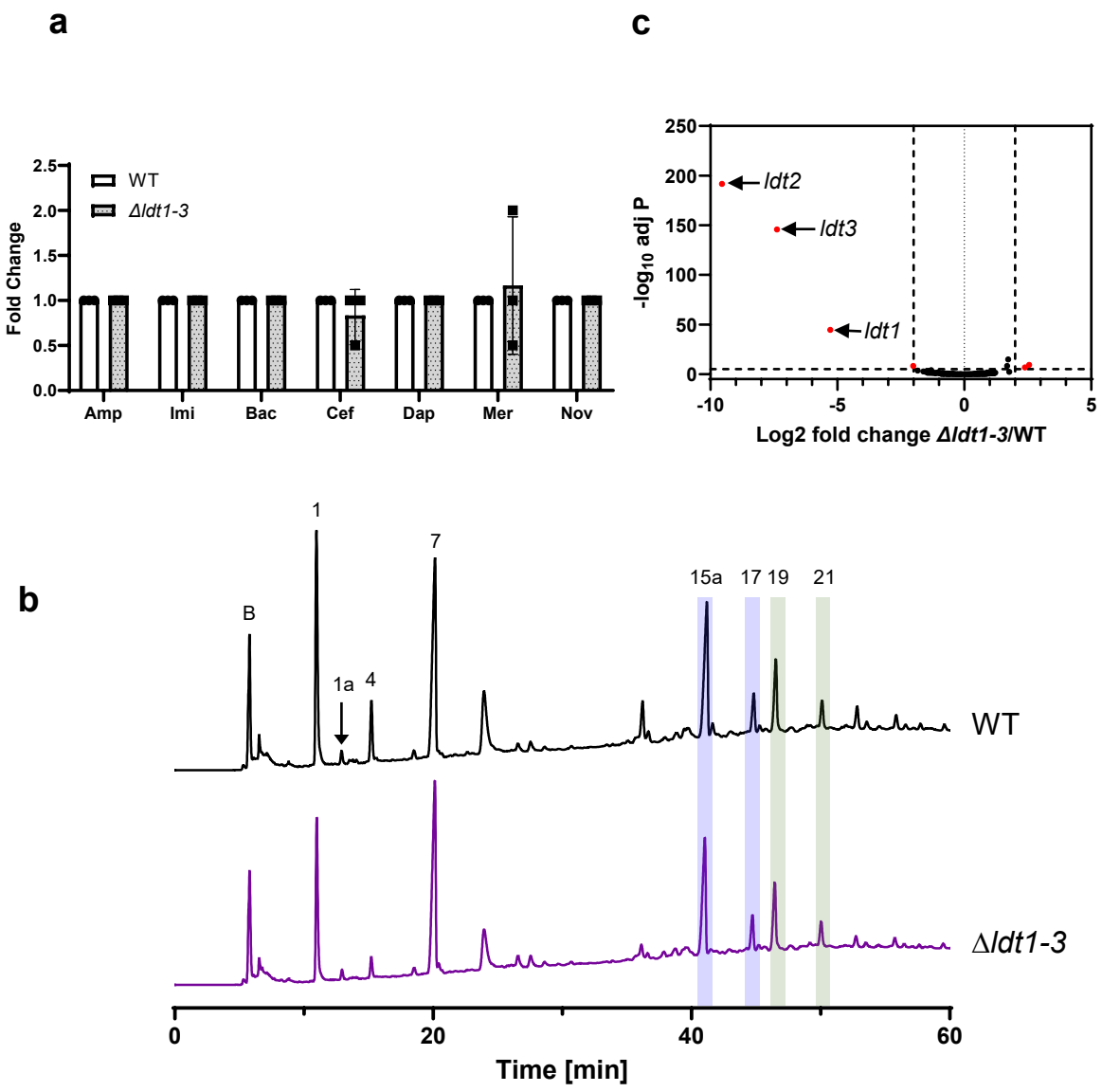
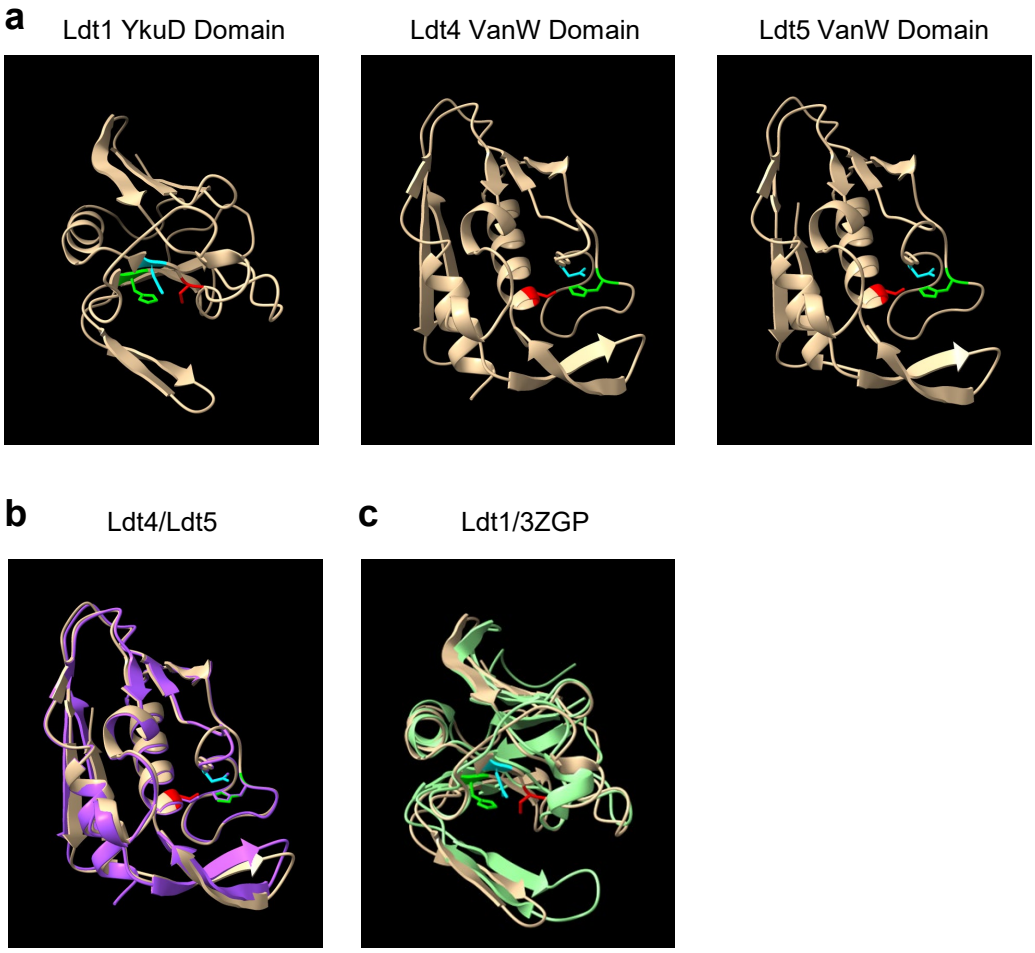


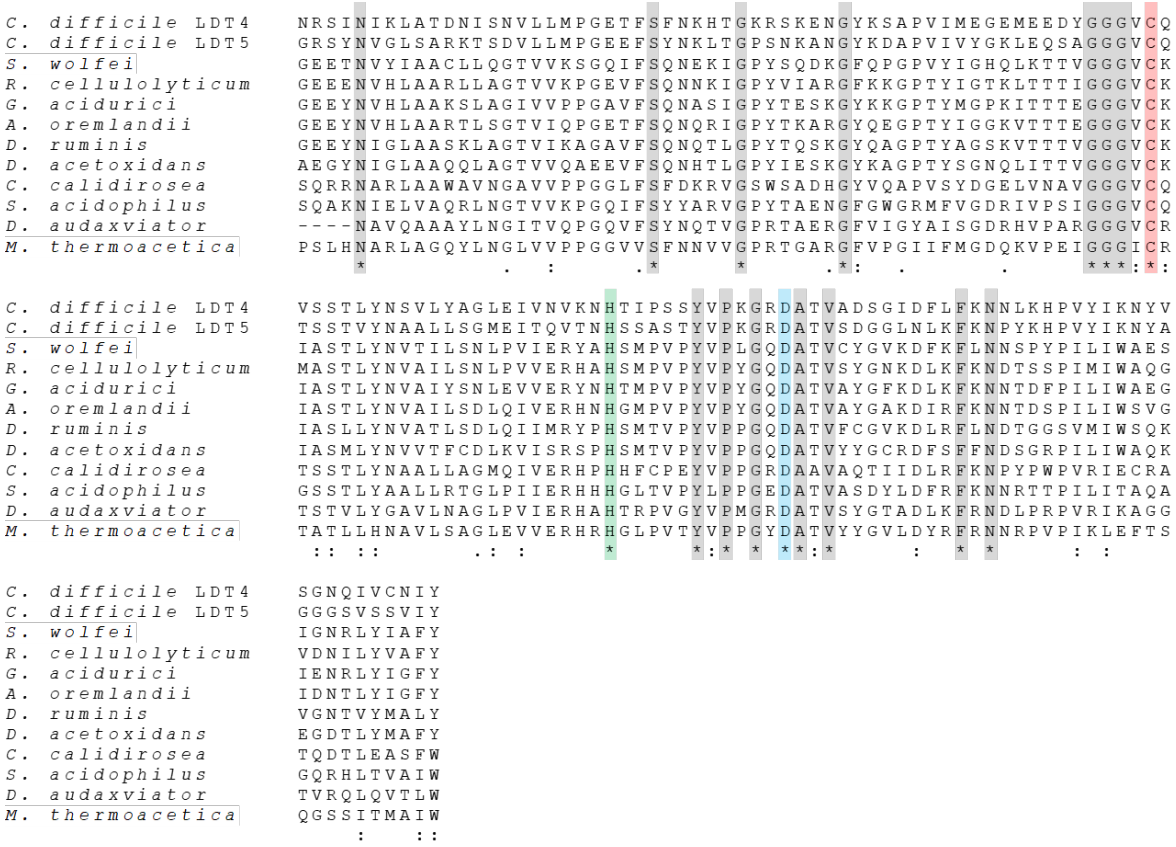
Supplemental Fig. 1. Validation of Δ*ldt1-3* deletion mutant. (**a**) PCR amplification products obtained using primers that flank the indicated gene deletion. Reaction mixtures were analyzed on a 1% agarose gel that was stained with ethidium bromide and photographed. Gel is representative of 2 experiments. (**b-d**) Western blots with antisera raised against soluble extracellular domain of the indicated Ldt protein. Blots are representative of at least three biological replicates. Overnight cultures of WT or the Δ*ldt1-3* mutant harboring a P_{xyl}::*ldt* expression plasmid were diluted 1:100 into TY or TY-thiamphenicol with or without xylose as indicated. Samples were taken for Western blotting at OD_{600} ~0.8. The predicted molecular masses are: Ldt1, 52.5 kDa; Ldt2, 71.9 kDa; Ldt3, 33.3 kDa. Note that Ldt3 is essentially not expressed in WT and this blot includes an inset (above) showing an unknown biotinylated protein detected with a red streptavidin probe as a loading control. The same control is also faintly visible in the Ldt1 blot. Strains used: WT, R20291; Δ*ldt1-3*, KB124; Δ*ldt1-3*/P_{xyl}::*ldt1*, KB210; Δ*ldt1-3*/P_{xyl}::*ldt2*, KB154; and Δ*ldt1-3*/P_{xyl}::*ldt3*, KB181.



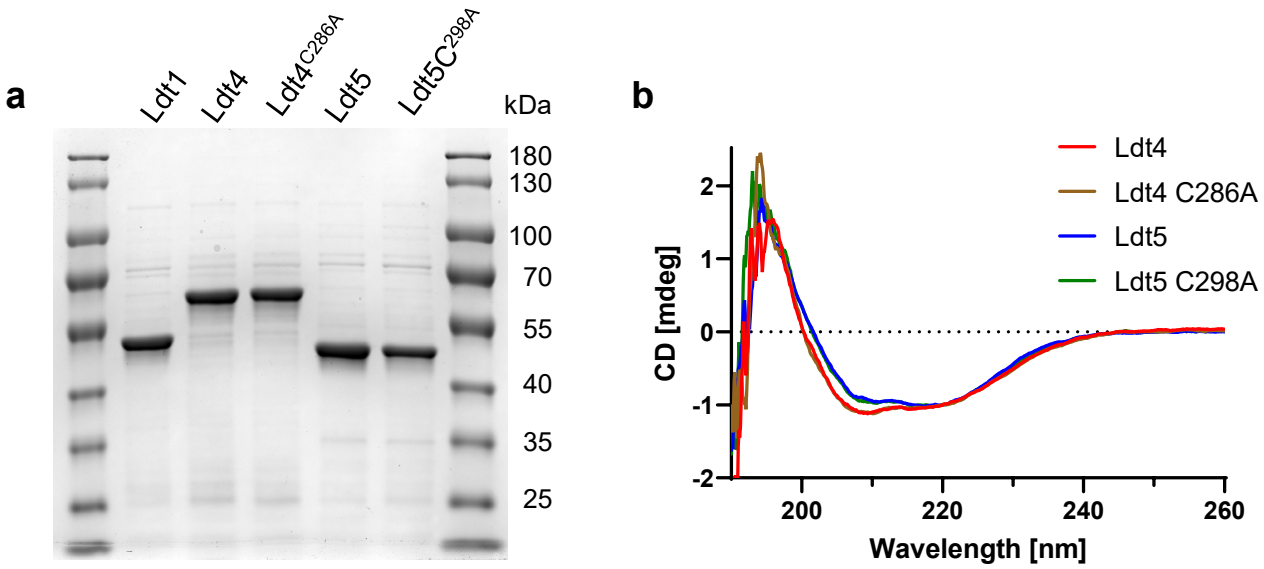
Supplemental Fig. 2. The Δ*ldt1-3* deletion mutant is similar to wild-type in antibiotic sensitivity, transcriptional profile, and PG composition. (**a**) Minimum inhibitory concentration of select antibiotics against the wild type (WT) strain and Δ*ldt1-3.* Data are from three biological replicates and reported as fold change difference between the strains. Abbreviations as follows with wild type MIC in brackets. Amp: ampicillin (3.1 µg/mL), Imi: imipenem (6.3 µg/mL), Bac: bacitracin (250 µg/mL), Cef: cefoxitin (200 µg/mL), Dap: daptomycin (1.6 µg/mL), Mer: meropenem (2.08 µg/mL), Nov: novobiocin (16.7 µg/mL). (**b**) Muropeptide analysis. HPLC chromatograms representative of three biological replicates are shown with the major 3-3 and 4-3 crosslinked muropeptides numbered as in Peltier et al.[15] and highlighted in blue (3-3) or green (4-3). (**c**) Volcano plot comparing transcriptome of the Δ*ldt1-3* strain to wild type growing in TY. Vertical dotted lines: log2-fold change=2; horizontal dotted line: -$\log_{10}$ adjusted P-value=5. Red dots indicate genes with log2-fold>2 and -$\log^{10}$ adjusted P-value>5. The genes encode the three deleted *ldts* and three more that just exceed the cut-offs, *cdr_2121-2122* (*sinR, sinR'*) and *cdr_2123,* a small hypothetical. Strains used: WT, R20291; Δ*ldt1-3*, KB124.



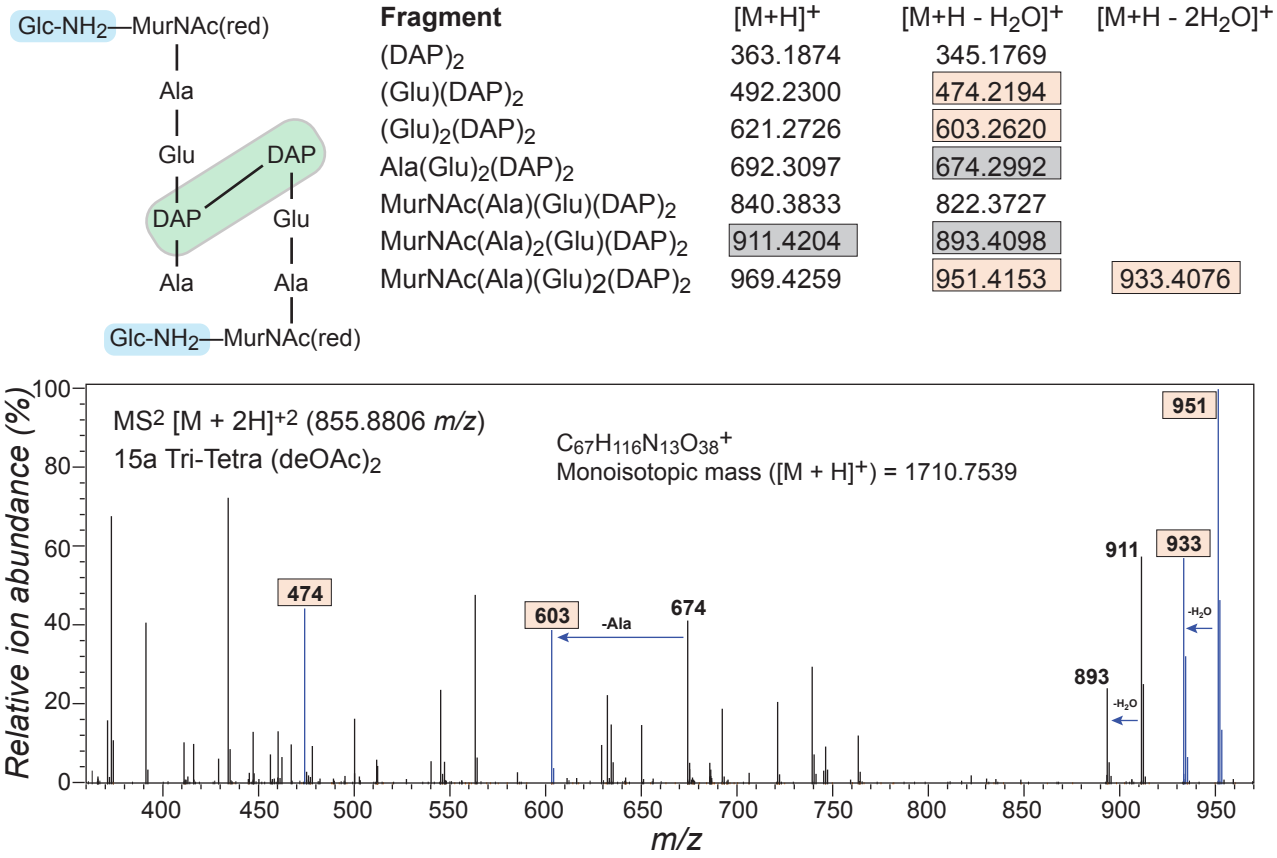
Supplemental Fig. 3. The YkuD and VanW domains have different folds. (**a**) AlphaFold2 structures of catalytic domains *C. difficile* Ldt1, Ldt4 and Ldt5. Catalytic triad residues are highlighted: Cys (red), His (green), Asp (cyan). (**b**) Overlay of the AlphaFold2 VanW domains from Ldt4 (purple) and Ldt5 (brown) show very similar folds. (**c**) Overlay of the AlphaFold2-predicted structure of the YkuD domain from Ldt1 (brown) with the NMR-determined structure of the YkuD domain from *E. faecium* Ldt (3ZGP, green).



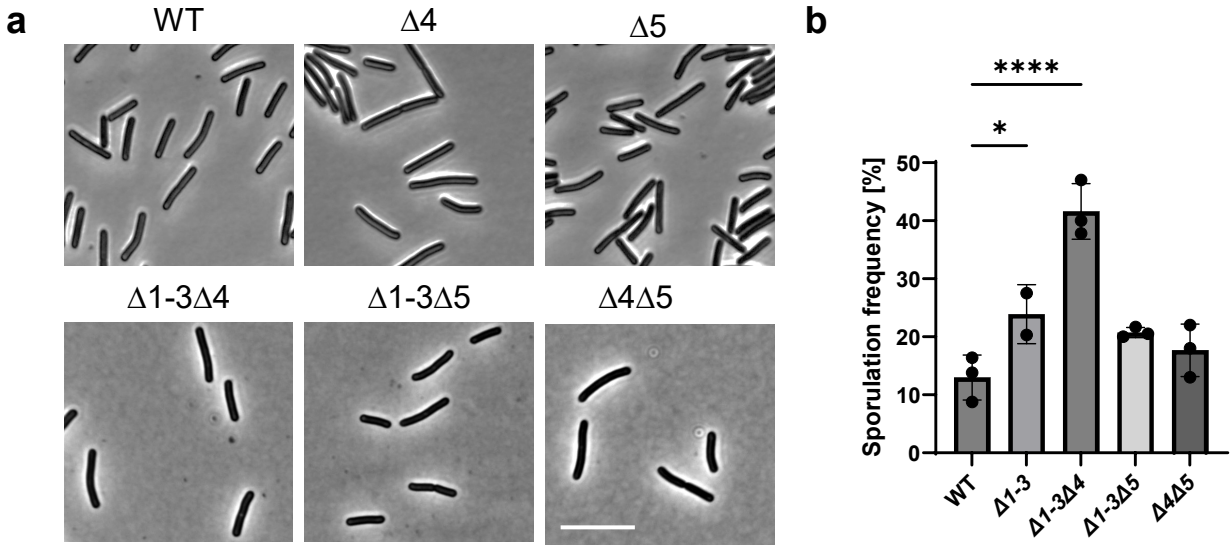
Supplemental Fig. 4. VanW domain amino acid sequence alignment. Proposed catalytic triad is highlighted with red, green and blue. Gray highlight and asterisks denote strict amino acid identity, colons and periods indicate other conserved positions. Sequences shown are from *C. diffficile*, *Desulforudis audaxviator, Moorella thermoacetica, Sulfobacillus acidophilus, Ruminiclostridium cellulolyticum, Gottschalkia acidurici, Alkaliphilus oremlandii, Syntrophomonas wolfei, Desulforamulus ruminis, Desulfofarcimen acetoxidans*, and *Chthonomonas calidirosea*.



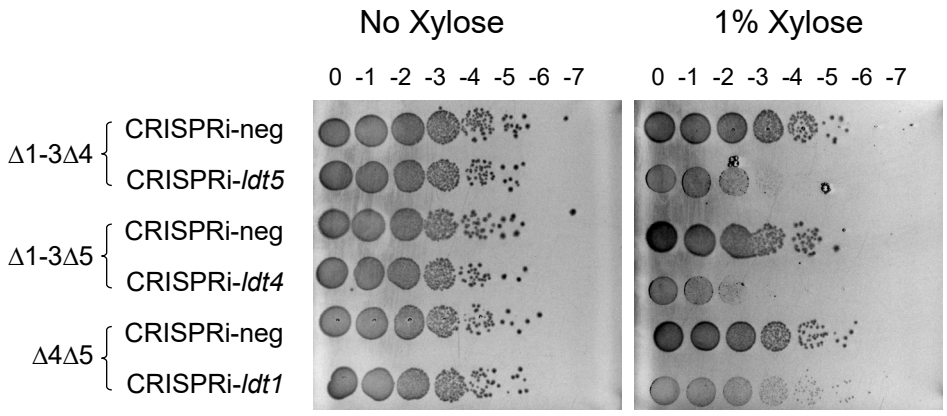
Supplemental Fig. 5. Purified Ldts. His-tagged Ldts without the N-terminal transmembrane region were expressed in *E. coli* and purified over a Nickel-affinity resin. (**a**) Purity determination by SDS-PAGE. About 4 µg of purified protein were separated by 10% SDS-PAGE and stained with Coomassie Blue. Molecular mass standards are indicated to the right of the gel. Predicted molecular weights: Ldt1, 51 kD; Ldt4 54 kD; Ldt5, 45 kD. (**b**) Circular dichromism (CD) spectroscopy to evaluate protein folding. CD spectra were generated for 5 µM enzyme and normalized to the absorbance at 220 nm. Ldts with the active site mutation showed the same degree of folding as the corresponding wild type enzyme.



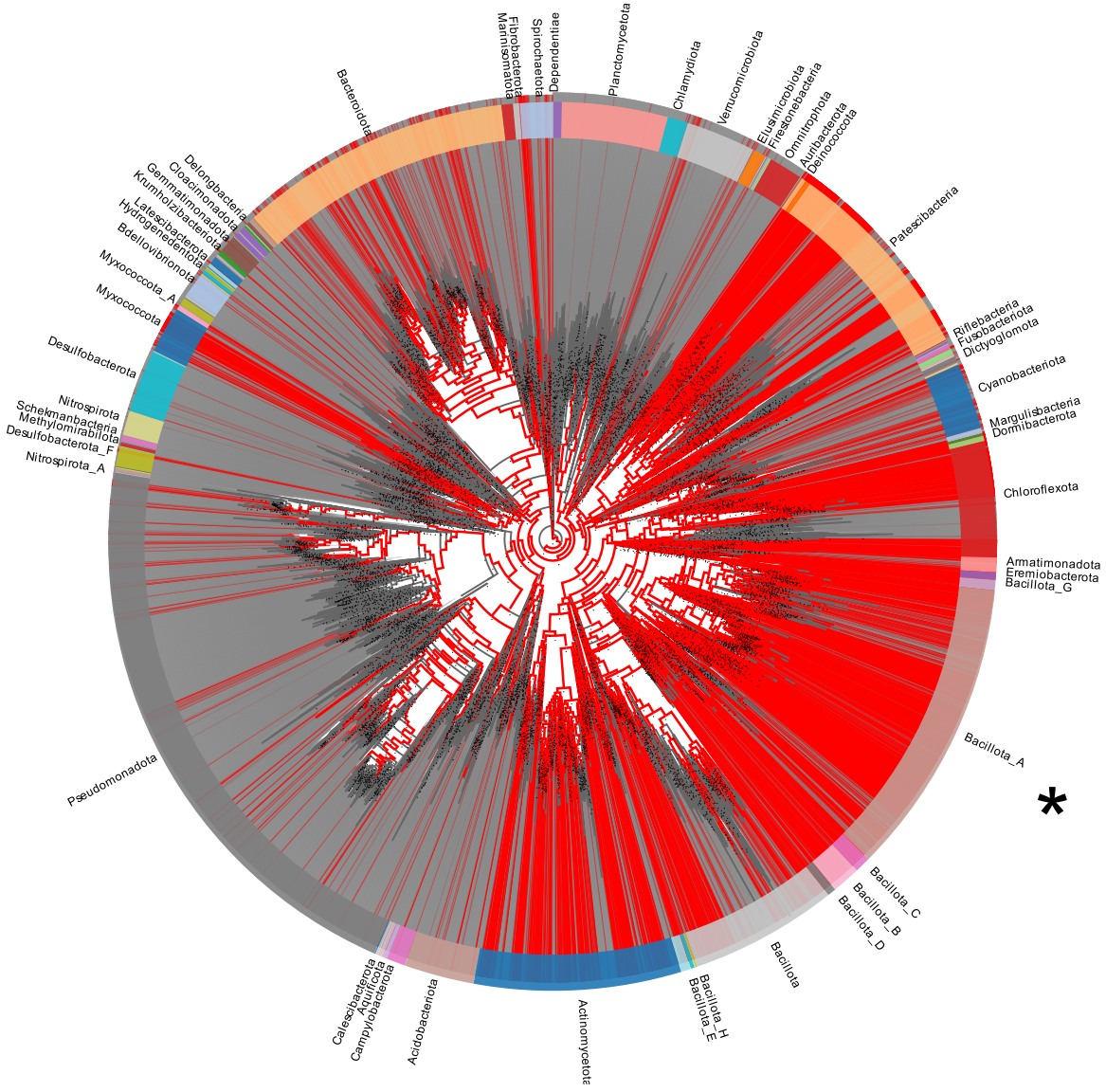
Supplemental Fig. 6. *Structural analysis of the predominant Ldt4 and Ldt5 reaction product.* Confirmation of the 3-3 crosslink was determined by manual interpretation of fragmentation data that solely supported DAP-DAP bonds (beige ions). The ions indicated in gray were present but not indicative of a 3-3 crosslink. While the DAP dimer (363 *m/z*) was observed, the ion intensity was too weak to be used for confirmation. However, ions associate with Glu and DAP (474, 603 *m/z*) fully supported the stucture shown along several ions containing an intact NAM(red) moiety and only one alanine (951, 933 *m/z*).



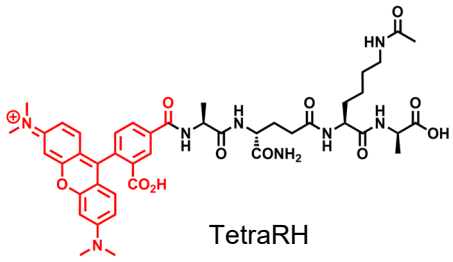
Supplemental Fig. 7: Morphology and sporulation of *ldt* deletion mutants. (**a**) Phase contrast images of strains grown to mid-log. Size bar: 10 µm. Images are representative of at least three biological replicates. (**b**) Sporulation efficiency. Dots indicate the values from two or three biological replicates. Bars and error bars indicate the mean ± s.d. *, $p < 0.05$. **** $p < 0.001$ in a two-way ANOVA. Frequency is calculated as the number of spores divided by the sum of spores plus vegetative cells [spores/(spores + vegetative cells)]. Strains shown: WT, R20291; Δ4, KB439; Δ5, KB440; Δ1-3Δ4, KB474 ; Δ1-3Δ5, KB502; and Δ4Δ5, KB529.



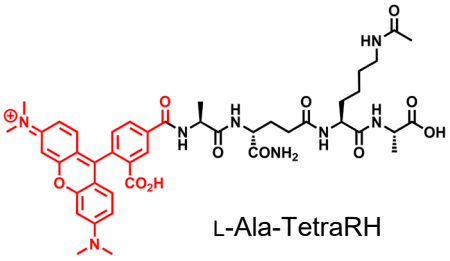
Supplemental Fig. 8: Ldt1, Ldt4, or Ldt5 is sufficient for viability and Ldt2 plus Ldt3 are not sufficient. CRISPRi plasmids targeting *ldt5, ldt4, or ldt1* were introduced into the indicated deletion strains. The negative control plasmid contained sgRNA against sequence not found in *C. difficile.* Serial dilutions of overnight cultures were spotted onto TY plates with or without 1% xylose, and plates were imaged after overnight incubation. Images are representative of three biological replicates. Strains shown: Δ1-3Δ4/neg, KB579; Δ1-3Δ4/CRISPRi-*ldt5*, KB508; Δ1-3Δ5/neg, KB580; Δ1-3Δ5/CRISPRi-*ldt4*, KB514 ; Δ4Δ5/neg, KB565; and Δ4Δ5/CRISPRi-*ldt1*, KB566



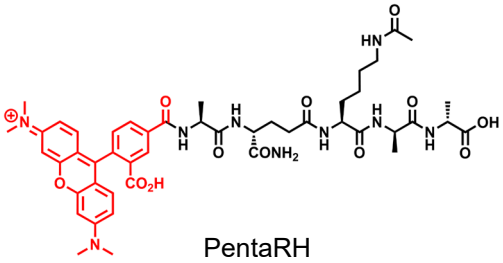
Supplemental Fig. 9: Taxonomic distribution of VanW domains. Red lines indicate the presence of VanW domais, gray lines the absence. Outer ring indicates bacterial phyla at the level of Class. *C. difficile* belongs to Bacillota A (asterisk). Some phyla names were pruned for clarity. Figure prepared using AnnoTree v1.3 Beta.



TetraRH



L-Ala-TetraRH



PentaRH

Supplemental Fig. 10. Fluorescent substrate analogs. TetraRH: L,D-transpeptidase specific substrate analog, Rhodamine-L-Ala-iso-D-Gln-L-Lys(Ac)-D-Ala. L-Ala-TetraRH: negative control, Rhodamine-L-Ala-iso-D-Gln-L-Lys(Ac)-L-Ala; PentaRH: PBP specific substrate analog, Rhodamine-L-Ala-iso-D-Gln-L-Lys(Ac)-D-Ala-D-Ala.

Supplemental Table 2. Strains used in this study.

Strain and species	Genotype and/or description	Alternate name	Source or reference
E. coli			
OmniMAX-2T1R	F′ [*proAB+ lacI* q *lacZΔ* M15 Tn*10* (Tetr) *Δ(ccdAB)*] *mcrA Δ* (*mrr-hsdRMS-mcrBC*) φ80(*lacZ*)*Δ* M15 *Δ* (*lacZYA-argF*)*U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD*		Invitrogen
HB101/pRK24	F− *mcrB mrr hsdS20* (rB− mB−) *recA13 leuB6 ara − 14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*		(1)
MG1655	Wild type isolate		(2)
Rosetta(DE3)	F- *ompT hsdS* $_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE (Cam^R)		Sigma-Aldrich
KB005	Rosetta(DE3)/pKB001		This study
KB007	Rosetta(DE3)/pKB003		This study
KB008	Rosetta(DE3)/pKB004		This study
CE4771	Rosetta(DE3)/pCE1169		This study
CE4772	Rosetta(DE3)/pCE1170		This study
CE4777	Rosetta(DE3)/pCE1173		This study
CE4778	Rosetta(DE3)/pCE1174		This study
B. subtilis			
BS49	Tn*916* donor strain, Tet^r		(3)
PY79	Wild type strain		(4)
C. difficile , all R20291 or derivatives			
R20291	Wild type strain from UK outbreak (ribotype 027)		(5)
KB071	*Δldt1*		This study
KB075	*Δldt2*		This study
KB130	*Δldt3*		This study
KB103	*Δldt1Δldt2*		This study
KB139	*Δldt1Δldt3*		This study
KB166	*Δldt2Δldt3*		This study
KB124	*Δldt1Δldt2Δldt3*	Δ1-3	This study
KB464	R20291/pBZ101		This study
KB465	Δ1-3/pBZ101		This study
KB154	Δ1-3/pCE0938		This study
KB181	Δ1-3/pCE0983		This study
KB210	Δ1-3/pKB025		This study
KB439	Δ*ldt4*	Δ4	This study
KB440	Δ*ldt5*	Δ5	This study
KB474	Δldt1Δldt2Δldt3Δ*ldt4*	Δ1-3Δ4	This study
KB502	Δldt1Δldt2Δldt3Δ*ldt5*	Δ1-3Δ5	This study
KB529	Δ*ldt4* Δ*ldt5*	Δ4Δ5	This study
KB547	Δ*ldt1-3*Δ*ldt4* P_{tet}::*ldt5*	Δ1-3Δ4 P_{tet}::ldt5	This study
KB548	Δ1-3Δ4 P_{tet}::*ldt5/* pBZ101		This study
KB549	Δ1-3Δ4 P_{tet}::*ldt5/* pKB025		This study
KB550	Δ1-3Δ4 P_{tet}::*ldt5/* pCE0938		This study
KB551	Δ1-3Δ4 P_{tet}::*ldt5/* pCE0983		This study
KB552	Δ1-3Δ4 P_{tet}::*ldt5/* pCE1175		This study
KB553	Δ1-3Δ4 P_{tet}::*ldt5/* pCE1176		This study

Supplemental Table 2. Strains used in this study.

Strain and species	Genotype and/or description	Alternate name	Source or reference
KB508	Δ1-3Δ4/pKB081		This study
KB514	Δ1-3Δ5/pKB083		This study
KB566	Δ4Δ5/pIA68		This study
KB565	Δ4Δ5/pIA34		This study
KB579	Δ1-3Δ4/pIA34		This study
KB580	Δ1-3Δ5/pIA34		This study
KB633	Δ1-3Δ4/pBZ101		This study
KB634	Δ1-3Δ4/pCE1176		This study
KB635	Δ1-3Δ4/pCE1186		This study
KB636	Δ1-3Δ4/pCE1191		This study
KB637	Δ1-3Δ4/pCE1198		This study
KB638	Δ1-3Δ4/pCE1199		This study
KB639	Δ1-3Δ4/pCE1200		This study
KB640	Δ1-3Δ4/pCE1201		This study
KB641	Δ1-3Δ4/pCE1202		This study

Supplemental Table 3. Plasmids used in this study.

Plasmid	Relevant features	Parent vector	Restriction enzymes to digest parent vector	PCR primers	PCR template	Assembly	Comments	Reference
pAP114	P_{xyl}::*mCherryOpt catP*						Parent plasmid for cloning under P_{xyl} control	(6)
pBZ101	P_{xyl} empty vector *catP*						Empty vector control for P_{xyl} induction	(7)
pCE678	P_{xyl}::*Cas9-opt* P_{gdh}::*sgRNA-pgdA-2,* homology to delete *pgdA catP*						Parent plasmid for CRISPR editing	(8)
pCE938	P_{xyl}::*ldt2 catP*	pAP114	BamHI, SacI	5609+5610	R20291	ITA[1]	*cdr_2601 (ldt2)* from *C. difficile* R20291 under xylose control	This study
pCE983	P_{xyl}::*ldt3 catP*	pAP114	BamHI, SacI	6243+5612	R20291	ITA	*cdr_2843 (ldt3)* from *C. difficile* R20291 under xylose control	This study
pCE1169	P_{tac}::*6xHis-ldt4* $^{28\text{-}489}$ *ampR*	pET21a-6xHis-rTEV	NcoI, EcoRI	6875+6876	R20291	ITA	Expression plasmid: N-terminal His tagged Ldt4$^{28\text{-}489}$, no transmembrane region	This study
pCE1170	P_{tac}:: *6xHis-ldt5* $^{38\text{-}419}$ *ampR*	pET21a-6xHis-rTEV	NcoI, EcoRI	6878+6879	R20291	ITA	Expression plasmid: N-terminal His tagged Ldt5$^{38\text{-}419}$, no transmembrane region	This study
pCE1172	P_{xyl}::*Cas9* P_{gdh}::*sgRNA-cdr_985, homology region to replace* P_{ldt5} with P_{tet}, *catP*	pIA123	PstI	6886+6887 6888+6889 6890+6891	R20291 R20291 pRPF185	ITA	Plasmid intermediate: inserts homology regions to replace P_{ldt5} with P_{tet} into CRISPR editing plasmid pIA123	This study
pCE1173	P_{tac}::*6xHis-ldt4* $^{28\text{-}489}$ *C286A ampR*	pET21a-6xHis-rTEV	NcoI, EcoRI	6875+6897 6896+6876	R20291 R20291	ITA	Expression plasmid: N-terminal His tagged Ldt4$^{28\text{-}489}$ catalytic mutant C286A, no transmembrane region	This study
pCE1174	P_{tac}::*6xHis-ldt5* $^{38\text{-}419}$ *C298A ampR*	pET21a-6xHis-rTEV	NcoI, EcoRI	6878+6899 6898+6879	R20291 R20291	ITA	Expression plasmid: N-terminal His tagged Ldt5$^{38\text{-}419}$ catalytic mutant C298A, no transmembrane region	This study
pCE1175	P_{xyl}::*ldt4 catP*	pAP114	BamHI, SacI	6892+6893	R20291	ITA	*cdr_1285 (ldt4)* from *C. difficile* R20291 under xylose control	This study
pCE1176	P_{xyl}::*ldt5 catP*	pAP114	BamHI, SacI	6894+6895	R20291	ITA	*cdr_2055 (ldt5)* from *C. difficile* R20291 under xylose control	This study
pCE1180	P_{xyl}::*Cas9* P_{gdh}::*sgRNA-ldt5,* homology region to replace P_{ldt5} with P_{tet}, *catP*	pCE1172	MscI, MluI	6906+4237	pIA33	ITA	Vector suitable for CRISPR replacing P_{ldt5} with P_{tet}; replaces *sgRNA-cdr_985* with *sgRNA-ldt5* in pCE1172	This study
pCE1186	P_{xyl}::*yoaR*	pAP114	BamHI, SacI	6922+6923	*Bacillus subtilis* PY79	ITA	*yoaR* from *Bacillus subtilis* PY79 under xylose control	This study
pCE1191	P_{xyl}::$vanW_{Ef}$	pAP114	BamHI, SacI	6932+6933	*Enterococcus faecalis* V583	ITA	*vanW* from *Enterococcus faecalis* V583 under xylose control	This study
pCE1198	P_{xyl}::$vanW_{Pb}$	pAP114	BamHI, SacI	7013+7014	*Paraclostridium bifermentans* 638	ITA	*vanW* (WP_021433578) from *Paraclostridium bifermentans* 638 under xylose control	This study
pCE1199	P_{xyl}::$vanW_{Rum}$	pAP114	BamHI, SacI	7015+7016	*Ruminococcaceae bacterium* D16	ITA	*vanW* (HMPREF0866_01899) from *Ruminococcaeae bacterium* D16 under xylose control	This study
pCE1200	P_{xyl}::$vanW_{Lac}$	pAP114	BamHI, SacI	7017+7018	*Lachnospiraceae bacterium* 5_1_57FAA	ITA	*vanW* (HMPREF0993_00855) from *Lachnospiraceae bacterium* 5_1_57FAA under xylose control	This study
pCE1201	P_{xyl}::$vanW_{Pep0521}$	pAP114	BamHI, SacI	7021+7022	*Peptostreptococcaceae bacterium* AS15	ITA	*vanW* (HMPREF1142_0521) from *Peptostreptococcaceae bacterium* AS15	This study
pCE1202	P_{xyl}::$vanW_{Pep1713}$	pAP114	BamHI, SacI	7023+7024	*Peptostreptococcaceae bacterium* AS15	ITA	*vanW* (HMPREF1142_1713) from *Peptostreptococcaceae bacterium* AS15	This study
pET21a-6xHIS-rTEV							pET21a vector (Novagen) with N-terminal His6 tag and tobacco etch virus (rTEV) protease cleavage site	(9)
pCI5492	P_{xyl}::*dCas9-opt* P_{gdh}::*sgRNA-cdr_985*	pIA33	MscI, NotI	5492+4084	pIA33	ITA	CRISPRi against *cdr_985 (pbp2)*	This Study
pIA33	P_{xyl}::*dCas9-opt* P_{gdh}::*sgRNA-rfp catP*						Parent plasmid for CRISPRi constructs	(6)
pIA34	P_{xyl}::*dCas9-opt* P_{gdh}::*gRNA-neg*						CRISPRi negative control plasmid	(6)

Supplemental Table 3. Plasmids used in this study.

Plasmid	Relevant features	Parent vector	Restriction enzymes to digest parent vector	PCR primers	PCR template	Assembly	Comments	Reference
pIA68	P_{xyl}::*dCas9-opt* P_{gdh}::*sgRNA-ldt1*	pIA34	MscI, MluI		pIA33	ITA	CRISPRi against *ldt1*	This study
pIA123	P_{xyl}::*Cas9* P_{gdh}::*sgRNA-cdr_985 catP*	pCI5492	SalI, XhoI	6269+6270	pCE678	ITA	CRISPR editing parent plasmid; sgRNA-*cdr_985 (pbp2)*; no homology region	This study
pKB001	P_{tac}::6xHis-*ldt1*$^{38\text{-}469}$ *ampR*	pET21a-6xHis-rTEV	NcoI, EcoRI	5467+5468	R20291	ITA	Expression plasmid: N-terminal 6x His tag on Ldt1$^{38\text{-}469}$, no transmembrane section	This study
pKB003	P_{tac}::6xHis-*ldt2*$^{38\text{-}617}$ *ampR*	pET21a-6xHis-rTEV	NcoI, EcoRI	5471+5472	R20291	ITA	Expression plasmid: N-terminal His tagged Ldt2$^{38\text{-}617}$, no transmembrane region	This study
pKB004	P_{tac}::*6xHis-ldt3*$^{7\text{-}289}$ *ampR*	pET21a-6xHis-rTEV	NcoI, EcoRI	5473+5474	R20291	ITA	Expression plasmid: N-terminal His tagged Ldt3$^{7\text{-}289}$, no transmembrane region	This study
pKB007	P_{xyl}::*Cas9-opt* P_{gdh}::*sgRNA-pgdA-2,* homology to delete *ldt2 catP*	pCE678	NotI, XhoI	5541+5542, 5543+5544	R20291 R20291	ITA	Plasmid intermediate to build *ldt2* deletion plasmid	This study
pKB009	P_{xyl}::*Cas9-opt* P_{gdh}::*sgRNA-pgdA-2,* homology to delete *ldt3 catP*	pCE678	NotI, XhoI	5463+5464, 5465+5466	R20291 R20291	ITA	Plasmid intermediate to build *ldt3* deletion plasmid	This study
pKB015	P_{xyl}::*Cas9-opt* P_{gdh}::*sgRNA-pgdA-2,* homology to delete *ldt1 catP*	pCE678	NotI, XhoI	5459+5460, 5461+5462	R20291 R20291	ITA	Plasmid intermediate to build *ldt1* deletion plasmid	This study
pKB019	P_{xyl}::*Cas9-opt* P_{gdh}::*sgRNA-ldt1,* homology to delete *ldt1 catP*	pKB15	MscI, MluI	5448+4237	pCE678	ITA	CRISPR edit plasmid to delete *ldt1*	This study
pKB022	P_{xyl}::*Cas9-opt* P_{gdh}::*sgRNA-ldt2,* homology to delete *ldt2 catP*	pKB07	MscI, MluI	5540+4237	pCE678	ITA	CRISPR edit plasmid to delete *ldt2*	This study
pKB024	P_{xyl}::*Cas9-opt* P_{gdh}::*sgRNA-ldt3,* homology to delete *ldt3 catP*	pKB09	MscI, MluI	5456+4237	pCE678	ITA	CRISPR edit plasmid to delete *ldt3*	This study
pKB025	P_{xyl}::*ldt1 catP*	pAP114	BamHI, SacI	5607+5608	R20291	ITA	*cdr_2797 (ldt1)* from *C. difficile* R20291 under xylose control	This study
pKB067	P_{xyl}::*Cas9* P_{gdh}::*sgRNA-cdr_985,* homology to delete *ldt5 catP*	pIA123	PstI	6737+6738, 6739+6740	R20291 R20291	ITA	Plasmid intermediate to build *ldt5* deletion plasmid	This study
pKB068	P_{xyl}::*Cas9* P_{gdh}::*sgRNA-cdr_985,* homology to delete *ldt4 catP*	pIA123	PstI	6733+6734, 6735+6736	R20291 R20291	ITA	Plasmid intermediate to build *ldt4* deletion plasmid	This study
pKB071	P_{xyl}::*Cas9* P_{gdh}::*sgRNA-ldt5,* homology to delete *ldt5 catP*	pKB67	MscI, MluI	6780+4237	pIA123	ITA	CRISPR edit plasmid to delete *ldt5*	This study
pKB073	P_{xyl}::*Cas9* P_{gdh}::*sgRNA-ldt4,* homology to delete *ldt4 catP*	pKB68	MscI, MluI	6774+4237	pIA123	ITA	CRISPR edit plasmid to delete *ldt4*	This study
pKB081	P_{xyl}::*dCas9-opt* P_{gdh}::*sgRNA-ldt5*	pIA34	MscI, MluI	6865+4237	pIA34	ITA	CRISPRi against *ldt5*	This study
pKB083	P_{xyl}::*dCas9-opt* P_{gdh}::*sgRNA-ldt4*	pIA34	MscI, MluI	6871+4237	pIA34	ITA	CRISPRi against *ldt4*	This study
pRPF185	P_{tet}::*gusA*						Source of P_{tet}	(10)

[1]: Isothermal assembly

Supplemental Table 4. Oligonucleotides used in this study.

Oligo	Sequence	Use
4084	AACTTATAGGATCCGCGGCCGCTAGTCAGACATCATGCTGATCTAGA	Cloning
4237	CTTATAGGATCCGCGGCCGCTAG	Cloning
5448	AATTAAACTGTAAATGGCCAAATTGTAATATCTTTACCTG GTTTTAGAGCTAGAAATAGC	Cloning
5456	AATTAAACTGTAAATGGCCAATTTTTAAGAAAGATAATGG GTTTTAGAGCTAGAAATAGC	Cloning
5459	AAACAGCTATGACCGCGGCCGCGTTGAAGACATTACGAAACTAG	Cloning
5460	AACTGTTAGCAACACATTTAAATTAAATCCTTCCTTACATTG	Cloning
5461	ATGTAAGGAAGGATTTAATTTAAATGTGTTGCTAACAGTT	Cloning
5462	TTATTTTTATGCTAGCTCGAGCCTCATTGTTAAAGTATAAACA	Cloning
5463	AAACAGCTATGACCGCGGCCGCTTAAAAGGTGAAATAATCTGT	Cloning
5466	TTATTTTTATGCTAGCTCGAGAGACTATGAAGGTATCAAC	Cloning
5467	TGTATTTTCAGGGCGCCATGAGAAATCATTTTTACTTTGGA	Cloning
5468	TCGACGTAGGCCTTTGAATTCTAGTATAAAATAATTGGTGTACC	Cloning
5471	TGTATTTTCAGGGCGCCATGAGTAAACATGTGATTATAGTAAA	Cloning
5472	TCGACGTAGGCCTTTGAATTCTATTTTGCAAGAATATCCA	Cloning
5473	TGTATTTTCAGGGCGCCATGAAATTAACTAAACTAAATATTAAAAAATATA	Cloning
5474	TCGACGTAGGCCTTTGAATTTTAATGAATTATAACTGTTGTTG	Cloning
5492	AATTAAACTGTAAATGGCCACTGCTATTGAAACACCAACA GTTTTAGAGCTAGAAATAGC	Cloning
5540	AATTAAACTGTAAATGGCCACCTATAATTGTATCCCATGT GTTTTAGAGCTAGAAATAGC	Cloning
5541	AAACAGCTATGACCGCGGCCGCAACTCAATAGTGGTTGAT	Cloning
5542	TTTTTCAATATATATTTTTATACTACATGTGTCTAATTATAACAT	Cloning
5543	ATAATTAGACACATGTAGTATAAAAATATATATTGAAAAATAATTATAATTGAG	Cloning
5544	TTATTTTTATGCTAGCTCGAGGCTCTACAATAGGAACTTC	Cloning
5607	CGATAGTTATGAAGTGAGCTGTAAGGAAGGATTTAATTATGATTGATG	Cloning
5608	TTATTAAAACTTATAGGATCCTTCGCTTAACTGTTAGCAAC	Cloning
5609	CGATAGTTATGAAGTGAGCTTAAGGAGGATGTAGTAATGTTTCTAAAAGAGGGGGA	Cloning
5610	TTTTATTAAAACTTATAGGATCCACATCTCAATTATAATTATTTTTCAATATATATTTTTACTATTTTGCA	Cloning
5612	AGTTTTATTAAAACTTATAGGATCTTGAGAATCATTATCCCAATAAAAACTC	Cloning
6243	AGATACCATAGATCCGGTACCATGAATTATAACTGTTGTTGTATCTG	Cloning
6269	AGGAGGGTAAAGAGGAGAGTCGACGCATGGATAAAAAATATAGTATAGGATTAGATATAG	Cloning
6270	CCGTATTTCTACGATGTTTTTGCTCGAGTTAATCACCACCTAATTGAGATAAA	Cloning
6733	CAGGAAGGGCGAATTCTGCATGGTAAGTGCAAAAACTAAA	Cloning
6736	GAGACCGGTCAGATCTGCACCTTGTTTATAAAGTTCATCTAGT	Cloning
6737	CAGGAAGGGCGAATTCTGCAAAAACAATTAGACGAAATAATAGA	Cloning
6738	ATATTTCTCTAAAAAATTATAACTTCACCTCATTTTGACA	Cloning
6739	TGTCAAAATGAGGTGAAGTTATAATTTTTTAGAGAAATATTTGTAATAT	Cloning
6740	GAGACCGGTCAGATCTGCACACATCTATTATTTTTAGTATATAAGGA	Cloning
6774	AATTAAACTGTAAATGGCCAAATGTGCATGCAATTTAAAG GTTTTAGAGCTAGAAATAGC	Cloning
6780	AATTAAACTGTAAATGGCCATTGTATGAAAATTCTTCACC GTTTTAGAGCTAGAAATAGC	Cloning
6865	AATTAAACTGTAAATGGCCATAAGTAAGATTTAAGTCTTC GTTTTAGAGCTAGAAATAGC	Cloning
6871	AATTAAACTGTAAATGGCCAATTTTAAGACCATCAGAATC GTTTTAGAGCTAGAAATAGC	Cloning
6875	TGTATTTTCAGGGCGCCATGATGCAATTTAAAGGGGAGAAAA	Cloning
6876	AGGCCTTTGAATTCCGGATCTTAAGCTTGCGGTTGTGGTT	Cloning
6878	TGTATTTTCAGGGCGCCATGAACAGTAAATTTCTGTACAATGGG	Cloning
6879	AGGCCTTTGAATTCCGGATCCTATTTTTTTAATTCTTTATAAGAACTATTTGATAT	Cloning
6886	CAGGAAGGGCGAATTCTGCATTTTAGCTTTAAAAGCTGTT	Cloning
6887	GCTTCTTATTTTTATGCTAGAATTTAAATGTAACCTATTACAGG	Cloning
6888	AGCGTTAACAGATCTGAGCTAAATGAGGTGAAGTTATGGG	Cloning
6889	CCGGTCAGATCTGCACTGCATTTTTCATCTTAACATTTATACTATC	Cloning
6890	TAATAGGTTACATTTAAATTCTAGCATAAAAATAAGAAGCCTGC	Cloning
6891	CCCATAACTTCACCTCATTTAGCTCAGATCTGTTAACGCT	Cloning
6892	CGATAGTTATGAAGTGAGCTAAGGAGAGTATGGGATGAGTAATGTGAACAAA	Cloning
6893	TTATTAAAACTTATAGGATCTTAAGCTTGCGGTTGTGGTT	Cloning
6894	CGATAGTTATGAAGTGAGCTAAGGAGGTGAAGTTATGGGCAGAAGA	Cloning
6895	TTATTAAAACTTATAGGATCCTATTTTTTTAATTCTTTATAAGAACTATTTG	Cloning
6896	GAAGATTATGGTGGAGGAGTTGCCCAAGTTTCATCTACG	Cloning
6897	GATGAAACTTGGGCAACTCCTCCACCATAATCTTCTTCC	Cloning
6898	CAGCAGGAGGAGGTGTTGCCCAAACATCTTCAACAGTTTA	Cloning
6899	CTGTTGAAGATGTTTGGGCAACACCTCCTCCTGCTGATTG	Cloning
6906	AATTAAACTGTAAATGGCCA AGCTATTCAAATATAATCTA GTTTTAGAGCTAGAAATAGC	Cloning
6922	CGATAGTTATGAAGTGAGCTAAGGAGACCGACTTATGATACAAATTTTGATC	Cloning
6923	TTATTAAAACTTATAGGATCTTACTGCTCTGCATTTATTTCT	Cloning
6932	CGATAGTTATGAAGTGAGCTAAGGAGATAATGCTATGAACAGAAAAAGATTGAC	Cloning

Supplemental Table 4. Oligonucleotides used in this study.

Oligo	Sequence	Use
6933	TTATTAAAACTTATAGGATCTCATTGGTTCGCCTCCTGAA	Cloning
7013	CGATAGTTATGAAGTGAGCTCTAAGGAGGGGAATGAAATGCAACAAAATGTAGCAGTA	Cloning
7014	TTTATTAAAACTTATAGGATCCTTATTTTTTCTTGTACGAGCTTGT	Cloning
7015	CGATAGTTATGAAGTGAGCTCTAAGGAGGAACGTCATATGGAAGGTAGTCGGGTCCAA	Cloning
7016	TTATTAAAACTTATAGGATCCTCAGGATGCCTGACTGCTGGC	Cloning
7017	CGATAGTTATGAAGTGAGCTCTAAGGAGGAGAATAAGATGGCTGCAGGAAGTCAGAGA	Cloning
7018	TTATTAAAACTTATAGGATCCTTACTGTGCCGGCTGTACCTG	Cloning
7021	CGATAGTTATGAAGTGAGCTCTAAGGAGGTCAGCTAAATGATTATTGTAATTATTATAAGA	Cloning
7022	TTATTAAAACTTATAGGATCCTTAAAATACTACATTAGAATCTGATGTTTCTGT	Cloning
7023	CGATAGTTATGAAGTGAGCTCTAAGGAGGATGATATTTTGCAACTTCTCAAAAAACTC	Cloning
7024	TTATTAAAACTTATAGGATCCTTACTGTCTTGAATACCAATCGC	Cloning
5465	AAAATGTTGTAAAAAGACATGAAAAATTTTGAGTTTTTATTGG	Cloning
5464	ATAAAAACTCAAAATTTTTCATGTCTTTTTACAACATTTTATG	Cloning
6734	AAATCCTATTCTTGAGAACTCCCATACTTCTCCTTACAAT	Cloning
6735	ATTGTAAGGAGAAGTATGGGAGTTCTCAAGAATAGGATTT	Cloning
5599	AAGGGATTTTGAAAGGGGTG	Check *ldt1* deletion
5600	CTGTCGGTGACTGCCTTCTC	Check *ldt1* deletion
5601	AGTAGCTGGAGGAGCAGGAT	Check *ldt2* deletion
5602	CCTGTCAATGTAAATGGGTC	Check *ldt2* deletion
5603	GCCAAAACTTGGGGAATTGA	Check *ldt3* deletion
5604	GGTTCTCCACAAGACTGTGG	Check *ldt3* deletion
6808	GCTGAAGAGGCAAATACTTCTGG	Check *ldt4* deletion
6809	CCACCATTCTCATAATAAAAAAGGTAATCC	Check *ldt4* deletion
6810	CGGGGAGCTCTTTGTATAACTATAGATGC	Check *ldt5* deletion
6811	CCCCATTATTTGCTTTTGTAATTCC	Check *ldt5* deletion

References Supplemental Information

1 Trieu-Cuot, P., Carlier, C., Poyart-Salmeron, C. & Courvalin, P. Shuttle vectors containing a multiple cloning site and a lacZ alpha gene for conjugal transfer of DNA from *Escherichia coli* to gram-positive bacteria. *Gene* **102**, 99-104 (1991). https://doi.org:10.1016/0378-1119(91)90546-n

2 Arends, S. J. & Weiss, D. S. Inhibiting cell division in *Escherichia coli* has little if any effect on gene expression. *Journal of bacteriology* **186**, 880-884 (2004). https://doi.org:10.1128/jb.186.3.880-884.2004

3 Christie, P. J., Korman, R. Z., Zahler, S. A., Adsit, J. C. & Dunny, G. M. Two conjugation systems associated with *Streptococcus faecalis* plasmid pCF10: identification of a conjugative transposon that transfers between *S. faecalis* and *Bacillus subtilis*. *Journal of bacteriology* **169**, 2529-2536 (1987). https://doi.org:10.1128/jb.169.6.2529-2536.1987

4 Youngman, P., Perkins, J. B. & Losick, R. Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. *Plasmid* **12**, 1-9 (1984). https://doi.org:10.1016/0147-619x(84)90061-1

5 Stabler, R. A. *et al.* Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome biology* **10**, R102 (2009). https://doi.org:10.1186/gb-2009-10-9-r102

6 Müh, U., Pannullo, A. G., Weiss, D. S. & Ellermeier, C. D. A Xylose-Inducible Expression System and a CRISPR Interference Plasmid for Targeted Knockdown of Gene Expression in *Clostridioides difficile*. *Journal of bacteriology* **201**, e00711-00718 (2019). https://doi.org:10.1128/JB.00711-18

7 Kaus, G. M. *et al.* Lysozyme Resistance in *Clostridioides difficile* Is Dependent on Two Peptidoglycan Deacetylases. *Journal of bacteriology* **202** (2020). https://doi.org:10.1128/JB.00421-20

8 Müh, U., Ellermeier, C. D. & Weiss, D. S. The WalRK Two-Component System Is Essential for Proper Cell Envelope Biogenesis in *Clostridioides difficile*. *Journal of bacteriology*, e0012122 (2022). https://doi.org:10.1128/jb.00121-22

9 Shepherd, T. R. *et al.* The Tiam1 PDZ domain couples to Syndecan1 and promotes cell-matrix adhesion. *Journal of molecular biology* **398**, 730-746 (2010). https://doi.org:10.1016/j.jmb.2010.03.047

10 Fagan, R. P. & Fairweather, N. F. *Clostridium difficile* has two parallel and essential Sec secretion systems. *The Journal of biological chemistry* **286**, 27483-27493 (2011). https://doi.org:10.1074/jbc.M111.263889