

**Table S1.** Chemical shift assignments for the dimer peptidoglycan fragment **a**.

<sup>1</sup> H signal	Chemical shift (ppm)
GlcNAc amide	8.35
GlcNAc methyl	2.08
GlcNAc anomeric	4.67
GlcNAc ring	3.74, 3.58, 3.48, 3.44
MurNAc amide	8.11
MurNAc methyl	2.01
MurNAc ring *	4.43, 4.32
Lac CH	4.28
Lac methyl	1.42
A NH	8.41
A C $\alpha$ H	4.32
A methyl	1.43
iE NH	7.92
iE C $\alpha$ H	4.20
iE C $\beta$ H <sub>2</sub>	1.90, 2.10
iE C $\gamma$ H <sub>2</sub>	2.26
DAP NaH **	8.11
DAP NbH **	8.04
DAP CaH **	4.43
DAP CbH **	4.26
DAP CaH <sub>2</sub> **	1.74
DAP CbH <sub>2</sub> **	1.95
DAP CcH <sub>2</sub> **	1.69

\* Note that the muramic acid sugar has been reduced, meaning that there is no anomeric proton for this sugar.

\*\* See structures for the locations of these protons

**Table S2.** Minimum inhibitory concentration (MIC) of beta lactams against *C. difficile* R20291 and *ldt<sub>Ca</sub>* isogenic mutants.

	WT	$\Delta ldt_{Ca1}$	$\Delta ldt_{Ca2}$	$\Delta ldt_{Ca3}$	$\Delta ldt_{Ca1}$ $\Delta ldt_{Ca2}$	$\Delta ldt_{Ca1}$ $\Delta ldt_{Ca3}$	$\Delta ldt_{Ca2}$ $\Delta ldt_{Ca3}$	$\Delta ldt_{Ca1}$ $\Delta ldt_{Ca2}$ $\Delta ldt_{Ca3}$
<b>Penicillins</b>								
Amoxicillin	4	4	4	4	4	4	2	2
Co-amoxicillin	2	2	1	1	2	2	2	2
Piperacillin	16	16	16	16	16	16	16	16
Piperacillin-tazobactam	16	16	16	16	16	16	16	16
<b>Cephalosporins</b>								
Cephalotin <sup>a</sup>	>256	>256	>256	>256	>256	>256	256	256
Cephalexin <sup>a</sup>	>256	>256	>256	>256	>256	>256	>256	>256
Cefuroxime <sup>b</sup>	>256	>256	>256	>256	>256	>256	>256	>256
Cefoxitin <sup>b</sup>	>256	>256	>256	>256	>256	>256	>256	>256
Ceftriaxone <sup>c</sup>	128	128	128	128	128	128	128	128
Cefotaxime <sup>c</sup>	>256	>256	>256	>256	>256	>256	>256	>256
<b>Carbapenems</b>								
Meropenem	8	8	8	8	8	8	8	8
Imipenem	32	32	16	32	16	16	16	16
<b>Glycopeptides</b>								
Vancomycin	1	1	1	1	1	1	1	2

<sup>a</sup> first generation cephalosporins

<sup>b</sup> second generation cephalosporins

<sup>c</sup> third generation cephalosporins

**Table S3.** Quantification of Ldt<sub>Cd2</sub> endopeptidase activity against a 3-4 crosslinked dimer <sup>a</sup>

	<b>3-4 dimer</b>	<b>3-4 dimer + Ldt2</b>
<b>gm-AEJA=gm-AEJA</b>	99.963%	0.093%
<b>gm-AEJ=gm-AEJA - H<sub>2</sub>O</b>	0	86.014%
<b>gm-AEJ=gm-AEJA</b>	0	13.861%
<b>gm-AEJ</b>	0	0.032% <sup>b</sup>
<b>gm-AEJA</b>	0.037%	0
	100.0%	100.0%

<sup>a</sup> Abundance was calculated based on Ion intensity.

<sup>b</sup> The low abundance of gm-AEJ corresponds to carboxy-peptidase activity on traces of gm-AEJA present in the substrate

**Table S4.** Bacterial strains, plasmids, and oligonucleotides.

Strains/plasmids/ oligonucleotides	Relevant properties/sequence	Source
<b>Strains</b>		
<i>Clostridioides difficile</i>		
R20291	Clinical isolate, ribotype 027	(1)
R20291 $\Delta$ ldt1	R20291 derivative with an in-frame deletion in <i>ldt<sub>ca1</sub></i>	This work
R20291 $\Delta$ ldt2	R20291 derivative with an in-frame deletion in <i>ldt<sub>ca2</sub></i>	This work
R20291 $\Delta$ ldt3	R20291 derivative with an in-frame deletion in <i>ldt<sub>ca3</sub></i>	This work
R20291 $\Delta$ ldt2 $\Delta$ ldt1	R20291 derivative with an in-frame deletion in <i>ldt<sub>ca2</sub></i> and <i>ldt<sub>ca1</sub></i>	This work
R20291 $\Delta$ ldt3 $\Delta$ ldt1	R20291 derivative with an in-frame deletion in <i>ldt<sub>ca3</sub></i> and <i>ldt<sub>ca1</sub></i>	This work
R20291 $\Delta$ ldt3 $\Delta$ ldt2	R20291 derivative with an in-frame deletion in <i>ldt<sub>ca3</sub></i> and <i>ldt<sub>ca2</sub></i>	This work
R20291 $\Delta$ ldt2 $\Delta$ ldt1 $\Delta$ ldt3	R20291 derivative with an in-frame deletion in <i>ldt<sub>ca2</sub></i> , <i>ldt<sub>ca1</sub></i> and <i>ldt<sub>ca3</sub></i>	This work
R20291 $\Delta$ Paloc	R20291 derivative with a deletion in the genes encoding toxins	(2)
<i>Escherichia coli</i>		
NEB5alpha	Cloning strain	NEB
BL21(DE3)	Expression strain	NEB
CA434	HB101 derivative carrying R702	(3)
<b>Plasmids</b>		
pJAK112	Plasmid for gene replacement in <i>C. difficile</i>	(4)
pNG007	pJAK derivative containing homology regions flanking a 1332bp <i>ldt<sub>ca1</sub></i>	This work
pNG008	pJAK derivative containing homology regions flanking a 1695bp <i>ldt<sub>ca2</sub></i>	This work
pNG009	pJAK derivative containing homology regions flanking a 753bp <i>ldt<sub>ca3</sub></i>	This work
pET2818	pET derivative for expression of His-tagged recombinant proteins	(5)
pET-Ldt1	pET2818 derivative expressing Ldt <sub>ca1</sub> (residues 3-289; C-terminal His-tag)	This work
pET-Ldt2	pET2818 derivative expressing Ldt <sub>ca1</sub> (residues 41-164; N-terminal His-tag)	This work
pET-Ldt3	pET2818 derivative expressing Ldt <sub>ca1</sub> (residues 38-469; N-terminal His-tag)	This work
<b>Oligonucleotides</b>		
RF_21 (pJAK112)	GGATTTTCACATTTGCCGTTTTGTAAAC	
RF_22 (pJAK112)	GATCTTTTCTACGGGGTCTGAC	
RF1795 ( <i>ldt<sub>ca1</sub></i> locus)	GCTTTTACTTTGATACTGTCTGCTG	
RF1796 ( <i>ldt<sub>ca1</sub></i> locus)	ATGACAAACTTAAGGAAAGATGGCC	
RF1797 ( <i>ldt<sub>ca2</sub></i> locus)	GGATTCAGTTCCTGAATAACTAGGT	
RF1798 ( <i>ldt<sub>ca2</sub></i> locus)	GCAGTTGGTGAATCAGTAGAAAAAC	
RF1799 ( <i>ldt<sub>ca3</sub></i> locus)	GGAGGAGATATTAGAGACTATGAAG	
RF1800 ( <i>ldt<sub>ca3</sub></i> locus)	CGTGTTTATGCACATCCAACATATG	

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