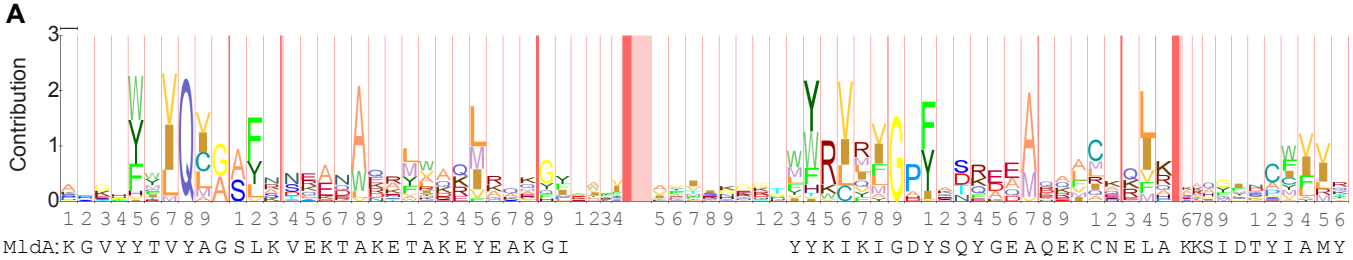
A



B

MldA

MKVLSENNII RNTVLNVTAS FLKQESKINE KLDGVLEKKF EKVEFNEAKY AELLKFNILF YKTLARNTEP
LIGKWIVDKY IPEIDELEKD LELTTAKCRK YVNKAMKEGL DSLKANDLNS FLAYDKMELS ERRRRLEKDY
KVLNLYKDLL NISLRKISLE KKECGDLFLK NQADAKRELK REIIFCVNKI LASNKDVVPN NVEENNEQDK
VEIKATEIED LQLGKDNSEN DMLLNEIKNS KVKHESKSND IESKFIDKLN SLNNESVSQT IHNEYKKLCG
LEELHSVEGY GFGKEIIKDF ACATVVLEFL KRRNRDLIEG AMRLTIIGEF GPENFKEFVD YVIKNKTEIS
EDTWNKAQNL IKDNYSELEN HEIASKRTRR NKDIDIEEYI YMIKNADKDI CFRSSISIED DAEEGVKEEI
DSNNQDIGDV VEDKDTTDKE YDSNKEDIIE PENKKSKKKA KLFGFIKKDN EEVEQEEENL NDISPDIILD
KPVENNQVKS EEIEQNELKE IKQEEPSQHI EEERSVKIEK PINNNLDEKV SSNNESKLEK ESKNLEDKKA
KEIKEEKLEN EKSVVIPIKK KENSNKKSKN SSKDKYRENK KEMKNYVSDK EDSLDDEEVV SKKSRLKETI
IAVVIVVIVG VGYFITVGNN KKNDKENIPK SSTQQQANNK LTEEEKKAQA EKEKKEAEEK AKAEKEAQEK
AQAEKKAKEM EAYKDGKGVY YTVYAGSLKV EKTAKETAKE YEAKGISSTI IQENGYYKIK IGDYSQYGEA
QEKCNELAKK SIDTYIAMYD KYYDYKLEEL KESAPSLSAE ELKQKYEDLR SELKNKSGYR EYVKHLDKLY
EEIVEGA

MldB

MLKLGEKIIY ELSDGFELSG ISRYSKEYKK LHITNLGNII ISKSDEVNED TGVKYIYSFS DNKEKLEAVL
EDSQIIVYGN NLPFYIKSEN SSKNVTANLT MKITLDEYDL ICKSQREFIF YLKDNMTILS LDDNKFYMGG
INKDNEKFIF ISGKNRFEIN FDDIERYILE DKRVSLKGYF HMEREGIIVR SVSIFNNNID SVVPSDLNER
VKDNQKIGNL PKDCEIVFCK ISGNIDGFDY KNTNMLLVKY QDQLIFINKK SKKTIVKSAK DNCSKLNLGE
DIILYDNKNV FNLHINDKNR EIMQIDDLKD IENEIVGYTL KHAPFFIQED FDSLTILKSF QKEIISIKNS
DIKDIVINKE FENENSNFVE TEIKFNNQKV LLNLSKSMVQ KLMQDVFIYA KQPLLKENSI EVIYKNWSKA
MNDMIIFNFF GNIYYMKSEF DKILEKELND EIRIEVINSL YKQIQEQRNN LDLLSAYMPR ILENQEIDLF
EKYNTKLDVQ VFKQIKNLLS DLSYNISSYL NEVEKSLDNI IFVISGEDKK KYNYRMLKES ESASLDVFLK
QAISRLNHLV ENMYPYYVDE TSREMFKLFE LLWKNYRNID DDSIKEILFE RITNTYVFKQ LTLNNSTKER
RKDIIEKIYN SVDYGTNKLD ENMFFTGGIK YVK

MldC

MSSKYNVWTF NYEFLGLDSG DEKNNEVYYE IDLDEILNEN YIDEDIDSGL DSDGNSILGE LISEMDIDED
VSVDMTYQEL EVDLEDLDSY LDEDIDSDIK GILDEI

FIG. S1. Sequences of Mld proteins. (A) The putative SPOR domain from MldA (residues 717-789) was aligned by hand to the Pfam HMM logo for SPOR domains (1). The alignment revealed matches to one of the top three amino acids at 28 out of 76 positions, consistent with previous evidence that SPOR domain sequences are highly degenerate (2). (B) The predicted amino acid sequences of MldA, MldB and MldC. Positively charged residues are in blue, negatively charged residues are in red. For MldA, the predicted coiled-coil regions are underlined in grey, the transmembrane helix is highlighted in grey and the SPOR domain is underlined in black. Predicted coiled-coil regions in MldB are underlined in grey (3).

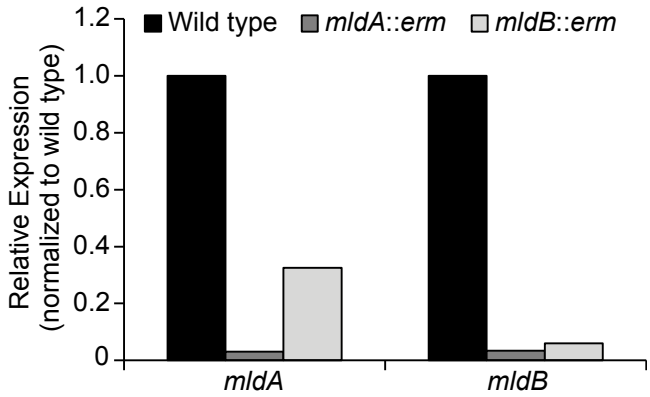


FIG. S2. Expression of *mldA* is reduced in an *mldB*::*erm* mutant. RNA was harvested from wild-type and mutant *C. difficile* strains in logarithmic growth, and qRT-PCR was used to assess mRNA levels for *mldA* and *mldB*. The housekeeping gene *rpoB* was used for normalization. Oligonucleotide primers are listed in Table S1.

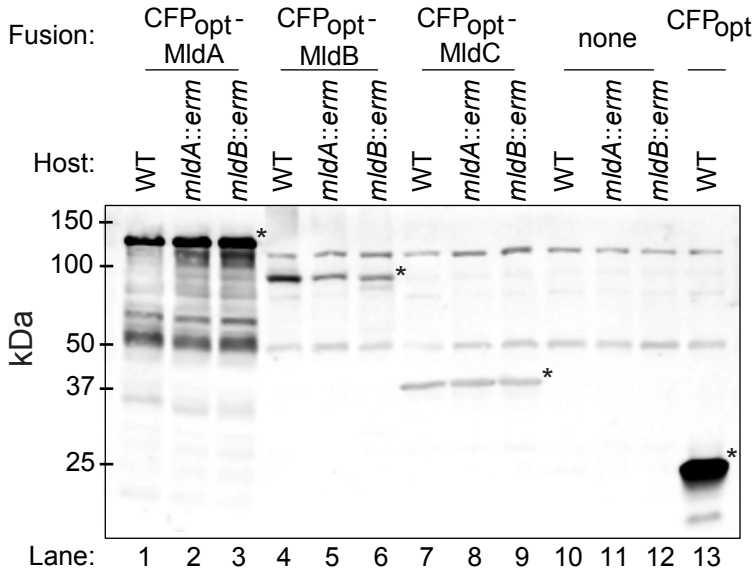


FIG. S3. Expression of CFP_{opt}-Mld fusion proteins in wild-type and *mld* mutant backgrounds. Whole cell extracts were prepared from the same cultures used for localization (Fig. 4). Steady-state levels of CFP_{opt} fusion proteins were determined by Western blotting with polyclonal anti-GFP antibodies that recognize CFP_{opt}. Note that the same amount of tetracycline (500 ng/ml) was used for induction in all cases, so the differences in fusion protein abundance may reflect differential stability. The Western results are consistent with the relative brightness of the CFP fusions in Figure 4. Molecular mass standards are indicated to the left of the blot. Asterisks denote the various CFP_{opt} fusion proteins, the predicted masses of which are CFP_{opt}-MldA = 126 kDa, CFP_{opt}-MldB = 106 kDa, CFP_{opt}-MldC = 39.5 kDa, CFP_{opt} = 27.7 kDa.

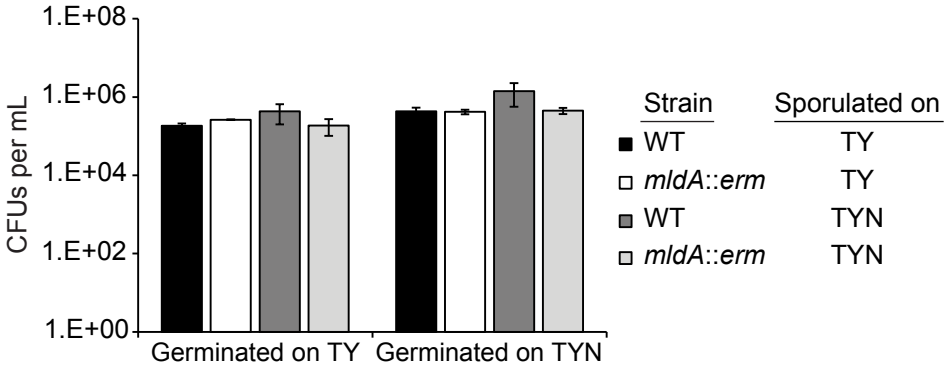


FIG. S4. Sporulation and germination. Wild type and mutant *C. difficile* strains were allowed to sporulate on TY or TYN plates. Spores were harvested using equal volumes of PBS and then heat-killed at 65°C for 10 min. Serial dilutions were spread onto either TY or TYN containing 0.1% taurocholate to stimulate germination of spores. No colonies were detected on plates that lacked taurocholate. Samples were normalized to volumes of PBS used to process each plate.

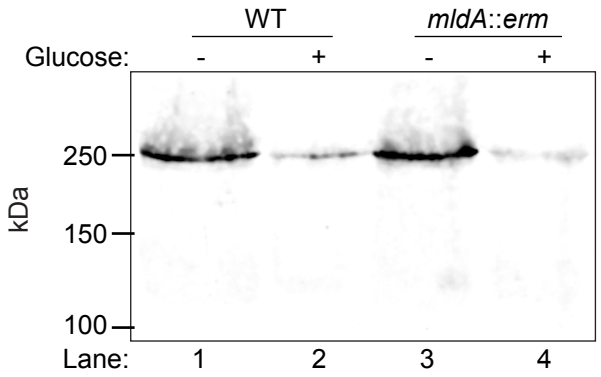


FIG. S5. Toxin A production. Cell-free spent medium was concentrated approximately 100-fold and analyzed by Western blotting with a monoclonal antibody against toxin A (predicted mass 306 kDa). Molecular mass standards are indicated to the left of the blot. Glucose in the growth medium is known to repress toxin A production and was included in some cultures to verify antibody specificity (4). Samples were normalized to volume of spent medium.

Table S1. Oligonucleotide primers used in this study.

Oligo	Purpose	Sequence 5`-3`*
CDE914	EBS universal primer	CGAAATTAGAAACTTGCGTTCAGTAAAC
NF1438	Reverse primer for sequencing inserts in pRPF185	GATCCAGCACACTGGCATCTTTTTATTTAGGGATTTCTCAC
P1447	IBS for constructing *mldA*$_{248}$::*erm*	AAAAAAGCTTATAATTATCCTTAGAAAAAGATTTAGTGCGCCCAGATAGGGTG
P1448	EBS1 for constructing *mldA*$_{248}$::*erm*	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGATTTAGATAACTTACCTTTCTTTGT
P1449	EBS2 for constructing *mldA*$_{248}$::*erm*	TGAACGCAAGTTTCTAATTTCGGTTTTTTCTCGATAGAGGAAAGTGTCT
RP1	Verifying insertion in *mldA* gene	GAAGCAAAATATGCAGAGC
RP2	Verifying insertion in *mldA* gene	GCGTCTTCTTCTCTCTGATAGTTCC
RP26	Intergenic PCR between *cd2718-mldA*	TAGGTCGCTTGAGCCTATTGGTGA
RP27	Intergenic PCR between *cd2718-mldA*	CCATTTGCCAATAAGTGGTTCTGT
RP28	Intergenic PCR between *mldA-mldB* Verifying insertion in *mldB* gene	GAATCAGCTCCTTCTTTGAGTGC
RP29	Intergenic PCR between *mldA-mldB* Verifying insertion in *mldB* gene	AAAGGCAAGTTATTTCCATAGAC
RP30	Intergenic PCR between *mldB-mldC*	GAAAGTGAAAGTGCAAGTCTTGATG
RP31	Intergenic PCR between *mldB-mldC*	ACACTGACATCCTCATCTATATCC
RP56	Intergenic PCR between *mldC-gtaB*	GAGTTCTTAGGATTGGACTCAGGG
RP33	Intergenic PCR between *mldC-gtaB*	GCACAGTAAATTGCATGTCCAAGAC
RP34	qRT-PCR for *mldA*	ACAGAACCACTTATTGGCAAATGG
RP35	qRT-PCR for *mldA*	GAGAGTCTAATCCCTCTTTCATAGCC
RP36	qRT-PCR for *mldB*	CAGATGGGTTTGAGCTTAGTGGCA
RP37	qRT-PCR for *mldB*	ACACCTGTATCCTCATTAACTTCATCTG
RP200	qRT-PCR for *mldC*	GAGTTCTTAGGATTGGACTCAGG
RP201	qRT-PCR for *mldC*	TCCATCAGAATCTAAACCACTATCT
RP58	qRT-PCR for *gtaB*	CCTGCAATTGAAGAAGCTCCATCTG
RP59	qRT-PCR for *gtaB*	TCTCCACCTTTACCAGGTGTCTGT
TEQ009	qRT-PCR for *rpoB*	AAGAGCTGGATTCGAAGTGCGTGA
TEQ010	qRT-PCR for *rpoB*	ACCGATATTTGGTCCCTCTGGAGT
RP47	Sequencing *mldA*	GTAGTATTTCAATAGAAGATGATGCTGAAGAAGGGG
RP48	Sequencing *mldA*	CTTCTTTATTGCTGTCGTACTCTTTATCTGTAG
RP80	Sequencing *mldB*	CGATAGCTTGACTATTCTAAAGTC
RP81	Sequencing *mldB*	CATCTTGCATAAGTTTTTGAACCATAC
RP114	IBS for constructing *mldB*$_{153}$::*erm*	AAAAAAGCTTATAATTATCCTTAAATGACGATACAGTGCGCCCAGATAGGGTG
RP115	EBS1 for constructing *mldB*$_{153}$::*erm*	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGATACAGGTAACTTACCTTTCTTTGT
RP116	EBS2 for constructing *mldB*$_{153}$::*erm*	TGAACGCAAGTTTCTAATTTCGGTTTCATTCCGATAGAGGAAAGTGTCT
RP160	Cloning CFP$_{opt}$ into pRPF185	CCCGGATCCTTACTTATATAATTCATCCATTCC
RP161	Cloning CFP$_{opt}$ into pRPF185	GGGGAGCTCCTGCAGTAAAGGAGAAAATTTTATGGTTTCAAAAGGAGAAGAATTATTTAC
RP164	Cloning *mldABC, mldAB and mldA* into pRPF185	GGGGAGCTCCTGCAGTAAAGGAGAAAATTTTAAGGTTTTGAGTGAAAATAATATAATAAG
RP165	Cloning *mldABC* and *mldC* into pRPF185	CCCGGATCCAATTTCATCTAAGATGCCTTTTATATC
RP166	Forward primer for sequencing inserts in pRPF185	CATTGATAGAGTTATTTGTCAAACTAG

RP171	Cloning *cfp*$_{opt}$ – MCS[†] into pRPF185	GGCGGATCCGGCGCGCCTCAGCTGTTTAATTAAGTCGACGCATGCGTTCATCTTATATAATTCATCCATTCC
RP178	Cloning *cfp*$_{opt}$–*mldA* fusion	AAAGCATGCGTGAAGGTTTTGAGTGAAAATAATATAATAAGAAACACTG
RP179	Cloning *cfp*$_{opt}$–*mldA* fusion	TTTGGCGCGCCCTAAGCACCTTCAACTATTTCTTCATAAAGTTTATC
RP184	Cloning *mldB* into pRPF185	GGGGAGCTCCTGCAGTAAAGGAGAAAATTTTATGTTGAAATTAGGAGAAAAAATCATATA
RP185	Cloning *mldC* into pRPF185	GGGGAGCTCCTGCAGTAAAGGAGAAAATTTTATGTCAAGTAAATACAATGTTTGGACTTT
RP186	Cloning *mldA* into pRPF185	CCCGGATCCCTAAGCACCTTCAACTATTTCTTCATAAAG
RP187	Cloning *mldB* and *mldAB* into pRPF185	CCCGGATCCTTACTTGACATACTTTATTCCTCCTGTG
RP196	Cloning *cfp*$_{opt}$–*mldB* fusion	AAAGCATGCATGTTGAAATTAGGAGAAAAAATCATATATGAGC
RP197	Cloning *cfp*$_{opt}$–*mldB* fusion	TTTGGCGCGCCTTACTTGACATACTTTATTCCTCCTGTG
RP198	Cloning *cfp*$_{opt}$–*mldC* fusion	AAAGCATGCATGTCAAGTAAATACAATGTTTGGACTTT
RP199	Cloning *cfp*$_{opt}$–*mldC* fusion	TTTGGCGCGCCCTAAATTTCATCTAAGATGCCTTTTATATC

* Restriction sites underlined.

[†] MCS, multicloning site.

Table S2. Sequence identities and E-values of MldABC homologs*

Organism	MldA	MldB	MldC
Clostridium difficile CD196	YP_003215577 (2559) 847 a.a. E-value 0.0	YP_003215576 (2558) 663 a.a. E-value 0.0	YP_003215575 (2557) 106 a.a. E-value 2e-65
Clostridium difficile Bl1	YP_006199787 (13235) 847 a.a. E-value 0.0	YP_006199786 (13230) 663 a.a. E-value 0.0	YP_006199785 (13225) 106 a.a. E-value 2e-65
Clostridium difficile R20291	YP_003219085 (2606) 847 a.a. E-value 0.0	YP_003219084 (2605) 663 a.a. E-value 0.0	YP_003219083 (2604) 106 a.a. E-value 2e-65
Clostridium difficile NAP07	EFH14798 (2596) 844 a.a. E-value 0.0	EFH14797 (2595) 663 a.a. E-value 0.0	EFH14796 (2594) 106 a.a. E-value 5e-63
Clostridium hiranonis	EEA85283 839 a.a. E-value 7e-113	EEA85284 665 a.a. E-value 0.0	EEA85285 104 a.a. E-value 1e-6
Peptostreptococcus anaerobius CAG:653-L	EFD05099 985 a.a. E-value 2e-38	EFD05083 660 a.a. E-value 3e-134	HMPREF0631_1876 58 a.a. E-value 4e-2
Peptostreptococcus stomatis DSM 17678	WP_007789343 947 a.a. E-value 7e-38	Unannotated ORF 575 a.a. E-value 2e-108	Unannotated ORF 58 a.a. E-value 2.2e-2

*Determined using BLAST searches (5)

Table S3. Complementation of *mld* mutants.

Strain		Cells	Cell	Septa per Cell					
host	plasmid	Scored	Length*	0	1	2	3	4-5	≥6
Wild type	vector	451	8.7 ± 2.9	36.8%	59.9%	3.1%	0.2%	0%	0%
mldA::*erm*	vector	315	11.7 ± 6.0	13.3%	30.5%	21.9%	14.3%	14.3%	5.7%
mldA::*erm*	*mldABC*	323	10.9 ± 4.0	20.7%	60.7%	14.9%	2.8%	0.9%	0%
mldA::*erm*	*mldAB*	328	13.1 ± 5.6	11.3%	48.8%	20.7%	14.6%	3.7%	0.9%
mldA::*erm*	*mldA*	217	11.6 ± 5.6	11.1%	38.2%	20.7%	15.2%	11.1%	3.7%
mldA::*erm*	*mldBC*	212	11.0 ± 5.6	12.3%	37.3%	17.5%	17.5%	10.4%	5.2%
mldA::*erm*	*mldB*	201	10.8 ± 4.9	12.9%	37.8%	15.4%	13.9%	15.9%	4.0%
mldA::*erm*	*mldC*	219	11.2 ± 5.1	9.1%	39.7%	20.1%	18.3%	9.1%	3.7%
mldB::*erm*	vector	211	13.9 ± 7.0	8.1%	38.4%	14.7%	11.4%	14.7%	12.8%
mldB::*erm*	*mldABC*	207	11.4 ± 5.0	41.1%	53.1%	4.3%	1.4%	0%	0%
mldB::*erm*	*mldAB*	229	14.6 ± 10.4	17.5%	55.0%	14.0%	11.8%	1.7%	0%
mldB::*erm*	*mldA*	109	15.5 ± 9.8	8.3%	31.2%	16.5%	13.8%	20.2%	10.1%
mldB::*erm*	*mldBC*	105	11.3 ± 6.4	12.4%	34.3%	13.3%	21.9%	12.4%	5.7%
mldB::*erm*	*mldB*	107	11.5 ± 8.5	14.0%	27.1%	12.1%	19.6%	15.0%	12.1%
mldB::*erm*	*mldC*	106	11.5 ± 6.6	9.4%	23.6%	16.0%	15.1%	19.8%	16.0%

* Mean ± standard deviation, in μm.

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