SUPPLEMENTAL MATERIALS

Material and Methods

Cloning of *slpA* alleles and construction of revertants.

The *slpA* genes from a panel of *C. difficile* strains with distinct SLCTs were amplified using the primers listed in Table S2 and cloned between *Bam*HI and *Sac*I sites in the inducible expression plasmid pRPF185 (*40*). Plasmids were transferred into *C. difficile* strain FM2.5 by conjugation from the *E. coli* donor CA434. For construction of *slpA* revertants, a watermark consisting of two adjacent synonymous mutations, was introduced into the SLCT-4 *slpA* using primers RF135 and RF136. Single crossover recombinant colonies were identified by an enhanced growth phenotype, and recombination was confirmed by PCR using primers RF33 and NF1323. Stable double crossover revertants were isolated following growth without selection for approximately 20 generations and confirmed by screening for plasmid loss, sequencing of the *slpA* gene, and confirmation of SLP expression by SDS-PAGE analysis (described below).

Construction and expression of Avidocin-CDs.

New Avidocin-CDs were constructed with bacteriophage RBPs identified from newly isolated bacteriophages and prophage insertions. The RBP genes along with the adjacent 3'end of the Baseplate attachment region (Bpar) and chaperone genes were cloned and inserted into an integration vector containing the Diffocin-4 gene cluster via a combination of restriction enzyme digests and Gibson assembly. The fusion sites used to replace the bacteriocin genes and integrate the bacteriophage derived genes are shown (Fig. S9). Avidocin constructs using the downstream fusion site 1 are denoted with a ".1" suffix (i.e. Av-CD681.1), while constructs using the downstream fusion site 2 are denoted with a ".2" (i.e. Av-CD027.2). The resulting integration vectors were sequence verified and transformed into the *B. subtilis* production strain as previously described (*12*). The nucleotide sequences for the Bpar fusion, RBP and downstream chaperones have been deposited in Genbank along with source information. Accession numbers are provided in Table S3. Expression of each Avidocin-CD was induced with IPTG as previously

described (*12*). Preparation of each Avidocin-CD and Diffocin-4 relied on a combination of Polyethlene-glycol-8000 precipitations and ultracentrifugation as described previously (*12*).

Isolation of Av-CD291.2-resistant clones.

A greater than 100-fold excess of active Av-CD291.2 was added to exponentially growing *C. difficile* strain R20291. After 75 min bacteria were harvested, resuspended in phosphate buffered saline (PBS), and spread on non-selective Brucella agar plates maintained under anaerobic conditions at 37°C overnight. CFU counts before and after addition of Av-CD291.2 were compared to determine resistance frequency. Resistant colonies were streaked on *C. difficile*-selective agar and Av-CD291.2-resistance was confirmed. Av-CD291.2-resistant strains FM2.5 and FM2.6 was sequenced by Illumina Miseq and identified *slpA* mutations were confirmed by Sanger sequencing.

Quantitative analysis of sporulation and germination.

Overnight bacterial cultures were diluted to 0.01 OD_{600nm} in BHI-S broth, incubated for 8 hours, diluted again to 0.0001 OD_{600nm} and grown overnight. The resulting stationary phase cultures (T=0) were then incubated for 5 days. The relative proportion of spores and vegetative cells was determined at 24 h intervals by counting total and heat-resistant (65°C for 30 min) CFUs on BHI-S agar supplemented with 0.1 % taurocholate. Assays were repeated in triplicate with biological triplicates. In order to test a range of different stress conditions, additional samples were taken on day 5 and treated at either 75°C for 30 min, 50% ethanol for 30 min or incubated aerobically for 18 h before enumerating viable spores as before. For the germination studies, spores were purified as previously described (*19*) and Ca^{2+}-DPA release and core rehydration was monitored by measuring changes in OD_{600nm} upon addition of 0.5% taurocholate.

For microscopic examination, bacterial samples were washed with PBS and fixed in 3.2% paraformaldehyde. For phase contrast microscopy, samples were mounted in 80% glycerol and visualised using a Nikon Ti Eclipse inverted microscope. For TEM, samples were additionally fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer followed by 1% OsO_4, then dehydrated in

ethanol and embedded in araldite resin. Embedded samples were sectioned at 85 nm on a Leica UC6 ultramicrotome, transferred onto coated copper grids, stained with uranyl acetate and lead citrate and visualized using a FEI Tecnai BioTWIN TEM at 80 kV fitted with a Gatan MS600CW camera.

Animal experiments.

All procedures were performed in strict accordance with the Animals (Scientific Procedures) Act 1986 with specific approval granted by the Home Office, U.K. (PPL60/4218) for the experiments outlined. Briefly, 3 weeks before bacterial challenge, telemetry chips were surgically inserted by laparotomy into the peritoneal cavity of female Golden Syrian hamsters. Animals were allowed to recover from surgery before being placed in cages above transponder receiver plates that allow direct monitoring of body temperature. All animals were orally dosed with clindamycin phosphate (30 mg/kg) five days prior to challenge with 10^4 spores of either *C. difficile* R20291, FM2.5 or FM2.5RW as previously described (*39*). Six animals per group were inoculated and animals monitored carefully for onset of symptoms. Animals were culled when core body temperature dropped below 35°C.

To quantify colonization at experimental endpoint, the caecum (CAE) and colon (COL) of each animal were removed, opened longitudinally and washed thoroughly in PBS to remove unbound bacteria; these organisms were designated luminal associated organisms (LA). The washed tissue was then homogenized and subsequently recovered material contained more intimately associated organisms; designated tissue associated (TA). Total viable counts were determined by plating serial dilutions on ChromID (Biomeuriex). Spores were enumerated following heat-treatment at 56°C for 20 min. Colonies from all plates were subjected to PCR amplification of both the *slpA* gene specifically, using primers RF110 and RF111, and seven repeat regions to confirm strain identity by MVLA analysis 2 to confirm strain identity.

Quantification of toxin expression.

Quantification of toxin activity was performed using a Vero cell based cytotoxicity assay. Dilutions of filtered (0.45 μm) post-challenge gut contents were added to confluent monolayers for 24h and the end-point titer was defined as the first dilution at which Vero cell morphology was indistinguishable from untreated wells. Each experiment was performed in duplicate on two separate occasions. For measurements of toxin production *in vitro*, whole cell lysates or culture supernatants were separated by SDS-PAGE, transferred to PVDF membrane and toxin B detected by Western immunoblot using a specific mouse monoclonal antibody (MA1-7413, Thermo Fisher).

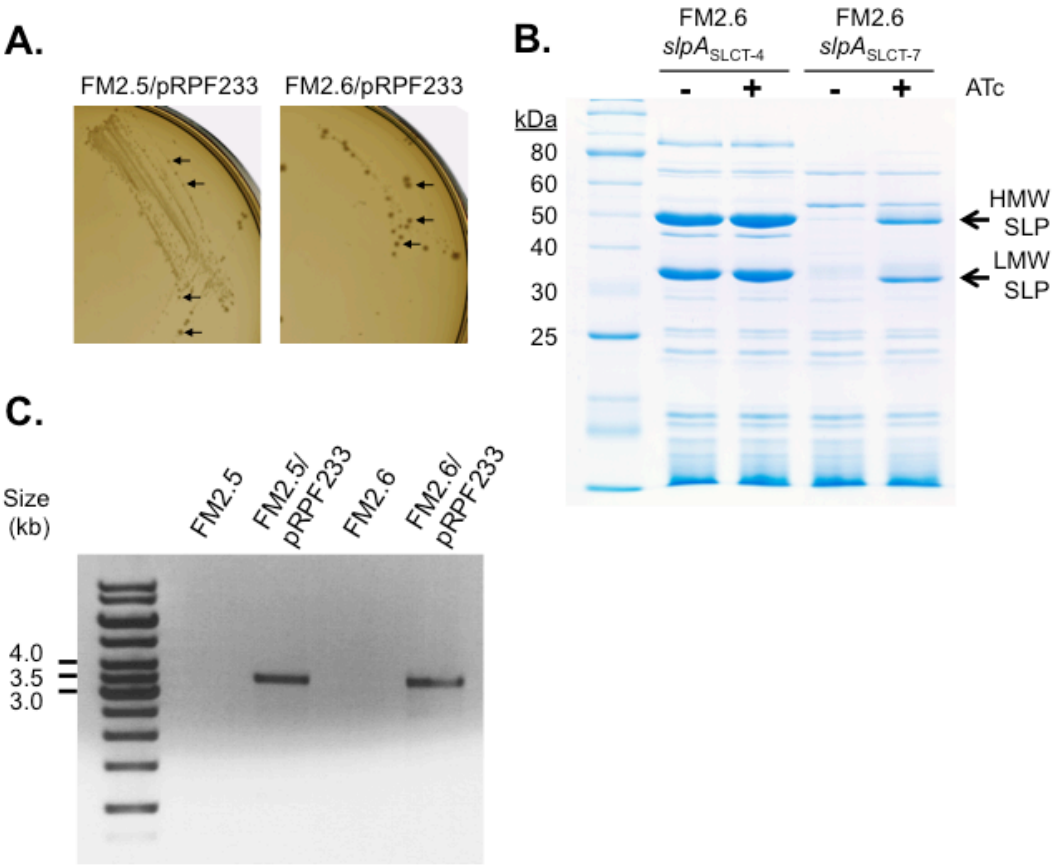

Fig. S1. Restoration of wild type *slpA* to the chromosome of FM2.5 and FM2.6. Plasmid pRPF233, carrying a wild type copy of the R20291 *slpA* gene under the control of a tetracycline-inducible promoter, was introduced into FM2.5 and FM2.6 by conjugation from an *E. coli* donor strain CA434. **(A)** When grown on solid media, supplemented with thiamphenicol to select for the plasmid, both FM2.5/pRPF233 and FM2.6/pRPF233 produced two distinct colony morphologies; small colonies that were indistinguishable from those typical of FM2.5 and FM2.6 and larger colonies that appeared to grow significantly faster than the *slpA* mutants. **(B)** When the S-layer profile of the larger colonies was analyzed by SDS-PAGE constitutive SlpA expression, independent of anhydrotetracycline (ATc) induction, was observed. However, when the *slpA* gene from strain 630 (SLCT-7), which shares 69.4% nucleotide identity with $slpA_{R20291}$ (SLCT-4), was introduced expression of SlpA remained inducible. **(C)** Analysis of these large colonies by PCR using one chromosome-specific primer (RF33, annealing 1,250 bp downstream of *slpA* in the *secA2* gene) and one plasmid-specific primer (NF1323, annealing in the P_{tet} promoter 122 bp upstream of *slpA* on pRPF233) confirmed that pRPF233 had integrated into the

chromosome at the *slpA* locus by a single crossover recombination event. Stable *slpA* revertants, FM2.5RW and FM2.6RW, were later generated from these single crossover mutants by culturing the bacteria without selection and then screening for plasmid loss and retention of the fast growth colony phenotype.

	Strain Designation	Source	Ribotype	SlpA-type	CD4 (12) RTB I Diffocin-4	R20291 (4) PI II-B Av-CD291.2	panel (4) phage II-B Av-CD027.2	M68 (7) PI II-A Av-CD681.1	M68 (7) PI II-B Av-CD682.1	M68 (7) PI/ Phage II-A Av-CD684.1	M68 (7) PI II-A Av-CD685.1	CD305 (11) PI II-A Av-CD305.1	19123 (6) phage III Av-CD123.2	CD62 (12) phage II-A Av-CD242.2	19147 (1) phage II-A Av-CD147.1	CD630 (7) PI II-A Av-CD630.1
1	Ox1424 (ST12)	Dingle	003	1		-	-	-		-	-	-	-	+	+	+
2	19108	Citron	053	1	+	-	-	-		-	-	-		+	+	+
3	19147	Citron	053	1	+	-	-	-		-	-			+	+	+
4	19110	Citron	053	1	+	-	-	-		-	-		-	+	+	+
5	19098	Citron	057	1	-	-	-	-	-	-	-		-	+	+	+
			Totals (Killed/Tested)		3/4	0/5	0/5	0/5	0/1	0/5	0/5	0/2	0/3	5/5	5/5	5/5
6	Ox858 (ST16)	Dingle	029	2		-	+	-		-	-	-	-	-	-	
7	Ox2404 (ST16)	Dingle	029	2		-	+	-		-	-	-	-	-	-	
8	19117	Citron	103	2	-	-	-	-	-	-	+		-	-	-	-
9	CD843	Riley	103	2		-	-	-			+		-	-	-	-
			Totals (Killed/Tested)		0/1	0/4	2/4	0/4	0/4	0/3	2/4	0/2	0/4	0/4	0/4	0/2
10	Ox1121 (ST35)	Dingle	284	3		-	-	-		-	-	-	-	-	-	
			Totals (Killed/Tested)			0/1	0/1	0/1		0/1	0/1	0/1	0/1	0/1	0/1	
11	19103	Citron	001	4	+	+	+	-		-	-		-	-	-	
12	19135	Citron	001	4	+	+	+	-	+	-	-	-				
13	LIV24	Lawley	001	4	+	+	+	-	+	-	-					
14	BI-9	Lawley	001	4	+	+	+	-	+	-	-	-				
15	19137	Citron	015	4	+	+	+	-	+	-	-		-	-	-	
16	19129	Citron	027	4	+	+	+	-		-	-		-	-	-	
17	19139	Citron	027	4	+	+	+	-		-	-		-	-	-	
18	19126	Citron	027	4	-	+	+	-	+	-	-		-	-	-	
19	20068	Citron	027	4	-	+	+	-	+	-	-		-	-	-	
20	20315	Citron	027	4	-	+	+									
21	CD196	Lawley	027	4	-	+	+									
22	R20291	Lawley	027	4	-	+	+	-	+	-	-	-	-	-	-	-
23	Ox160 (ST1)	Dingle	027	4		+	+	-		-	-	-	-	-	-	
24	ATCC43255	ATCC	087	4	+	+	+	-	+	-	-					
			Totals (Killed/Tested)		8/13	14/14	14/14	0/12	8/8	0/12	0/12	0/4	0/8	0/8	0/8	0/1
25	Ox1437a (ST7)	Dingle	026	5		-	-	-		-	-	-	-	-	-	
			Totals (Killed/Tested)			0/1	0/1	0/1		0/1	0/1	0/1	0/1	0/1	0/1	

Strain Source (SLCT)

RBP type

Construct Designation

26	19123	Citron	014	6	-	-	+	+	+	-	-	-	-	-	-	
27	Ox1896 (ST17)	Dingle	018	6		-	+	+		-	-	-	-	-	-	
28	CD4	Fortier	024	6	w	-	+	+	+	-	-	-	-	-	-	
29	M120	Lawley	078	6H	-	-	+	+	-	-	-	-	-	-	-	
30	8119	Cepheid	078	6H			+	+								
31	8401	Cepheid	078	6H			+	+								
32	8905	Cepheid	078	6H			+	+								
33	10399	Cepheid	078	6H			+	+								
34	10411	Cepheid	078	6H			+	+								
35	Ox575 (ST11)	Dingle	078	6H		-	+	+		-	-	-	-	-	-	
				Totals (Killed/Tested)	0/2	0/4	10/10	10/10	2/3	0/5	0/5	0/5	0/5	0/5	0/5	
36	CD630	Lawley	012	7	-	-	-	-	-	+	+	-	-	-	-	-
37	M68	Lawley	017	7	+	-		-	-	+	+					
38	CF5	Lawley	017	7	+	-	-	-	-	+	+		-	-	-	
39	19119	Citron	019	7	+	-	-	-	-	+	+		-	-	-	
40	19121	Citron	019	7	+	-		-	-	+	+					
41	19134	Citron	137	7	-	-		-	-	+	+	-				
42	Ox1145 (ST4)	Dingle	137	7		-	-	-		+	+	-	-	-	-	
				Totals (Killed/Tested)	4/6	0/7	0/4	0/7	0/6	7/7	7/7	0/3	0/4	0/4	0/4	0/1
43	Ox1396 (ST54)	Dingle	012	8		+	-	+		+	-	+	+	+	-	
				Totals (Killed/Tested)		1/1	0/1	1/1		1/1	0/1	1/1	1/1	1/1	0/1	
44	19099	Citron	002	9	-	-		+	-	-	-	-				
45	19131	Citron	002	9	-			+	-	-	-					
46	TL178	Lawley	002	9	-	-		+	-	-	-	-				
47	Ox1192c (ST8)	Dingle	002	9		-	-	+		-	-	-	-	-	-	
48	TL174	Lawley	015	9	+			+	-	-	-	-				
49	19145	Citron	153	9	-	-		+	-	-	-		-	-	-	
				Totals (Killed/Tested)	1/5	0/4	0/1	6/6	0/5	0/6	0/6	0/4	0/2	0/2	0/2	
50	TL176	Lawley	014	10	-	-		-	-	-	-	-	+			-
51	19102	Citron	106	10	-			-	-	-	-		+			
52	LIV22	Lawley	106	10	-			-	-	-	-	-	+			
53	Ox1533 (ST13)	Dingle	129	10		-	-	-		-	-	-	+	-	-	
				Totals (Killed/Tested)	0/3	0/2	0/1	0/4	0/3	0/4	0/4	0/3	4/4	0/1	0/1	0/1
54	CD305	Lawley	023	11	-	-				-	-	+				
55	Ox1523 (ST5)	Dingle	023	11		-	-	-		-	-	+	-	-	-	
56	19155	Citron	080	11	-			-	-	-	-	+				
				Totals (Killed/Tested)	0/2	0/2	0/1	0/2	0/1	0/3	0/3	3/3	0/1	0/1	0/1	
57	19146	Citron	010	12	-									+		
58	CD62	Riley	010	12	-	-	-	-	-	-	-	-	-	+	-	-
59	CD242	Riley	010	12		-	-	-	-	-	-	-	-	+	-	
60	Ox1342 (ST15)	Dingle	012	12		-	-	-		-	-	-	-	+	-	
61	F200	Young	12w	12				-	-	-	-			+		
				Totals (Killed/Tested)	0/2	0/3	0/3	0/4	0/3	0/4	0/4	0/3	0/3	5/5	0/3	0/1
62	19142	Citron	046	13	-	+	-	-	-	-	-	-	-	-	-	-
				Totals (Killed/Tested)	0/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1

Figure S2. *C. difficile* strain sensitivity patterns to Avidocin-CDs and Diffocin-4. Preparations of each Avidocin-CD and Diffocin-4 were serially diluted and spotted on bacterial lawns for each strain. If a zone of clearance correlating with killing was detected, the strain was deemed sensitive to the agent tested and is demarked by a "+" in the figure. If no zone of clearance was observed, the strain was deemed insensitive and is demarked with "-" in the figure. Faint zones of clearance are demarked with a "w". Not all strains were tested against each Avidocin-CD preparation. The number of strains killed per the total number of strains assayed is listed for each SLCT. Totals in red indicate less than 100% sensitivity. Boxes highlighted in red indicate testing on RBP strain source. The strain designation, source, ribotype and SLCT are listed. Source designations: Dingle = Kate Dingle, University of Oxford; Riley = Tom Riley, University of Western Australia; Lawley = Trevor Lawley, Wellcome Trust Sanger Institute; Tenover = Fred Tenover, Cepheid; Young = Vincent Young, University of Michigan; R.M.A.R. = R.M. Alden Research Laboratory.

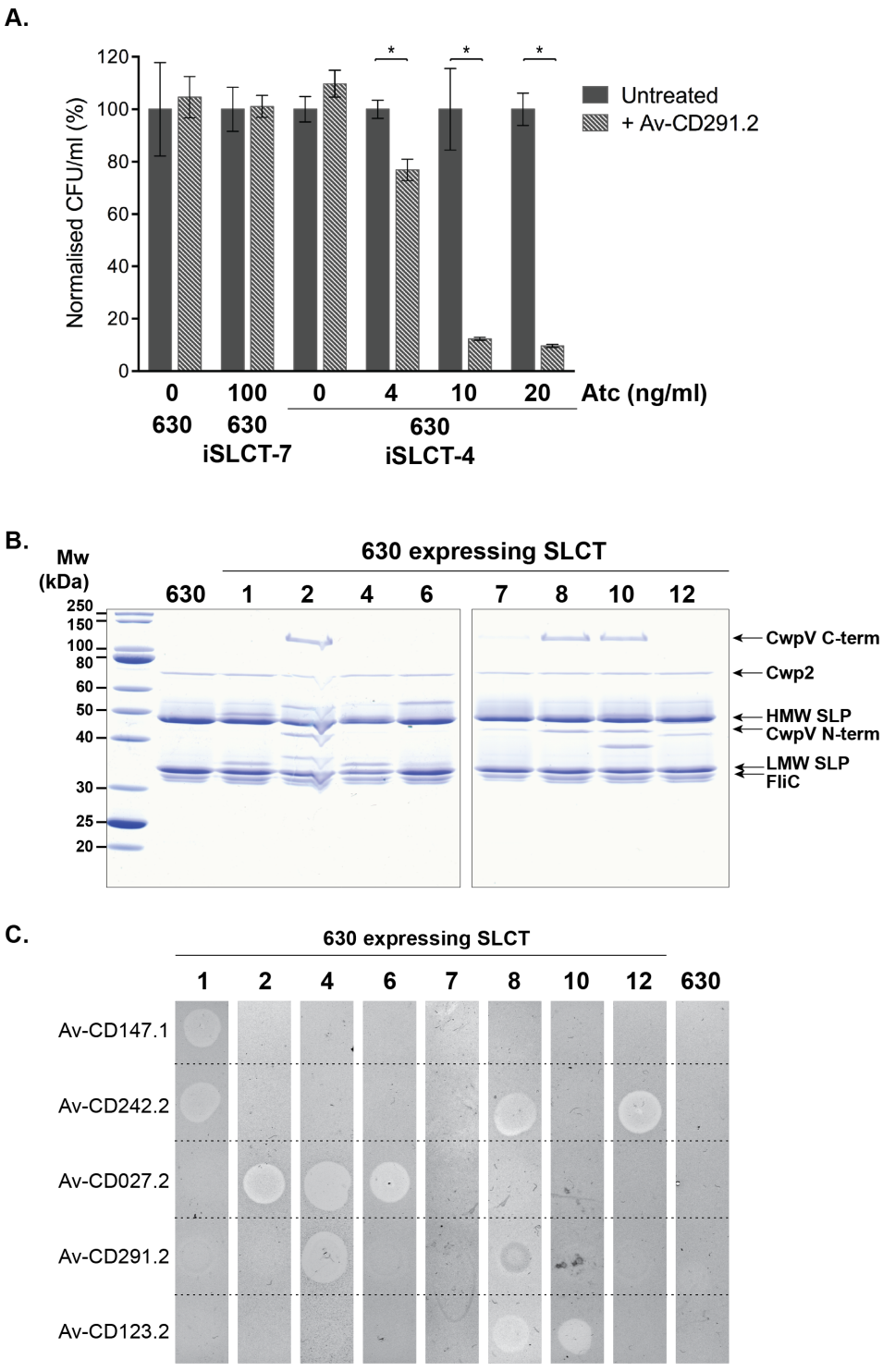

Figure S3. Avidocin-CD sensitivity correlates with SLCT. **(A)** Av-CD291.2 killing efficiency is dependent on the level of SLCT-4 SlpA induction. Strain 630 (SLCT-7) or 630 carrying a plasmid-borne inducible copy of SLCT-7 SlpA or SLCT-4 SlpA were subcultured to an OD_{600nm}

of 0.05, grown for two hours, induced with the indicated concentration of anydrotetracycline (ATc) and then challenged with Av-CD291.2 following two hours of induction. Identical untreated cultures were included as controls. Shown are means and standard deviations of biological duplicates assayed in triplicate. p =<0.01, determined using using two-tailed *t*-tests with Welch's correction. **(B)** SDS-PAGE analysis of S-layer extracts from 630 (SLCT-7) and a panel of eight 630 derivative strains expressing SLCT-1, 2, 4, 6, 7, 8, 10 and 12 under the control of a tetracycline-inducible promoter (*40*). Heterologous SlpA expression was induced with 20 ng/ml anhydrotetracycline. The positions of the LMW and HMW SLPs, the minor cell wall proteins CwpV and Cwp2, and the flagellar subunit FliC are indicated. **(C)** Spot bioassays with 5 Avidocin-CDs on the *C. difficile* strains used in panel B following induction with anhydrotetracycline (20 ng/ml). The zone of clearance caused by each Avidocin-CD is shown along with the SLCT.

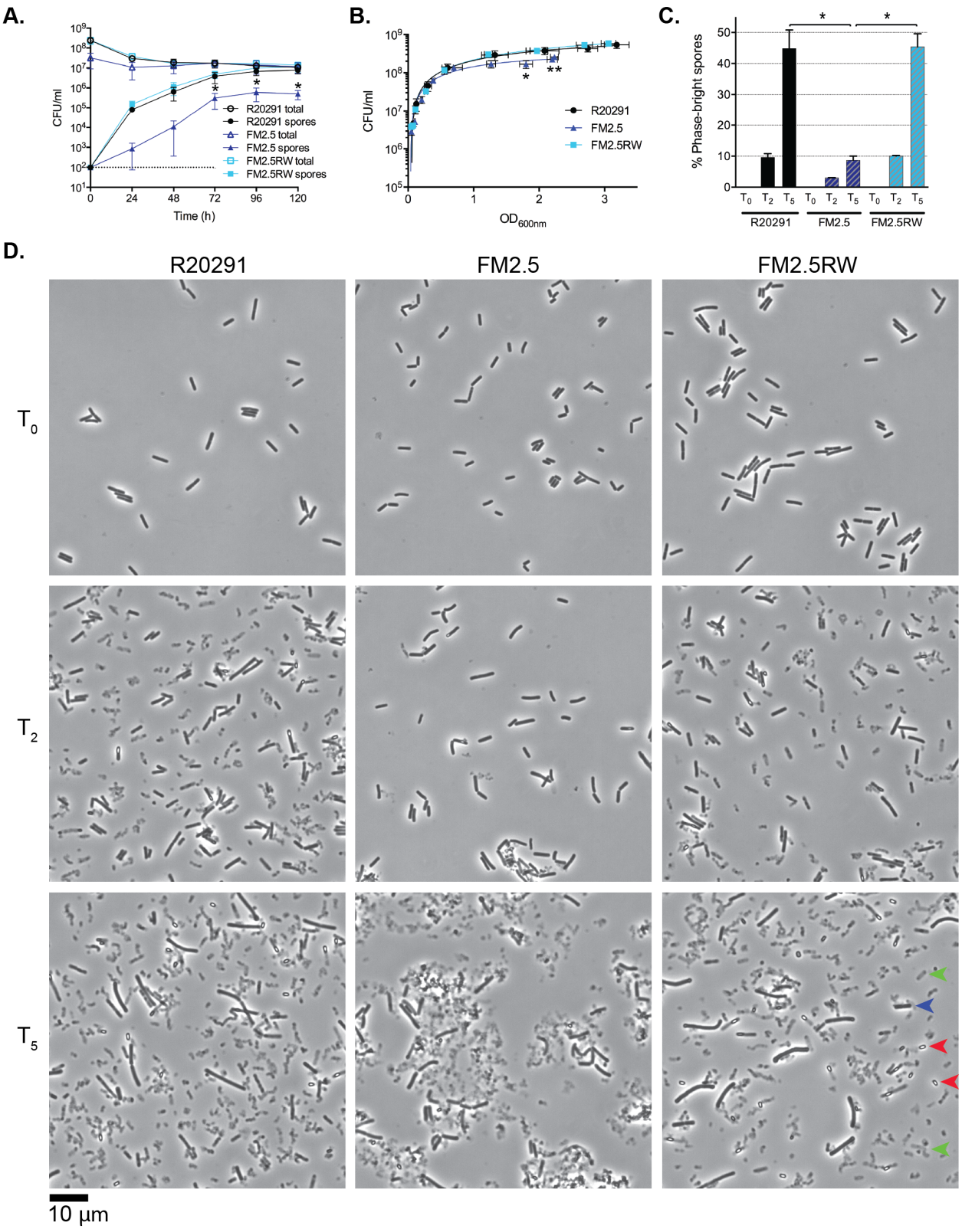
A.
CFU/ml
Time (h)
R20291 total
R20291 spores
FM2.5 total
FM2.5 spores
FM2.5RW total
FM2.5RW spores
B.
CFU/ml
OD600nm
R20291
FM2.5
FM2.5RW
C.
% Phase-bright spores
R20291
FM2.5
FM2.5RW
D.
R20291
FM2.5
FM2.5RW
T0
T2
T5
10 µm

Figure S4. Characterization of growth and sporulation. **(A)** Sporulation of R20291, FM2.5, and FM2.5RW over 5 days. Total CFUs and heat-resistant spore CFUs (65°C for 30 min) were determined at 24 h intervals. Experiments were carried out in duplicate on biological duplicates. Mean and standard deviation are shown. The dotted line indicates the limit of detection. **(B)** Overnight cultures of strains R20291, FM2.5, and FM2.5RW were subcultured to an OD_{600nm} of 0.05 and growth was monitored hourly for 9 hours by measuring the OD_{600nm} and direct counting of CFUs. Shown are the means and standard deviations of biological duplicates with technical triplicates. Growth of all 3 strains is identical until an OD_{600nm} of 1.25. FM2.5 prematurely enters stationary phase, reaching a maximum OD_{600nm} of 2.2 compared with 3.2 for R20291. **(C)** Sporulation of R20291, FM2.5, and FM2.5RW was monitored by direct counting vegetative cells and phase bright spores by phase contrast microscopy at T=0, 48 and 120 of a standard 5 day sporulation assay. 20 microscope fields were counted for each strain at each timepoint. The number of spores is expressed as a percentage of total with the mean and standard deviations shown. * = $p<0.01$, determined using using two-tailed *t*-tests with Welch's correction. **(D)** Example microscope fields representative of those counted for panel C. For clarity only 40% of each field is shown. In the bottom right image representative spores are marked with red arrow heads, a representative vegetative cell with a blue arrow head and representative cell debris with green arrow heads.

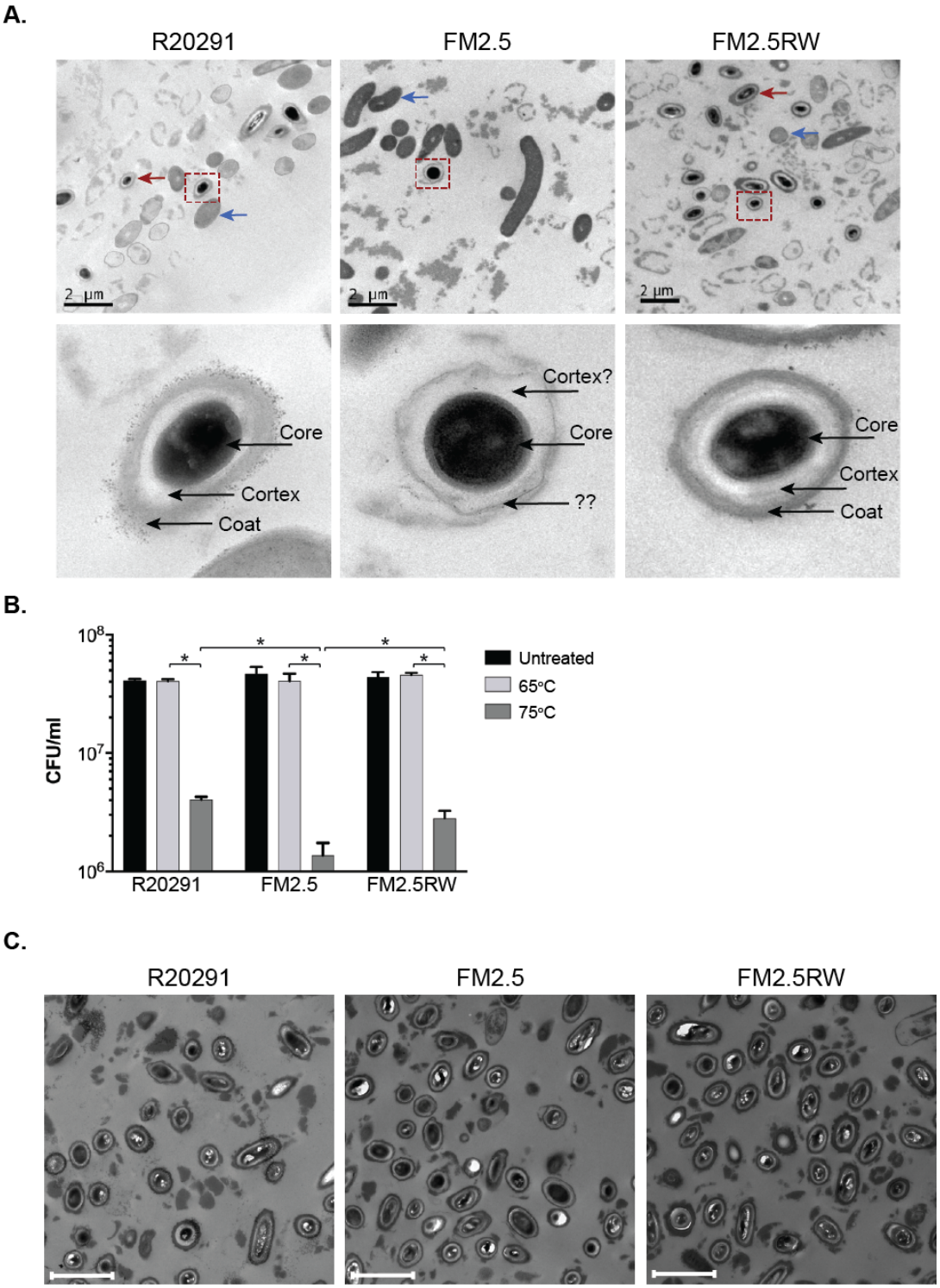

Figure S5. Spore morphology and thermal sensitivity. (**A)** Electron micrographs of cultures on day 5 of the sporulation assay shown in Figure S4A. Spore-containing representative fields are shown. Example spores (red arrows) and vegetative cells (blue arrows) are indicated. Higher magnification views of individual spores are shown below (boxed in top panels). The core,

cortex and coat layers are indicated. **(B)** Spores were purified on a 20-50% Histodenz gradient and CFUs were quantified prior to (Untreated) and following heat stress at 65°C or 75°C for 30 min. Shown are the mean and standard errors of biological triplicates assayed in triplicate. * = $p<0.01$, determined using using two-tailed *t*-tests with Welch's correction. **(C)** Electron micrographs of spores used in panel B. The scale bar on each image indicates 2 μm.

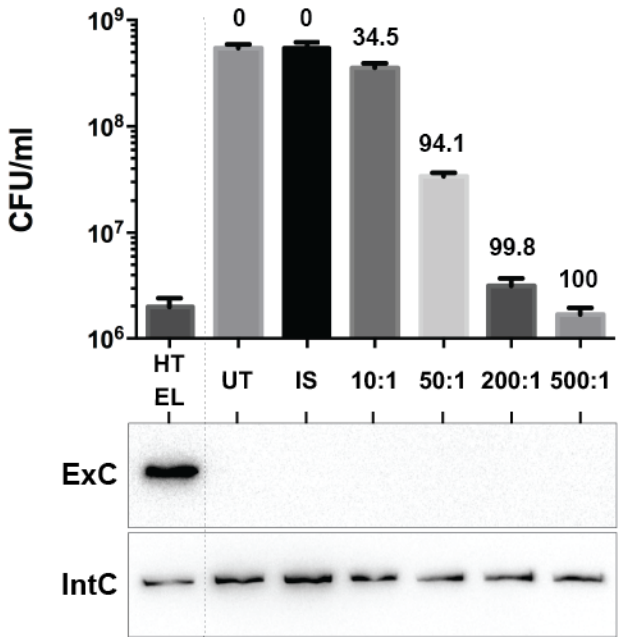

Figure S6. Bactericidal activity by Avidocin-CD does not result in intracellular toxin release. For toxin producing pathogens, antibiotics that lyse bacteria or increase toxin production can exacerbate disease severity and lessen the effectiveness of the treatment by inadvertently releasing toxins (*41*). We examined culture supernatants following treatment with Avidocin-CDs for the presence of extracellular Toxin B to determine if killing by an Avidocin-CD could result in unwanted toxin release. (A) R20291 (RT027) bacteria were incubated for an hour with either Av-CD291.2 at the indicated ratio of agent to cells, Av-CD684.1 (which does not kill strain R20291) at a 500:1 ratio, or left untreated. After incubation, surviving bacteria (CFU/mL) were enumerated. The percentage of CFU following each treatment relative to untreated control is shown. The number of spores present in the untreated sample was determined following heat treatment at 65 °C for 30 min to kill vegetative cells (lane HK). Av-CD291.2 killing occurred in a dose-dependent manner, while Av-CD684.1 treated cells behaved like untreated controls. (B) Following Avidocin-CD treatment released toxin B in culture supernatants (ExC) was detected by Western immunoblot using a specific monoclonal antibody. As a positive control for toxin release, R20291 was treated with a bacteriophage (CD27L) endolysin, which effectively lyses *C. difficile* (*42*). (C) Following Avidocin-CD treatment, the amount of remaining intracellular toxin B was determined by lysing cells with CD27L endolysin and detection by Western immunoblot as before (IntC). A fresh sample of untreated R20291 was lysed with CD27L endolysin to show

normal intracellular toxin quantities (lane EL). Despite effective killing, intracellular Toxin B remained constant with no detectable release observed in the culture supernatants (ExC) at any concentration, including samples that exhibited total killing of all vegetative bacterial cells due to a large excess (500:1) of Avidocin-CD to bacteria. These results confirm that Avidocin-CDs are bactericidal but neither lyse the target cell nor release harmful intracellular stores of toxin B. As a result, the use of Avidocin-CD to treat or prevent CDI is unlikely to exacerbate disease symptoms.

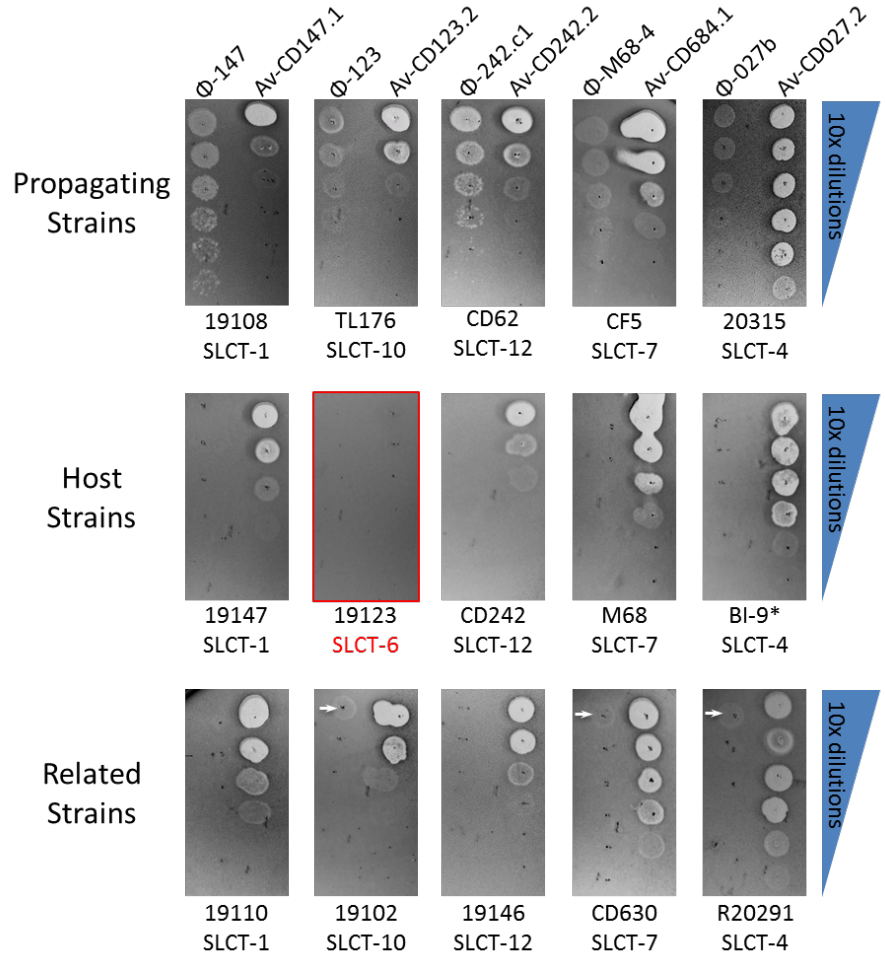

Figure S7. Comparison of *C. difficile* bacteriophage host range vs. Avidocin-CD sensitivity. Preparations of each bacteriophage and corresponding Avidocin-CD were serially diluted and spotted on bacterial lawns corresponding to the bacteriophage propagating strains, bacteriophage host strains and strains with same SLCT as propagating strain. Bacteriophage plaques are small and numerous. Avidocin-CD killing results in a large zone of clearance. Only one host strain, denoted in red, had a different SLCT than propagating strain. At high bacteriophage titers, bacteriophage are observed to cause "lysis from without" and is denoted by small white arrows.

```
SlpA-CD630      MNKKNIAIAMSGLTVLASAAPVFAATTGTQGYTVVKNDWKKAVKQLQDGLKDNSIGKITV    60
SlpA_M68        MNKKNLAMAMAAVTVVGSAAPIFADS-TTPGYTVVKNDWKKAVKQLQDGLKNKTISTIKV    59
                *****:*:**:.:**:.****:** :  * **********************:::*..*.*

SlpA-CD630      SFNDGVVGEVAPKSA-NKKADRDAAAEKLYNLVNTQLDKLGDGDYVDFSVDYNLENKIIT    119
SlpA_M68        SFNGNSVGEVTPASSGAKKADRDAAAEKLYNLVNTQLDKLGDGDYVDFEVTYNLATQIIT    119
                ***   ****:* *:  ******************************.* *** .:***

SlpA-CD630      NQADAEAIVTKLNSLNEKTLIDIATKDTFGMVSKTQDSEGKNVAATKALKVKDVATFGLK    179
SlpA_M68        K-AEAEAVLTKLQQYNDKVLINSATDTVKGMVSDTQVDSK-N-VAANPLKVSDMYTIPSA    176
                : *:***::***:. *:*.**: **. . ****.** ..  * .*:: ***.*: *:

SlpA-CD630      SGGSEDTGYVVEMKA-GAVEDKYGKVGDSTAGIAINLPST-GLEYAGKGTTIDFNKTLKV    237
SlpA_M68        ITGSDDSGYSIAKPTEKTTSLLYGTVGDATAGKAITVDTASNEAFAGNGKVIDYNKSFKA    236
                  **:*:** :   :  :..  **.***:*** **.: ::    :**:*..**:**::*.

SlpA-CD630      DVTGGSTPSAVAVSGFVTKDDTDLAKSGTINVRVINAKEESIDIDASSYTSAENLAKRYV    297
SlpA_M68        TVQGDGT---VKTSGVVLKDASDMAATGTIKVRVTSAKEESIDVDSSSYISAENLAKKYV    293
                 * * .*   * .**.* ** :*:* :***:*** .*******:*:*** *******:**

SlpA-CD630      FDPDEISEAYKAIVALQNDGIESNLVQLVNGKYQVIFYPEGKRLETKSANDTIASQDTPA    357
SlpA_M68        FNPKEVSEAYNAIVALQNDGIESDLVQLVNGKYQVIFYPEGKRLETKSA-DIIADADSPA    352
                *:*.*:****:************:************************ * **. *:**

SlpA-CD630      KVVIKANKLKDLKDYVDDLKTYNNTYSNVVTVAGEDRIETAIELSSKYYNSDDKNAITDK    417
SlpA_M68        KITIKANKLKDLKDYVDDLKTYNNTYSNVVTVAGEDRIETAIELSSKYYNSDDKNAITDD    412
                *:.**************************************************.

SlpA-CD630      AVNDIVLVGSTSIVDGLVASPLASEKTAPLLLTSKDKLDSSVKSEIKRVMNLKSDTGINT    477
SlpA_M68        AVNNIVLVGSTSIVDGLVASPLASEKTAPLLLTSKDKLDSSVKSEIKRVMNLKSDTGINT    472
                ***:************************************************************

SlpA-CD630      SKKVYLAGGVNSISKDVENELKNMGLKVTRLSGEDRYETSLAIADEIGLDNDKAFVVGGT    537
SlpA_M68        SKKVYLAGGVNSISKDVENELKNMGLKVTRLSGEDRYETSLAIADEIGLDNDKAFVVGGT    532
                ************************************************************

SlpA-CD630      GLADAMSIAPVASQLKDGDATPIVVVDGKAKEISDDAKSFLGTSDVDIIGGKNSVSKEIE    597
SlpA_M68        GLADAMSIAPVASQLKDGDATPIVVVDGKAKEISDDAKSFLGTSDVDIIGGKNSVSKEIE    592
                ************************************************************

SlpA-CD630      ESIDSATGKTPDRISGDDRQATNAEVLKEDDYFTDGEVVNYFVAKDGSTKEDQLVDALAA    657
SlpA_M68        ESIDSATGKTPDRISGDDRQATNAEVLKEDDYFKDGEVVNYFVAKDGSTKEDQLVDALAA    652
                *********************************.**************************

SlpA-CD630      APIAGRFKESPAPIILATDTLSSDQNVAVSKAVPKDGGTNLVQVGKGIASSVINKMKDLL    717
SlpA_M68        APIAGRFKESPAPIILATDTLSSDQNVAVSKAVPKDGGTNLVQVGKGIASSVINKMKDLL    712
                ************************************************************

SlpA-CD630      DM      719
SlpA_M68        DM      714
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Figure S8. Clustal Omega alignment of SlpA sequences from strains 630 and M68. Identical residues are demarked with an asterisk. Residues with strongly similar properties are demarked with colon. Residues with weakly similar properties are demarked with a period. An 81.7% sequence identity was found between the two SLCT-7 SlpA variants.

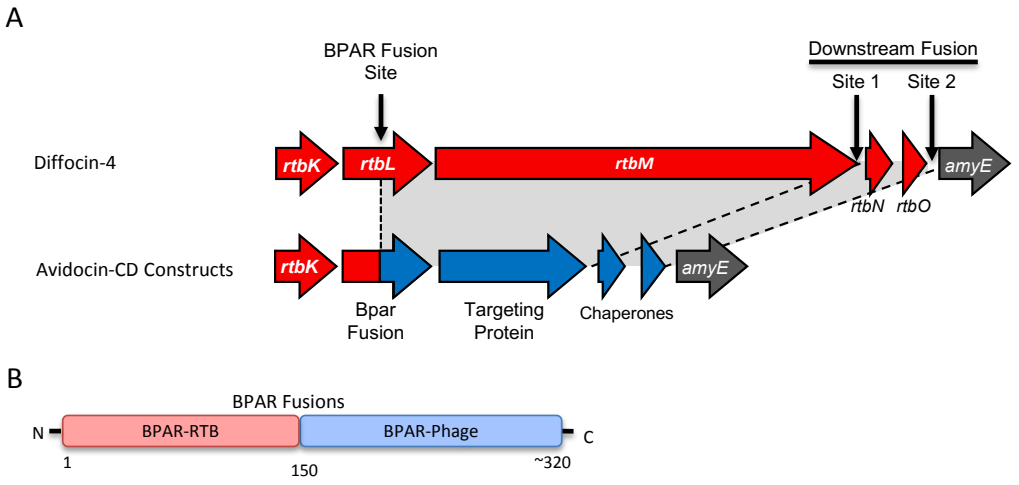

Figure S9. Schematic describing Avidocin-CD construction. (**A**) The fusion sites used to integrate the bacteriophage and prophage genes into the Diffocin-4 gene cluster are shown. There is a Bpar fusion site in *rtbL* as well as alternative downstream fusion sites before or after the predicted Diffocin-4 chaperones. The nucleotide sequences for the fusion region created in each Avidocin-CD construct fusion have been deposited in Genbank and given the following accession numbers (Table S3). (**B**) Schematic depicting R-type Bacteriocin and Bacteriophage regions for fused BPAR protein.

Table S1: Newly identified *C. difficile* phages.

Phage	Source	Propagating Strain	PtsM-like RBP
Phi-027b	panel of 027 strains	20315 (SLCT-4)	Yes
Phi-123	19123 (SLCT-6)	TL176 (SLCT-10)	No
Phi-147	19147 (SLCT-1)	19108 (SLCT-1)	Yes
Phi-242.c1	CD242 (SLCT-12)	CD62 (SLCT-12)	Yes
Phi-68.4	M68 (SLCT-7)	CF-5 (SLCT-7)	Yes

Table S2: Primers used in this study

Primer name	Primer sequence	Use
023-F	ATGAAAAAAAGAAATTTAGCA	Amplification of *slpA* variable region
023_010-R	CTATAGCTGTTTSTATTCTGTC	Amplification of *slpA* variable region
014+++-F	ATGAATAAGAAAARTTTRGCA	Amplification of *slpA* variable region
014_002+-R	CTATWGCAGTCTCTATTCTATC	Amplification of *slpA* variable region
RF1	GATCGGATCCTTACATACTTAATAAATC TTTTAATTTATTTATAACTG	Cloning of SLCT-4 *slpA* from strain R20291 with RF2
RF2	GATCGAGCTCTATAATGTTGGGAGGAAT TTAAGAAATG	Cloning of SLCT-4 *slpA* from strain R20291 with RF1
RF6	GATCGGATCCTTACATATCTAATAAATC TTTTATTTTACTTACAAC	Cloning of SLCT-H2/6 *slpA* from strain Ox575 with RF2
RF7	GATCGGATCCCTACATATCTAATAAGTC TTTTATCTTAGTG	Cloning of SLCT-10 *slpA* from strain Ox1533 with RF2
RF8	GATCGGATCCTTACATATCTAATAAATC TTTTAATTTGCTAACAAC	Cloning of SLCT-1 *slpA* from strain Ox1424 with RF2
RF33	GGCTTCTATAGCTTGGTGAA	Confirmation of *slpA* recombination with NF1323. Chromosome-specific
RF99	GATCGGATCCTTACATATCTAATAAATC TTTTATTTTATTTATTACTG	Cloning of SLCT-6 *slpA* from strain Ox1896 with RF2
RF101	GATCGGATCCTTATAATTCTAATAAATC TTTTATTCTCTTAATAAC	Cloning of SLCT-9 *slpA* from strain Ox1192c with RF134
RF110	GACATAACTGCAGCACTACTTG	Amplification of SLCT-4 *slpA* region containing point mutations and watermark
RF111	CAGGATTAACAGTATTAGCTTCTGC	Amplification of SLCT-4 *slpA* region containing point

		mutations and watermark
RF132	GATCGGATCCTTACATATTTAATAAATC TTTAAGCTTAGTTAC	Cloning of SLCT-8 *slpA* from strain Ox1396 with RF2
RF133	GATCGGATCCTTACATTCCTAATAAATC TTTTAATTTATTAATAAC	Cloning of SLCT-12 *slpA* from strain Ox1342 with RF134
RF134	GATCGAGCTCTATAATGTTGGGAGGAAT TTAAGGAATG	Cloning of SLCT-9 and 12 *slpA* with RF101 and RF133 respectively
RF135	CAGGTTGATAATAAATTAGACAATTTAG GTGATGG	Introduction of *slpA* watermark with RF136
RF136	TGTAAACAATAATTTACTTGCATCTTCT G	Introduction of *slpA* watermark with RF135
RF231	GATCGGATCCTTACATATCTAATAAATC TTTCATTTTGCTTATTAC	Cloning of SLCT-2 *slpA* from strain Ox858 with RF2
NF1323	CTGGACTTCATGAAAAACTAAAAAAAA TATTG	Confirmation of *slpA* recombination with RF33. Plasmid-specific
NF1414	GATCGAGCTCTATAATGTTGGGAGGAAT TTAAGAAATG	Cloning of SLCT-7 *slpA* from strain 630 with NF1415
NF1415	GATCGGATCCTTACATATCTAATAAATC TTTCATTTTG	Cloning of SLCT-7 *slpA* from strain 630 with NF1414

Table S3: Genbank accession identifiers.

Construct	**Source**	**Accession Id:**
Diffocin-4	CD4	KX557294
Av-CD291.2	R20291 genome	KX592438
Av-CD027.2	Phi-027b	KX592434
Av-CD681.1	M68 genome	KX592441
Av-CD682.1	M68 genome	KX592442
Av-CD684.1	M68 genome/phi-M68.4	KX592443
Av-CD685.1	M68 genome	KX592444
Av-CD305.1	CD305 genome	KX592439
Av-CD123.2	Phi-123	KX592435
Av-CD242.2	Phi-242.c1	KX592437
Av-CD147.1	Phi-147	KX592436
Av-CD630.1	630 genome	KX592440