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Johann Peltier, Audrey Hamiot, Julian R Garneau, Pierre Boudry, Anna Maikova, et al.. Type I toxin-antitoxin systems contribute to the maintenance of mobile genetic elements in *Clostridioides difficile*. *Communications Biology*, 2020, 3 (1), 10.1038/s42003-020-01448-5 . hal-03028201

HAL Id: hal-03028201

<https://hal.science/hal-03028201>

Submitted on 27 Nov 2020

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Type I toxin-antitoxin systems contribute to mobile genetic elements maintenance in *Clostridioides difficile*

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Keywords: toxin-antitoxin, small noncoding RNA, *cis*-antisense RNA, prophage stability

Running title: new type I TA systems in *C. difficile*

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34

35 **ABSTRACT**

36 Toxin-antitoxin (TA) systems are widespread on mobile genetic elements as well as in
37 bacterial chromosomes. According to the nature of the antitoxin and its mode of action for
38 toxin inhibition, TA systems are subdivided into different types. In type I TA, synthesis of the
39 toxin protein is prevented by the transcription of an antitoxin RNA during normal growth.
40 The first type I TA modules were recently identified in the human enteropathogen
41 *Clostridioides* (formerly *Clostridium*) *difficile*. Here, we report the characterization of five
42 additional type I TA systems present within phiCD630-1 and phiCD630-2 prophage regions
43 of *C. difficile* strain 630. Toxin genes encode 34 to 47 amino acid peptides and their ectopic
44 expression in *C. difficile* induces growth arrest. Growth is restored when the antitoxin RNAs,
45 transcribed from the opposite strand, are co-expressed together with the toxin genes. In
46 addition, we show that type I TA modules located within the phiCD630-1 prophage
47 contribute to its stability and mediate phiCD630-1 heritability. Type I TA systems were
48 found to be widespread in genomes of *C. difficile* phages, further suggesting their functional
49 importance. We have made use of a toxin gene from one of type I TA modules of *C. difficile*
50 as a counter-selectable marker to generate an efficient mutagenesis tool for this bacterium.
51 This tool enabled us to delete all identified toxin genes within the phiCD630-1 prophage, thus
52 allowing investigation of the role of TA in prophage maintenance.

53

54 INTRODUCTION

55 *Clostridioides difficile* is a medically important human enteropathogen that became a key
56 public health concern over the last two decades in industrialized countries ^{1, 2}. This strictly
57 anaerobic spore-forming Gram-positive bacterium is a major cause of antibiotic-associated
58 nosocomial diarrhoea in adults ³. The main virulence factors of *C. difficile* are two toxins,
59 TcdA and TcdB, produced by all toxigenic strains ⁴ and some isolates produce a binary toxin
60 *Clostridium difficile* transferase (CDT). Additional factors, such as adhesins, pili, and
61 flagella, involved in the interactions with the host during colonization have also been
62 identified ⁵. However, many questions remain unanswered regarding the success of this
63 pathogen and its adaptation within the phage-rich gut environment.

64 *C. difficile* genome sequencing revealed the mosaic nature of its chromosome, which is
65 composed of more than 10 % of mobile genetic elements including integrated bacteriophages
66 (prophages) ⁶. Recent studies revealed a high prevalence of prophages in *C. difficile* genomes,
67 each genome harbouring between one and up to five prophages, either integrated into the
68 chromosome or maintained as stable extrachromosomal circular DNA elements ⁷. For
69 example, the largely used laboratory strain 630 carries two homologous prophages,
70 phiCD630-1 and phiCD630-2, while the NAP1/B1/027 epidemic strain R20291 carries one
71 prophage (phi-027). The importance of prophages in the evolution and virulence of many
72 pathogenic bacteria has clearly been demonstrated ⁸. In *C. difficile*, all phages identified so
73 far are temperate and can adopt a lysogenic lifecycle, and some of them have been shown to
74 contribute to virulence-associated phenotypes. This includes modulation of toxin production
75 and complex crosstalk between bacterial host and phage regulatory circuits ^{7, 8, 9}. When
76 integrated into *C. difficile* genomes, prophages are stably maintained and replicated along
77 with the host chromosome. However, when they are excised, either spontaneously or
78 following induction by antibiotics or the exposure to other stress conditions, prophages can

79 sometimes be lost during cell division and segregation. The rate of spontaneous phage loss
80 under natural conditions has been estimated for *Escherichia coli* phages to range between 10^{-5}
81 for phage P1 to $< 10^{-6}$ for phage lambda^{10, 11}. To our knowledge, no experiments have been
82 conducted to evaluate prophage loss rates in *C. difficile*.

83 TA modules are widespread in bacteria and archaea. These loci comprise two genes encoding
84 a stable toxin and an unstable antitoxin¹². Overexpression of the toxin has either bactericidal
85 or bacteriostatic effects on the host cell while the antitoxin is able to neutralize the toxin
86 action or production. For all identified TA modules, the toxin is always a protein. The RNA
87 or protein nature and the mode of action of the antitoxin led to the classification of TA
88 modules into six types¹². In type I systems, the antitoxin is a small antisense RNA targeting
89 toxin mRNA for degradation and/or inhibition of translation, while in type III systems, the
90 antitoxin RNA binds directly to the toxin protein for neutralization^{13, 14}. For other TA types,
91 both the toxin and the antitoxin are proteins. In most studied type II TA systems, the
92 proteinaceous antitoxin forms a complex with its cognate toxin leading to toxin inactivation
93¹⁵. Major functions suggested for TA modules include plasmid maintenance, abortive phage
94 infection and persistence, however, their role in persister cell formation in the presence of
95 antibiotics remains a subject of controversy^{16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26}. TA loci are
96 commonly found on mobile genetic elements, in particular plasmids in which they were
97 initially discovered and extensively studied. However, the roles of chromosomally-encoded
98 TA modules, including those within prophage genomes, remain largely unexplored.

99 We recently reported the identification of the first type I TA systems associated with CRISPR
100 arrays in *C. difficile* genomes²⁷. The co-localization and co-regulation by the general stress
101 response Sigma B factor and biofilm-related factors of TA and CRISPR components
102 suggested a possible genomic link between these cell dormancy and adaptive immunity
103 systems. Interestingly, two of these functional type I TA pairs are located within the

homologous phiCD630-1 and phiCD630-2 prophages in *C. difficile* strain 630. In the present work, we characterize additional type I TA modules highly conserved within *C. difficile* prophages and provide experimental evidence of their contribution to prophage maintenance and stability. Moreover, we demonstrate here that inducible toxicity caused by type I toxins can be used as a counter selection marker in allele exchange genome editing procedures by promoting the elimination of plasmid-bearing cells, largely improving their efficiency.

RESULTS

Identification of novel type I TA pairs in *C. difficile*

Multiple TA modules have been discovered in bacterial chromosomes including prophage regions¹². In *C. difficile*, we have recently identified several type I TA pairs adjacent to CRISPR arrays, two of them being located inside the phiCD630-1 and phiCD630-2 prophages of the strain 630 (*CD0956.2*-RCd10 and *CD2907.1*-RCd9, respectively)²⁷. To determine whether other type I TA modules might be present within phiCD630-1, we performed a bioinformatics analysis on the phiCD630-1 sequence. Due to the small size of the toxin-encoding genes, standard methods of open reading frame (ORF) detection and gene annotation can hinder the identification of all toxin homologs. Moreover, prophages are characterized by a very high gene density, which can impede such detection of small and often overlapping coding regions. We therefore used the tBlastn program using the previously identified type I toxin *CD0956.2* as a query, as it does not depend on annotation and ORF detection. We identified gene *CD0977.1* and two other novel putative genes, unannotated on the genome, that we named *CD0904.1* and *CD0956.3*. These genes code for small proteins of 47, 35 and 34 amino acids, respectively (Fig. 1A). Prophages phiCD630-1 and phiCD630-2 share a large region of homology with almost identical sequences, which include a duplication of *CD0977.1* and *CD0956.3* (named *CD2889* and *CD2907.2* in

phiCD630-2, respectively) (Fig. 1B). In contrast, *CD0904.1* is unique to phiCD630-1 and no other toxin gene homolog could be identified within phiCD630-2. Transcript reads were detected in regions of these putative genes by RNA-seq²⁸ (Fig. S1A-C). The presence of a consensus RBS sequence (AGGAGG) 7-8 nucleotides upstream of the respective ATG start codons suggest that the corresponding proteins are produced (data not shown). In addition, all three putative proteins carried a hydrophobic *N*-terminal region and a positively charged tail, which are characteristic features of type I toxins (Fig. 1A)²⁹. Analysis of our previous TSS mapping data²⁸ and sequence alignments (Fig. S1) suggested the presence of potential antisense RNAs of these toxin-encoding genes with the presence of TSS associated with Sigma A- and Sigma B-dependent promoter elements for both the toxin and antitoxin genes (Fig. S1 and data not shown)³⁰. Antitoxins of *CD0977.1*, *CD0904.1* and *CD0956.3*, located on phiCD630-1, were hereafter named RCd11, RCd13 and RCd14, respectively, and those of *CD2889* and *CD2907.2*, found in phiCD630-2, were named RCd12 and RCd15.

To determine whether these novel potential TA pairs are functional, pRPF185-derivatives with anhydrotetracycline (ATc)-inducible *P_{tet}* promoter were constructed to overexpress *CD0904.1*, *CD0956.3* and *CD0977.1* toxin genes (pT) or toxin-antitoxin modules (pTA) in *C. difficile* 630Δ*erm*. Antisense RNAs are expressed from their own promoter in pTA. Growth of 630Δ*erm* carrying the different pT and pTA vectors on BHI plates was indistinguishable in the absence of ATc inducer (Fig. 1C). In contrast, growth of the 630Δ*erm*/pT strains was completely inhibited when ATc was present in the medium, while strains 630Δ*erm*/pTA showed a reversion of the growth defect. These results demonstrate that *CD0904.1*, *CD0956.3* and *CD0977.1* encode potent toxins and are associated with antisense RNAs that function as antitoxins.

Detailed characterization of the *CD0977.1*-RCd11 TA pair

Intriguingly, predicted riboswitches responding to the c-di-GMP signalling molecule, *cdi1_4* and *cdi1_5*, precede RCd11 and RCd12 antisense RNAs²⁸. Most of these type I c-di-GMP-responsive riboswitches negatively control downstream genes by premature termination of transcription in the presence of c-di-GMP^{28, 31}. We therefore sought to further characterize the *CD0977.1*-RCd11 TA pair. In agreement with the data above, addition of ATc to liquid cultures in exponential growth phase led to an immediate growth arrest of strain 630 Δ *erm*/pT, unlike 630 Δ *erm*/p (Fig. S2A). In addition, the growth arrest was accompanied by a drop of colony-forming units (CFUs) (Fig. S2B). Similarly to previous observations with other *C. difficile* type I TA modules²⁷, the analysis of liquid cultures by light microscopy showed that toxin overexpression was accompanied by an increase in cell length in about 10% of the cells (Fig. S2C). Their length was above the mean length value of 630 Δ *erm*/p control strain with two standard deviations (10.5 μ m). The co-expression of the entire TA module led to the partial reversion of this phenotype.

Using Northern blotting, we detected both toxin and antitoxin transcripts in the 630 Δ *erm*/p, 630 Δ *erm*/pT (*CD0977.1*) and 630 Δ *erm*/pTA (*CD0977.1*-RCd11) strains (Fig. 2A and 2B). In the absence of ATc inducer, a major transcript of about 300 nt was detected in all three strains with a *CD0977.1*-specific probe. When using an RCd11-specific probe, transcripts of about 150, 300 and 400 nt were observed. Under inducing conditions, a reverse correlation between the relative toxin and antitoxin transcript abundance was noticed. The toxin overexpression in the presence of ATc inducer resulted in a decreased amount of the major 150-nt RCd11 antitoxin expressed from chromosomal location (lanes “pT” compared in the absence and in the presence of ATc). Similarly, for the strain carrying the entire TA locus on pTA plasmid expressing the antitoxin from its own strong promoter, the toxin overexpression after ATc induction led to a decrease in the 150-nt RCd11 antitoxin level (lanes “pTA” compared under conditions “-ATc” and “+ATc”). To determine the impact of c-di-GMP on

the antitoxin transcripts, we elevated c-di-GMP intracellular levels in the 630 Δ *erm* wild type strain by expressing the gene *dccA*, coding for a diguanylate cyclase involved in c-di-GMP production, from a plasmid (*pdccA*) (Fig. 2C), as previously reported²⁸. A c-di-GMP-regulated read-through transcript of about 400 nt, as well as a terminated transcript of about 140 nt were detected in this strain by Northern blotting using a riboswitch-specific probe. In contrast, abundance of the 150-nt RCd11 antitoxin and toxin transcripts was not affected by fluctuations of c-di-GMP levels. Elevated ci-di-GMP intracellular level could be associated with biofilm growth conditions. As for some other type I TA transcripts in our previous study²⁷, we detected by qRT-PCR analysis up to 20-fold increase in *CD0977.1* toxin gene expression in biofilms as compared to planktonic culture, but no difference for short RCd11 form amount (data not shown).

We then mapped the transcriptional start (TSS) and termination sites for the genes of the potential RCd11/RCd12-*CD0977.1/CD2889* TA modules by 5'/3'RACE analysis (Fig. S3, Table S1). The results obtained agreed well with the transcript lengths deduced from TSS mapping, RNA-seq and Northern blot. Taken together, these data suggest the presence of two tandem TSS for RCd11, i.e. P_1 associated with c-di-GMP-dependent riboswitch, yielding a premature terminated transcript of ~140 nt, primary read-through transcript of ~400 nt (referred to as long transcript hereafter) and a processed transcript of ~300 nt, and P_2 located downstream from the riboswitch, yielding a transcript of ~150 nt (referred to as short transcript hereafter) (Fig. S1). All these transcripts except for riboswitch-associated terminated transcript shared the same Rho-independent terminator (Fig. S3). According to the position of the Northern blotting probes, the long 400-nt transcript could be detected with both riboswitch- and RCd11-specific probes, while terminated 140-nt transcript could be revealed only with riboswitch-specific probe and RCd11-specific probe hybridized to 300-nt and 150-nt transcripts (Fig. 2A).

We investigated the interaction between *CD0977.1* toxin mRNA and the short and long RCd11 RNAs to determine whether they form kissing complexes as in the case of the RCd9/CD2907.1 TA pair ²⁷. The results shown in Figure S4 reveal no difference in duplex formation with toxin mRNA for long and short antitoxin forms under native or full RNA duplex conditions suggesting that no kissing intermediate is formed during binding in native conditions *in vitro*. It should be noticed that only a fraction of *CD0977.1* can interact with both antitoxin forms even when they are in excess, indicating that it is tightly folded in these experimental conditions (Fig. S4).

It is in the nature of type I antitoxins to be short-lived in contrast to the stable toxin mRNA ¹⁴. To determine the half-lives of toxin and antitoxin RNAs of the *CD0977.1*-RCd11 module, *C. difficile* strains were grown in TY medium until late-exponential phase and rifampicin was added to block transcription. Samples were taken at different time points after rifampicin addition for total RNA extraction and Northern blot analysis with toxin and antitoxin-specific probes. In a control strain 630Δ*erm*/p carrying an empty vector, the half-life of the major short transcript for RCd11 was estimated to be about 8 min while the half-life of *CD0977.1* toxin mRNA was estimated to about 89 min (Fig. 2D). Interestingly, depletion of the RNA chaperone protein Hfq, which generally increases the intracellular half-life of sRNAs and stabilizes the interactions between sRNAs and their target mRNAs, resulted in a moderate destabilization of *CD0977.1* toxin mRNA and antitoxin RCd11 RNA with the half-life of 64 min and 4 min, respectively (Fig. S5). By contrast, the stable *CD0977.1* toxin mRNA was further stabilized to over 120 min half-life in the strains depleted for the ribonucleases RNase III, RNase J and RNase Y, that could be involved in toxin and antitoxin RNA decay. For antitoxin RCd11 RNA, we also observed a stabilization in strain depleted for RNase Y (Fig. S5) suggesting that this ribonuclease contributes to antitoxin RNA degradation.

To confirm the protein nature of CD0977.1 and assess its subcellular localization, we constructed a derivative of CD0977.1 with an HA tag fused to the C-terminus of CD0977.1 expressed from a plasmid under the control of the inducible P_{tet} promoter (pT-HA). *C. difficile* strain carrying pT-HA was grown to mid-exponential phase, induced with ATc for 90 min, and whole cell extracts were prepared. Induction of *CD0977.1* expression immediately stopped the growth, as revealed by OD₆₀₀ measurements (data not shown), suggesting that the HA-tag does not interfere with toxin activity. HA-tagged CD0977.1 was detectable by Western blotting with anti-HA antibodies (Fig. 2E). No band was observed in a whole cell extract of a control strain producing untagged CD0977.1 protein. The distribution of HA-tagged CD0977.1 within supernatant, cell wall, membrane and cytosolic compartments was then studied (Fig. 2E). HA-tagged CD0977.1 was only detected in the membrane fraction, indicating the association of CD0977.1 with the cell membrane of *C. difficile*.

The antitoxin transcript controlled by *cdi1_4* riboswitch is dispensable for efficient toxin inactivation

To get further insights into the function of abundant short (transcribed from P_2) and less abundant long RCd11 antitoxin transcripts (transcribed from P_1) (Fig. S1), we generated new plasmid constructs that allowed the inducible expression of the *CD0977.1* toxin gene under the control of the P_{tet} promoter and the expression of different forms of the RCd11 antisense RNA (Fig. 3A). The first construct, yielding pDIA6816, lacked the *cdi1_4* riboswitch and its associated promoter (P_1) but retained the P_2 promoter of the antitoxin. On the opposite, the second construct, yielding pDIA6817, retained the P_1 promoter and the associated riboswitch but had a disrupted P_2 promoter. The construct in which both promoters of RCd11 and the riboswitch were present (pDIA6785) and the one in which only the toxin gene is expressed (pDIA6335) served as a positive and as a negative control for the assay, respectively. All plasmids were introduced into *C. difficile* 630 Δ *erm* and the corresponding strains were grown

on BHI plates supplemented with 10 and 100 ng/ml ATc to induce *CD0977.I* toxin expression. Growth of the strain carrying pDIA6816 was similar to that observed for the control strain carrying pDIA6785 in the presence of 10 ng/ml ATc and was slightly defective in the presence of 100 ng/ml ATc (Fig. 3A and Fig. S6A). In contrast, the strain carrying pDIA6817 did not grow in the presence of 10 or 100 ng/ml ATc, similarly to the negative control strain. Similar results were obtained when the strains were grown in an automatic plate reader for 20 h in liquid medium in the presence of 5 ng/ml ATc (Fig. 3A). Interestingly, induction of toxin expression on BHI plate with a lower dose of ATc (5 ng/ml) led to a partial reversion of the growth defect of the strain carrying pDIA6817 unlike the negative control strain (Fig. S6A). To further investigate the promoters functionality, a series of promoter fragments fused to the *phoZ* reporter gene was created in the wild type strain, and alkaline phosphatase (AP) activity was measured. After 4 h of growth in TY broth, the P_2 promoter fragment exhibited a reporter activity 1.4-fold lower than that of the full length promoter region, comprising P_1 and P_2 , while the AP activity from the P_1 promoter associated with the Cdi1_4 riboswitch fragment was 5.7-fold lower (Fig. 3B). This suggests that the P_1 promoter activity is weaker than that of P_2 , providing a rationale for the incompetence of the long antitoxin form expressed from P_1 for toxin inhibition. Of note, the promoter fragment comprising P_1 and the disrupted P_2 retained AP activity (Fig. S6B), suggesting that the nucleotide substitutions in P_2 do not prevent expression of the antitoxin transcripts. To determine whether promoters activity differs in various growth conditions, AP activity was next measured after 10 h of growth in TY broth and under nutrient starvation conditions. In these conditions, the full length promoter region (P_1 and P_2) exhibited a slight decrease in activity while activity from the P_1 or P_2 promoter fusions was not significantly different to that observed during the exponential growth phase (Fig. 3B), suggesting that the expression of the antitoxin transcripts was not strongly modulated in these conditions. Taken

together, these results suggest that the short antitoxin transcript driven by promoter P_2 is crucial for the efficient inactivation of the toxin, while the longer antitoxin transcript directed by P_1 is dispensable.

RCd12 counteracts toxic activity of non-cognate CD0977.1 toxin

Nucleotide sequences of short RCd11, lying within phiCD630-1 and short RCd12, lying within phiCD630-2, are almost identical with only 3 mismatches located near the 3' end, in the region overlapping with the toxin transcript (Fig. S7A and S8). The structure prediction suggested that the 3' part folded similarly with two conserved hairpin structures in both antitoxin short and long form predictions (Fig. S8). We therefore wondered whether RCd12 could cross-react with the transcript of the non-cognate toxin CD0977.1. To answer this question, we generated constructs in which *CD0977.1* toxin gene under the control of the P_{tet} promoter and different antitoxin genes with their own promoter were co-expressed from the same plasmid but from distant locations (Fig. 3C). As anticipated, expression of RCd11 in *trans* (pDIA6791) counteracted the toxicity associated with the expression of the cognate toxin both on plate and in liquid culture (Fig. 3C and Fig. S6C). Replacement of RCd11 with RCd12 (pDIA6792) led to the same result (Fig. 3C and Fig. S6C). By contrast, *in trans* expression of the more divergent RCd10 (the antitoxin of CD0956.2 toxin from a previously characterized TA module lying within phiCD630-1²⁷) (pDIA6793) (Fig. S7B) failed to revert the growth defect induced by *CD0977.1* expression (Fig. 3C and Fig. S6C). These data indicate that antitoxins act in a highly specific manner to repress their cognate toxins, not only when they are expressed from the native convergent TA configuration, but also when expressed in *trans*. However, the specificity of interaction is permissive for at least 3 mismatches allowing RCd12 expressed from phiCD630-2 to efficiently prevent CD0977.1 toxin production from phiCD630-1.

TA modules confer plasmid stabilization

TA systems have been initially discovered on plasmids where they confer maintenance of the genetic element¹⁸. Plasmid loss results in a rapid decrease in the levels of the unstable antitoxin, which allows the stable toxin to inhibit cell growth. To test whether the TA modules located on phiCD630-1 could contribute to plasmid maintenance, we assessed the stability of pMTL84121-derived plasmids in which each TA module of phiCD630-1 was cloned and expressed under the control of their respective native promoter in *C. difficile* 630Δ*erm*. *C. difficile* 630Δ*erm* harbouring the empty vector pMTL84121 was used as a control. After 7 passages in TY broth in the absence of antibiotic pressure, pMTL84121 was maintained by only 1.0% (+/-0.4%) of the bacterial population (Fig. 4). In contrast, plasmids expressing TA pairs were still present in 22.3 (+/- 5.4%) to 63.6 % (+/- 14.3%) of total cells. These results indicate that the four TA pairs can confer plasmid maintenance.

Deletion of phiCD630-1 toxin genes in *C. difficile* 630Δ*erm*

In order to determine whether the TA modules contribute to phiCD630-1 stability, we undertook the construction of mutants deleted for toxin genes of TA modules in *C. difficile* 630Δ*erm*. For this purpose, we constructed a new Allele-Coupled Exchange (ACE) vector, derived from pMTL-SC7315, a *codA*-based “pseudosuicide” plasmid³². The *codA* cassette was here replaced with the *CD2517.1* toxin gene placed under the control of the *P_{ter}* inducible promoter (Fig. S9A). The functionality of RCd8-*CD2517.1* type I TA module in *C. difficile* was previously demonstrated²⁷. In our new vector, designated pMSR, the inducible toxic expression of *CD2517.1* is used as a counter-selection marker to screen for plasmid excision and loss (see Materials and Methods), greatly facilitating the isolation of *C. difficile* deletion mutant generated by double cross-over allele exchange (Fig. S9C and D). We also constructed a second vector, pMSR0, for allele exchange in *C. difficile* ribotype 027 strains

and other ribotypes (see Materials and Methods and Fig. S9B). Using this new tool, we first deleted the 49.3 kb phiCD630-2 locus to prevent any interfering cross-talk with phiCD630-1 (Fig. S10). A multiple deletion mutant of toxin-encoding genes *CD0904.1*, *CD0956.2*, *CD0956.3* and *CD0977.1* (phiCD630-1ΔT4) was then generated in the ΔphiCD630-2 background.

TA systems are involved in maintenance of phiCD630-1 in the host cells

Because the loss of an integrated phage from cells first requires its excision from the host genome, we sought to determine whether spontaneous excision of phiCD630-1 from chromosomal DNA occurred. To do so, we performed a PCR on genomic DNA from *C. difficile* 630Δ*erm* ΔphiCD630-2 with primers flanking the *attL* and *attR* sites of phiCD630-1 (Fig. S11A). A PCR product with a size of 88 bp corresponding to a region with the excised prophage was detected (Fig. S11B) and DNA sequencing of this amplicon confirmed the complete removal of phiCD630-1 from the host chromosome. A second PCR-based assay showed that the excised prophage (PCR product of 117 bp) was present as an extrachromosomal circular form in the host cell (Fig. S11A and B). We could also deduce the *attB*, *attP*, *attL* and *attR* sites from the sequencing of the PCR products (Fig. S11C). However, the frequency of phiCD630-1 excision, as measured by quantitative PCR (qPCR), was very low (~ 0.015 %) (Fig. S12). To screen for the presence/absence of phiCD630-1 in the host cells, we introduced, using our new ACE vector, the *ermB* gene placed under the control of the strong *thl* promoter of *Clostridium acetobutylicum* as previously described³³, into an innocuous location (between *CD0946.1* and *CD0947*) of phiCD630-1 and phiCD630-1-ΔT4 (Fig. 5A). Starting from the overnight cultures, the ΔphiCD630-2 phiCD630-1::*erm* and ΔphiCD630-2 phiCD630-1-ΔT4::*erm* strains were subcultured four times in fresh medium and cells were screened for erythromycin resistance by plating onto non-selective and erythromycin-containing agar plates. Nearly 100% of cells from both strains were found

to still be resistant to erythromycin in these conditions, indicating that they had retained the prophage (Fig. S11D).

In an attempt to artificially increase the excision rate of phiCD630-1, we ectopically expressed the putative excisionase *CD0912* of phiCD630-1 from the inducible P_{tet} promoter, yielding pDIA6867. *CD0912*, identified in a bioinformatics search, is a 109 amino acid protein with a predicted DNA-binding domain similar to the HTH-17 superfamily and the excisionase (Xis) family³⁴. Induction of *CD0912* expression with 10 ng/ml ATc in *C. difficile* 630Δ*erm* resulted in a high excision rate of phiCD630-1 (~ 70 %), indicating that *CD0912* functions as an excisionase for phiCD630-1 (Fig. S12). Expression of *CD0912* in strains ΔphiCD630-2 phiCD630-1::*erm* and ΔphiCD630-2 phiCD630-1ΔT4::*erm* caused excision at a similar rate, suggesting that TA systems do not affect phiCD630-1 excision (Fig. S12).

Strains ΔphiCD630-2 phiCD630-1::*erm* and ΔphiCD630-2 phiCD630-1-ΔT4::*erm* carrying pDIA6867 or a control vector were then grown in TY supplemented with 7.5 μg/ml Tm and 10 ng/ml ATc. After 16 and 24 hrs of incubation at 37°C, measurement of the OD₆₀₀ revealed a dramatic growth defect of ΔphiCD630-2 phiCD630-1::*erm* expressing the excisionase compared to the strains carrying the control vector (Fig. 5B). Expression of the excisionase in ΔphiCD630-2 phiCD630-1-ΔT4::*erm* also resulted in a growth defect, although at a lesser extent. In addition, plating of the cells bearing pDIA6867 on non-selective and erythromycin-containing agar plates revealed that phiCD630-1::*erm* was still present in more than 90% of the total population while phiCD630-1-ΔT4::*erm* remained in less than 10 % of the cells (Fig. 5C). These results thus show that TA modules are important for phiCD630-1 maintenance after its excision and highlight the impact of the toxin expression on the cell growth upon the loss of prophage. Together, these data demonstrate that TA modules contribute to phiCD630-1 heritability.

Type I TA are prevalent in *C. difficile* phage genomes

Since we identified additional toxin variants of type I TA systems after careful inspection of phiCD630-1 full genome, we decided to re-scan for possible ORFs in every available phage genomes of *C. difficile* using the permissive algorithm of the NCBI ORFfinder software. ORFs with minimal length of 60 nucleotides as well as nested ORFs were detected. A blastP search against the corresponding proteins allowed the identification of toxin homologs in all *C. difficile* prophage genomes (functional phages) (Fig. 6). Moreover, toxin sequence alignments revealed the high conservation of the hydrophobic *N*-terminal region, as well as the lysine-rich, positively charged region at the C-terminus. Hence, these data suggest the functionality of the toxins and reinforce their proposed role for phage maintenance and preservation.

Despite these conserved regions, alignment of toxins also revealed small variations among sequences. We therefore sought to explore the possible relationship between phage phylogeny and the observed toxin variants. A whole genome comparison of all phages included in this study was performed to create phylogenetic groups (phiCD119-like viruses, phiCD38-2-like viruses and phiMMP04-like viruses), as previously described³⁵. A clear link between phage groups and specific toxin variants could be established, suggesting an independent acquisition of TA systems in different groups of phages (Fig. 6 and Fig. S13). Interestingly, an extended search outside *C. difficile* phages revealed the presence of other toxin homologs inside plasmids of *C. difficile* and *Paenibacillus sordellii*, a closely related species (Fig. S14). These findings imply that *C. difficile* phages could recombine with plasmids to exchange genetic material, as already proposed for *E. coli* phages^{36,37}.

DISCUSSION

In this study, we identified and characterized novel functional type I TA modules in *C. difficile* 630 prophages. Although these modules share characteristic features of known TA systems, i.e. (i) the toxins are membrane-associated proteins having a positively charged tail, (ii) the toxin mRNA is much more stable than the antitoxin RNA, (iii) artificial expression of the toxin genes inhibits bacterial growth unless their cognate antitoxin RNA is co-expressed; they do not present sequence homology with other TA modules identified to date in other bacteria.

RCd11-*CD0977.1* and RCd12-*CD2889* TA pairs are duplications respectively located within the homologous regions of phiCD630-1 and phiCD630-2 prophages. Two tandem TSS were identified for RCd11 (and RCd12), with the first one associated with the *cdi1_4* (and *cdi1_5*) c-di-GMP-responsive riboswitch. C-di-GMP is a second messenger in bacterial systems and a key signal in the control of critical lifestyle choices, such as the transition between planktonic and biofilm growth^{28, 38}. C-di-GMP has been found to regulate important functions in *C. difficile*, including motility, production of type IV pili, cell aggregation and biofilm formation, through control of gene expression by c-di-GMP-dependent riboswitches³⁸. Sixteen predicted c-di-GMP sensing riboswitches are encoded in the *C. difficile* 630 genome and the regulatory function of five of them has been investigated so far. *Cdi1_4* and *cdi1_5* riboswitches were recently reported to be insensitive to an elevation of the c-di-GMP levels, and only transcript reads corresponding to the terminated transcript were detected, and no read-through seemed to occur³⁹. However, our RACE-PCR and Northern-blot analysis indicated the presence of a transcript downstream from these riboswitches (Fig. 2, Fig. S3 and Table S1). Moreover, our data suggested that *cdi1_4* and *cdi1_5* are functional riboswitches responding to c-di-GMP since the abundance of the downstream transcripts was significantly reduced in the presence of high levels of c-di-GMP (Fig. 2). *In vitro* interaction assays showed that both long and short RCd11 antitoxin RNAs could form a duplex with

CD0977.1 mRNA with the same efficiency and only a fraction of toxin mRNA was included in these complexes probably due to extensive RNA folding (Fig. S4 and S8). Despite these data, we found that the shorter and more abundant RCd11 transcript alone was sufficient to ensure complete *CD0977.1* toxin inactivation under our conditions. In accordance, the analysis of promoter activities showed that c-di-GMP-independent P_2 promoter driving the short RCd11 transcription was much stronger than the P_1 promoter. In contrast, the longer antitoxin transcript associated with the *cdi1_4* riboswitch could only counteract the *CD0977.1* toxicity when the toxin gene was expressed at low levels. This suggests that this antitoxin transcript might be involved in the tight regulation of *CD0977.1* production and might be crucial to prevent toxin translation under conditions where expression levels of the toxin gene would be slightly higher than those of the short antitoxin transcript. The c-di-GMP levels would then be critical in this regulation since elevated levels would result in a decreased abundance of the short RCd11 transcript and consequently in growth inhibition. From our previous studies of type I TA pairs, a larger link with biofilm-related control could be suggested for these TA systems since biofilm conditions affected the expression of several other TA transcripts independently from their association with c-di-GMP-responsive riboswitch²⁷.

In this work, we detected a natural background excision of the ϕ CD630-1 prophage and we identified the phage excisionase gene, *CD0912*. Expression of *CD0912* from a plasmid promoted high levels of prophage excision from the host chromosome, mimicking prophage induction under stressful conditions. While ϕ CD630-1 and ϕ CD630-2 of *C. difficile* 630 share a large region of duplicated sequence, it is worth noting that *CD0912* is located in the variable region and has no homolog in ϕ CD630-2. Interestingly, no obvious putative excisionase-encoding gene could be identified in ϕ CD630-2 although natural excision of this prophage could also be detected in the course of our experiments. Moreover,

expression of *CD0912* had no impact on the excision rate of phiCD630-2, suggesting that phiCD630-2 might encode an atypical, yet to be identified excisionase. TA systems have been suggested to play three important biological functions, i.e., stabilization of mobile genetic elements (post-segregational killing), abortive phage infection and persister cell formation⁴⁰. Prophage maintenance is among the suggested function of TA including a recent example of type II TA system that stabilizes prophage in *Shewanella oneidensis*⁴¹ and another type II TA system promoting the maintenance of an integrative conjugative element in *Vibrio cholerae*⁴². This physiological function in prophage stabilization was also suggested for type I TA modules but had never been experimentally demonstrated prior to this study⁴³. Prophage excision upon expression of *CD0912* made phiCD630-1 prone to be lost by the host cells and we could thus show that type I TA systems are important to maintain the episomal form of the phage into the host cell. In the *C. difficile* cells expressing the excisionase gene, the frequency of phiCD630-1ΔT4 loss was higher than that of wildtype phiCD630-1 and excision of phiCD630-1 was associated with a strong growth defect, which can be attributed to the post-segregational killing mechanism. The unstable antitoxin is likely degraded in daughter cells where the phage has been lost after cell division upon excision, leading to the toxin production from its stable mRNA and to the growth inhibition of the new cell. Expression of the excisionase gene in cells carrying the prophage devoid of the toxin genes also resulted in a moderate growth defect, suggesting that a supplementary TA system might be present in phiCD630-1 or that the excisionase has an additional function affecting the cell growth. Several experimental conditions were tested in this work to induce the loss of the phage from the cells. Surprisingly, four passages of the strain carrying phiCD630-1 with the intact toxin genes grown in TY broth with constant expression of the excisionase gene from a plasmid resulted in approximately 99% of loss of this prophage (data not shown). This is likely due to a progressive enrichment of the cell population surviving the loss of the phage

since the growth rate of this population is higher than that of the population bearing the phage. In any case, these data suggest that the identification and the overexpression of the phage excisionase-encoding genes could provide an easy and efficient way to cure *C. difficile* strains from their prophages.

Toxins of type I TA systems are relatively small proteins, and this is probably one of the reasons why they have remained uncharacterized and unexplored in *C. difficile* and in other organisms. In this study, we have come to realize that standard methods of annotation are unable to detect all toxin homologs present in prophage genomes. Novel toxin homologs, previously unannotated, could thus be detected inside plasmids of *C. difficile* and *P. sordellii*. It has been proposed that phages could recombine with plasmids during infection of the same or different bacterial species to exchange genetic material^{9, 36, 37}. It is therefore tempting to speculate that this TA system has the ability to disseminate, through horizontal gene transfer involving conjugation and recombination, from one species to another. Intriguingly, it was previously noticed that a 1.9-kb region could have been transferred from the plasmid of a *C. difficile* strain 630 to the phiCD38-2 prophage⁹. It was suggested that this recombination event had led to the acquisition of *parA*, a gene assumed to help the newly created chimeric phage to autonomously replicate and segregate as a circular plasmid. Our *in silico* search for TA systems in *C. difficile* phages reveals that this 1.9-kb region in phiCD38-2 also carried a TA (gp33) that presumably contributes to the phage maintenance and stability. It is interesting to observe that TA encoding regions can relocate from one mobile genetic element to another in this fashion, and that genes in proximity to the TA being transferred (i.e. *parA* gene) have more chances to become fixed in the newly integrated DNA. In the latter case, the region transferred seems to provide two complementary and beneficial features to the phage, i.e. the capacity to segregate successfully to the daughter cell, and the death of the cells upon curing if the phage has not been sequestered in both dividing cells. However, since TA

498 systems behave as selfish elements that promote their propagation within bacterial genomes
499 at the expense of their host ^{44, 45}, they are likely to be maintained and observed after their
500 transfer by recombination events, even if they bring no selective advantage.

501 Thus, the large distribution of type I TA modules within *C. difficile* prophages argues in
502 favour of their functional importance for prophage acquisition and transfer between *C.*
503 *difficile* strains. The position of these TA modules in the extremities of the prophage is also
504 consistent with their role in the entire prophage maintenance. In addition to prophage
505 excision, other genetic events could lead to potential prophage loss such as recombination
506 with other homologous phages. The presence of phage-like elements, cryptic phage
507 rudiments and incomplete prophages in bacterial chromosomes including *C. difficile* genome
508 attests on the frequency of such events. We could hypothesize that TA systems will
509 contribute not only to episomal prophage stability, but also to the maintenance of integrated
510 prophage.

511 Besides their biological functions, TA modules are also versatile tools for a multitude of
512 purposes in basic research and biotechnology ⁴⁶. For example, the MazF toxin-encoding gene
513 from *E. coli* is used as a counter-selection marker for chromosomal manipulation in *Bacillus*
514 *subtilis* and *C. acetobutylicum* ^{47, 48}. In this study, we engineered an inducible counter-
515 selection marker based on the *C. difficile* *CD2517.1* toxin gene of the *CD2517.1*-RCd8 TA
516 module. Artificial expression of *CD2517.1* from a plasmid in *C. difficile* leads to an
517 immediate interruption of the bacterial growth ²⁷. Taking advantage of this feature, we
518 generated novel vectors for allele exchange in *C. difficile* 630 (pMSR) and in *C. difficile*
519 ribotype 027 strains and other ribotypes strains (pMSR0). It should be noted that expression
520 of the RCd8 antitoxin from the pMSR0 vector was required to counteract the basal expression
521 of *CD2517.1* toxin gene due to the *P_{tet}* leakiness. In contrast, expression of the RCd8
522 antitoxin from the pMSR vector was not required since the *CD2517.1*-RCd8 TA module is

naturally present within the chromosome of *C. difficile* 630. Native expression of RCd8 was therefore sufficient to prevent CD2517.1 production from the plasmid. Our vectors are derived from those developed by Cartman *et al.*, which use the *codA* gene coding for cytosine deaminase as a counter-selection marker for allelic exchange mutations³². However, *codA*-based counter-selection was somewhat ineffective in our hands and false-positive counter-selected colonies with the plasmid still integrated into the chromosome were repeatedly found. This was reported by the authors as the consequence of loss-of-function mutations in genes leading to the bypass of the counter-selection. Our system proved to be much more efficient than all the others we have tested so far, and we did not observe any false-positive clones so far. The false-positive rate could be estimated to less than 0.1% since all counter-selected clones tested during about 50 mutant constructions attempts were thiamphenicol-sensitive, indicative of the plasmid loss. We successfully used this system to construct multiple mutants in various *C. difficile* strains, including the Δ T4 mutant, as well as deletion of a large chromosomal region of 50 kb corresponding to the phiCD630-2 prophage, and gene insertion into the bacterial chromosome (*ermB* gene). We therefore expect these new vectors to become invaluable genetic tools that will foster research in *C. difficile*.

MATERIALS AND METHODS

Plasmids, bacterial strains construction and growth conditions

C. difficile and *Escherichia coli* strains and plasmids used in this study are presented in Table S2. *C. difficile* strains were grown anaerobically (5 % H₂, 5 % CO₂, and 90 % N₂) in TY⁴⁹ or Brain Heart Infusion (BHI, Difco) media in an anaerobic chamber (Jacomex). When necessary, cefoxitin (Cfx; 25 µg/ml), cycloserine (Cs; 250 µg/ml) and thiamphenicol (Tm; 7.5 µg/ml) were added to *C. difficile* cultures. *E. coli* strains were grown in LB broth, and when needed, ampicillin (100 µg/ml) or chloramphenicol (15 µg/ml) was added to the culture medium. The non-antibiotic analogue anhydrotetracycline (ATc) was used for induction of

the P_{tet} promoter of pRPF185 vector derivatives in *C. difficile*⁵⁰. Strains carrying pRPF185 derivatives were generally grown in TY medium in the presence of 250 ng/ml ATc and 7.5 µg/ml Tm for 7 h, unless stated otherwise. Growth curves were obtained using a GloMax plate reader (Promega).

All primers used in this study are listed in Table S3. Details of vector construction are described in the Supplementary materials.

The resulting derivative plasmids were transformed into the *E. coli* HB101 (RP4) and subsequently mated with the appropriate *C. difficile* strains (Table S2). *C. difficile* transconjugants were selected by sub-culturing on BHI agar containing Tm (15 µg/ml), Cfx (25 µg/ml) and Cs (250 µg/ml).

Mutagenesis approach and mutant construction

To improve the efficiency of the allele exchange mutagenesis in *C. difficile*, we made use of the inducible toxicity of the CD2517.1 type I toxin that we previously reported²⁷. To construct the pMSR vector, used for allele exchange in *C. difficile* 630Δ*erm*, the *codA* gene was removed from the “pseudosuicide” vector pMTL-SC7315³² by inverse PCR and replaced by a 1169 bp fragment comprising the entire P_{tet} promoter system and the downstream *CD2517.1* toxin gene. This fragment was amplified from pDIA6319 plasmid²⁷ and the purified PCR product was cloned into the linearized plasmid. In parallel, the pMSR0 vector, for allele exchange in *C. difficile* ribotype 027 strains and other ribotypes, was constructed by removing the *codA* gene from the vector pMTL-SC7215 by inverse PCR and replacing it with the *CD2517.1*-RCd8 TA region with *CD2517.1* under the control of the P_{tet} promoter, as described above, and RCd8 under the control of its own promoter. For deletions, allele exchange cassettes were designed to have between 800 and 1050 bp of homology to the chromosomal sequence in both up- and downstream locations of the sequence to be altered.

The homology arms were amplified by PCR from *C. difficile* strain 630 genomic DNA (Table S3) and purified PCR products were directly cloned into the PmeI site of pMSR vector using NEBuilder HiFi DNA Assembly. To insert *P_{thl}-ermB* into the phiCD630-1 prophage, within the intergenic region between *CD0946.1* and *CD0947* genes, homology arms (~900 bp up- and downstream of the insertion site) were amplified by PCR from strain 630 genomic DNA (Table S3). The *P_{thl}-ermB* cassette was amplified from the Clostron mutant *cwp19*^{33, 51}. Purified PCR products were all assembled and cloned together into the PmeI site of pMSR vector using NEBuilder HiFi DNA Assembly.

All pMSR-derived plasmids were initially transformed into *E. coli* strain NEB10 β and all inserts were verified by sequencing. Plasmids were then transformed into *E. coli* HB101 (RP4) and transferred by conjugation into the appropriate *C. difficile* strains. The adopted protocol for allele exchange was similar to that used for the *codA*-mediated allele exchange³², except that counter-selection was based on the inducible expression of the *CD2517.1* toxin gene. Transconjugants were selected on BHI supplemented with Cs, Cfx and Tm, and then restreaked onto fresh BHI plates containing Tm. After 24 h, faster-growing single-crossover integrants formed visibly larger colonies. One such large colony was restreaked once or twice on BHI Tm plate to ensure purity of the single crossover integrant. Purified colonies were then restreaked onto BHI plates containing 100 ng/ml ATc inducer to select for cells in which the plasmid had been excised and lost. In the presence of ATc, cells in which the plasmid is still present produce CD2517.1 at toxic levels and do not form colonies. Growing colonies were then tested by PCR for the presence of the expected deletion.

Light microscopy

For light microscopy, bacterial cells were observed at 100x magnification on an Axioskop Zeiss Light Microscope. Cell length was estimated for more than 100 cells for each strain using ImageJ software⁵².

Subcellular localization of HA-tagged toxins by cell fractionation and Western blotting

C. difficile cultures were inoculated from overnight grown cells in 10 ml of TY medium at an optical density at 600 nm (OD₆₀₀) of 0.05. Cultures were allowed to grow for 3 hours before the addition of 250 ng/ml ATc and incubation for 90 min. Then, cells were centrifuged and proteins were extracted. Cell lysis, fractionation and protein analysis were performed as previously described⁵³. Coomassie staining was performed for loading and fractionation controls. Western blotting was performed with anti-HA antibodies (1:2, 000) (Osenses) using standard methods.

Alkaline phosphatase activity assays

C. difficile strains containing the *phoZ* reporter fusions were grown and harvested in exponential growth phase, at the onset of stationary phase and under nutrient starvation conditions. Starvation conditions correspond to a 1 h incubation of exponentially grown cells in PBS buffer at 37 °C in anaerobic conditions. Alkaline phosphatase assays were performed as described previously⁵⁴.

RNA extraction, Northern blot and 5'/3'RACE

Total RNA was isolated from *C. difficile* strains after 4, 6 or 10 hrs of growth in TY medium, or 7.5 hrs in TY medium containing 7.5 µg/ml of Tm and 250 ng/ml of ATc for strains carrying pRPF185 derivatives, as previously described⁵⁵. Starvation conditions corresponded to a 1 h incubation of exponentially grown cells (6 h of growth) in PBS buffer at 37°C. Northern blot analysis and 5'/3'RACE experiments were performed as previously described²⁸.

RNA band shift assay

Templates for the synthesis of RNA probes were obtained by PCR amplification using the Term and T7 oligonucleotides (Table S3). The three RNAs (*CD0977.1* toxin mRNA, the short and the long RCd11 antitoxin forms) were synthesized by T7 RNA polymerase and RNA concentrations were monitored by measuring the absorbance at 260 nm. Just before use, *CD0977.1* was also transcribed with (α -³²P) UTP yielding uniformly labelled RNA and traces were added to the unlabelled *CD0977.1*. This strategy was used because of the very low efficiency of 5' labelling of all these transcripts. *CD0977.1* transcript was incubated with increasing concentrations of RCd11 short or long RNAs under two different conditions referred as Native and Full RNA duplex conditions as in ²⁷. The complexes were immediately loaded on native polyacrylamide gels to control for hybridization efficiency. RNA levels were quantified by phosphoimagery.

Measurement of RNA decay by rifampicin assay

For determination of toxin and antitoxin RNA half-lives the *C. difficile* strains were grown in TY medium supplemented with 250 ng/ml ATc and 7.5 μ g/ml Tm for 7.5 h at 37°C. Samples were taken at different time points after the addition of 200 μ g/mL rifampicin (0, 2, 5, 10, 20, 40, 60 and 120 min) and subjected to RNA extraction and Northern blotting.

Plasmid stability assays

Overnight cultures of *C. difficile* cells containing the pMTL84121 empty vector or the pMTL84121 derivatives were grown in TY broth with Tm and used to inoculate (at 1%) 5 ml of fresh TY broth without antibiotic. Every 10 to 14 hrs, 1% of the cultures were reinoculated into fresh TY broth without antibiotic. After seven passages, CFUs were estimated on TY plates supplemented or not with Tm to differentiate between the total number of cells and the plasmid-containing cells.

Quantification of the frequency of prophage excision

The frequency of prophage excision in different *C. difficile* strains was estimated by quantitative PCR on genomic DNA extracted using the NucleoSpin Microbial DNA kit (Macherey-Nagel). The total chromosome copy number was quantified based on the reference gene *dnaF* (*CD1305*) encoding DNA polymerase III. The number of chromosomes devoid of phiCD630-1 was quantified by PCR amplification using primers flanking phiCD630-1 (Table S3), which only results in PCR products when the prophage is excised.

PhiCD630-1 stability assays

Overnight cultures of *C. difficile* strain 630 Δ phiCD630-2 phiCD630-1::*erm* and Δ phiCD630-2 phiCD630-1- Δ T4::*erm* were used to inoculate 10 ml of TY broth at an initial OD₆₀₀ of 0.05. Every 10 to 14 h, cultures were subcultured at an initial OD₆₀₀ of 0.05. After four passages, the cultures were serially diluted and plated on BHI plates to estimate the total CFUs and on BHI plates supplemented with 2.5 μ g/ml erythromycin to determine the number of CFUs in which phiCD630-1 was still present. For cells expressing the excisionase gene, overnight cultures of *C. difficile* strain 630 Δ phiCD630-2 phiCD630-1::*erm* and Δ phiCD630-2 phiCD630-1- Δ T4::*erm* carrying pDIA6867 were used to inoculate fresh TY broth with Tm and 10 ng/ml ATc at an initial OD₆₀₀ of 0.005. After 24 hrs of incubation at 37°C, cultures were serially diluted and plated on BHI plates to estimate the total CFUs and on BHI plates supplemented with 2.5 μ g/ml erythromycin to determine the number of CFUs in which phiCD630-1 was still present. The OD₆₀₀ of the cultures was also measured to monitor cell growth.

ACKNOWLEDGEMENTS

This work was supported by Agence Nationale de la Recherche (“CloSTARn”, ANR-13-JSV3-0005-01 to O.S.), the Institut Universitaire de France (to O.S.), the University Paris-

Saclay, the Institute for Integrative Biology of the Cell, the Pasteur Institute, the DIM-
1HEALTH regional Ile-de-France program (LSP grant no. 164466), the CNRS-RFBR PRC
2019 (grant no. 288426, research project № 19-54-15003) to O.S., and a Vernadski
fellowship to A.M.

We would like to thank **Shonna McBride for the gift of the pMC358 vector** and Marc Monot
for helpful discussions.

AUTHOR CONTRIBUTIONS

J.P. and O.S. conceived and coordinated the study, which was initiated by P.B. J.P. and O.S.
performed the majority of the experiments. A.H. constructed vectors and deletion mutants.
J.R.G. performed the *in-silico* analyses, A.M. performed growth curves and light microscopy.
L-C.F. and B.D. provided scientific insight into the design of the experiments. J.P. and O.S.
wrote the paper and all authors reviewed and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

FIGURE LEGENDS

Figure 1. Identification and functionality of novel toxin genes within phiCD630-1. (A)
Protein alignment of toxin CD0977.1 with the newly identified CD0904.1, CD0956.2 and
CD0956.3. The hydrophobic and positively charged amino acids are indicated in red and
blue, respectively. (B) Maps and alignment of the phiCD630-1 and phiCD630-2 genomes.
The location of toxin genes in both prophages is indicated. (C) Growth of *C. difficile*
630Δ*erm* strains harbouring the pRPF185-based plasmids on BHI agar plates supplemented
with Tm and with (+ATc) or without 10 ng/ml of ATc inducer (-ATc) after 24 hrs of
incubation at 37°C. Schematic representations of the constructs are shown.

Figure 2. Detection of *CD0977.1* and RCd11 transcripts and CD0977.1-HA protein. (A)

A schematic of the *CD0977.1*-RCd11 TA pair genomic region and of the corresponding transcripts as identified by 5'/3'RACE and Northern blot. The Cdi1_4 riboswitch and the identified promoters are represented. The position of the different probes used in the Northern blot experiments is shown. (B) Northern blot of total RNA from *C. difficile* carrying p (empty

vector), pT (expression of *CD0977.1*) or pTA (expression of *CD0977.1* and its antitoxin) in the absence (- ATc) or in the presence (+ ATc) of 250 ng/ml of the inducer ATc. (C)

Northern blot of total RNA from *C. difficile* carrying p (empty vector) or *pdccA* (expression of the diguanylate cyclase encoding gene *dccA*) in the presence of 250 ng/ml ATc. (D)

Northern blot of total RNA from *C. difficile* 630 Δ *erm* carrying an empty vector (wt/p) collected at the indicated time after addition of rifampicin. All Northern blots were probed

with a radiolabelled oligonucleotide specific to the toxin (T CD0977.1/CD2889), the antitoxin (AT RCd11/RCd12) or the Cdi1_4/Cdi1_5 riboswitch (Riboswitch RCd11/RCd12)

transcript and 5S RNA at the bottom serves as loading control. The arrows show the detected transcripts with their estimated size. The relative intensity of the bands was quantified using

the ImageJ software. The half-lives for toxin and antitoxin transcripts were estimated from three independent experiments. (E) Detection and subcellular localization of the CD0977.1-

HA protein. Immunoblotting with anti-HA detected a major polypeptide of ~12 kDa in whole cell extracts of *C. difficile* carrying pT-HA (CD0977.1-HA) grown in the presence of 250

ng/ml of ATc but not in extracts of *C. difficile* carrying pT (non-tagged CD0977.1) (left panel). The culture of *C. difficile* carrying pT-HA was fractionated into supernatant (SN), cell

wall (CW), membrane (Mb) and cytosolic (Cy) compartments and immunoblotted with anti-HA antibodies. Proteins were separated on 12% Bis-Tris polyacrylamide gels in MES buffer.

Figure 3. Impact of toxin-antitoxin co-expression on growth. The effect on the toxicity of

CD0977.1 of long and short antitoxin transcripts expressed *in cis* (A) and *in trans* (C) was

assessed. (A) and (C) Growth of *C. difficile* 630 Δ *erm* strains harbouring the pRPF185-based plasmids on BHI agar plates supplemented with Tm and 10 ng/mL of ATc inducer after 24 hrs of incubation at 37°C and in TY broth at 37°C in the presence of 5 ng/mL ATc. Schematic representations of the constructs are shown. Plotted values represent means \pm standard deviations ($N = 3$). (B) Alkaline phosphatase activity of the RCd11 promoter::*phoZ* reporter fusions measured after 4 (exponential) and 10 h (stationary) of growth in TY broth or under nutrient starvation conditions. A schematic of the *CD0977.1*-RCd11 TA pair genomic region and of the locations and sizes of promoter fragments constructed for the *phoZ* reporter fusions is shown. Values represent means \pm standard deviations ($N = 4$). ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$ by a two-way ANOVA followed by a Dunnett's or Tukey's multiple comparison test.

Figure 4. Impact of TA modules on plasmid loss in the absence of selection pressure.

The stability of pMTL84121 (p, empty vector) and pMTL84121-derived vectors expressing the different TA modules in *C. difficile* 630 Δ *erm* was determined after seven passages (every 12 hours) in TY broth without thiamphenicol. Values represent means \pm standard deviations ($N = 3$). ** $P \leq 0.05$ and *** $P \leq 0.001$ by an unpaired *t* test.

Figure 5. Impact of TA modules on prophage maintenance. (A) Schematic representation

of the method used to quantify prophage maintenance. A cassette containing an erythromycin resistance gene (*ermB*) under control of the strong *thl* promoter of *C. acetobutylicum* was introduced into an innocuous location of phiCD630-1, within the intergenic region between *CD0946.1* and *CD0947* genes, encoding a hypothetical protein and a putative scaffold protein, respectively. Cultures grown for 24 h were plated on erythromycin-containing agar plates and cells that lost the prophage were selectively killed. (B) Strains Δ phiCD630-2 phiCD630-1::*erm* and Δ phiCD630-2 phiCD630-1- Δ T4::*erm* carrying a vector control or pDIA6867 (overproducing the excisionase CD0912) were inoculated at an initial optical

density at 600 nm (OD_{600nm}) of 0.005 in TY medium supplemented with 7.5 μ g/ml Tm and 10 ng/ml ATc. Cultures were incubated at 37°C and bacterial growth was determined by measurement of the OD_{600nm} after 16 h and 24 h. ** $P \leq 0.01$ and **** $P \leq 0.0001$ by a two-way ANOVA followed by a Tukey's multiple comparison test. (C) Maintenance of prophages in strains Δ phiCD630-2 phiCD630-1::erm and Δ phiCD630-2 phiCD630-1- Δ T4::erm carrying pDIA6867 after 24 h of growth as in (B) was quantified by plating serial dilutions on agar plates supplemented or not with 2.5 μ g/ml Erm.. Values represent means \pm standard deviations ($N = 3$). *** $P \leq 0.001$ by an unpaired t test.

Figure 6. Relationship between phage phylogeny and toxin variants. Putative toxin protein sequences detected in all available phage genomes were aligned using MUSCLE (v3.8) algorithm (EMBL-EBI). All phage genomes were re-scanned for potential ORFs using the NCBI ORFfinder software and detected ORFs were translated into their corresponding protein sequence. Protein sequences were combined to create a local BLASTp database and confirmed functional toxins CD0956.2 (large variant) and CD0904.1 (short variant) were used as queries to search for putative toxins in the database. Significant hits (min 45% identity, min 28% coverage) were retrieved and the corresponding proteins were aligned using MUSCLE (v3.8) algorithm (EMBL-EBI). The protein sequence consensus is shown. A phylogenetic tree was built using the Poisson distance method and neighbour joining implemented in Seaview (v4.4.2). Residues were coloured according to high hydrophobicity (red) and low hydrophobicity (blue).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. RNA-seq and TSS mapping profiles for the TA loci in *C. difficile* strain 630.

The TAP-/TAP+ profile comparison for 5'-end RNA-seq data is aligned with RNA-seq data

for *CD0904.1*-RCd13 (A) and *CD0956.3*-RCd14 (B) and *CD0977.1*-RCd11 (C) TA genomic regions. The TSS are indicated by red broken arrows in accordance with the positions of 5'-transcript ends shown by vertical green lines on the sequence read graphs corresponding to TSS. Potential processing site is shown in (C) by vertical green arrow. TSS corresponds to positions with significantly greater numbers of reads in TAP+ samples. 5'-end sequencing data show 51-bp reads matching to the 5'-transcript ends, while RNA-seq data show reads covering the whole transcript. Coding sequences are indicated by blue arrows and the regulatory RNAs are indicated by grey arrows. The promoter regions of the antitoxins are shown. The promoter region of RCd14 antitoxin was deduced from the alignment in (D). (D) Alignment between the sequences of *CD0904.1*-RCd13 (top line) and *CD0956.3*-RCd14 (bottom line) using EMBOSS Needle. Green boxes show the open reading frames of *CD0904.1* and *CD0956.3* and green arrows indicate the direction of the genes. Blue boxes show the -10 and the -35 boxes of the promoter regions and black boxes show the transcription start site of RCd13 and RCd14. In (A), (B) and (C), the red boxes show the Sigma A-dependent promoter -10 and -35 elements and the yellow boxes show the Sigma B-dependent promoter element.

Figure S2. Impact of toxin CD0977.1 expression on cell viability and morphology.

Growth (A) and viability (B) of *C. difficile* 630 Δ *erm* strain carrying the pRPF185-based plasmids (empty: p or with *CD0977.1* toxin gene under the control of the P_{tet} promoter: pT) in TY broth in presence of 200 ng/ml ATc. The time point of ATc addition is indicated by a vertical arrow. Values represent means \pm standard deviations ($N=3$). * $P \leq 0.05$ by a Student's t test. (C) Selected images from light microscopy observations of 630/p, 630/pT and 630/pTA strains grown in TY broth for 1 h at 37°C after the addition of 250 ng/mL ATc. Cell length was estimated using the ImageJ software for at least 115 cells per strain. The mean values with standard deviations are indicated for each strain, as well as the proportion

788 of cells with length above 2 standard deviations relative to the 630/p control strain mean
789 length.

790 **Figure S3. Sequence of type I *CD0977.1*-RCd11 TA locus in *C. difficile*.** The thick
791 horizontal arrows below the double-stranded sequences show the toxin and antitoxin
792 transcripts and the direction of transcription. The transcriptional start sites for sense and
793 antisense transcripts identified by 5'/3'RACE and TSS mapping are indicated by vertical
794 arrows with their genomic location. Line thickness corresponds to the proportion of observed
795 extremities. The genomic location of 5'- and 3'-ends of the transcripts are indicated above the
796 sequence. Potential processing site is shown by vertical green arrow. The inverted repeats at
797 the position of transcriptional terminators are indicated by thin black arrows. The positions of
798 Sigma A-dependent -10 and -35 promoter elements of antitoxin (AT) are shown in red boxes.
799 The positions of Sigma A-dependent -10 and -35 elements promoter, ribosome binding site,
800 translation initiation codon and stop codon of toxin (T) mRNA are shown in blue boxes. The
801 positions of Sigma B-dependent promoter elements are shown in green boxes for both TA
802 genes.

803 **Figure S4. Analysis of TA RNA duplex formation by RNA band shift assay.**
804 Radiolabeled *CD0977.1* transcript was incubated with increasing concentrations of RCd11
805 short or long RNAs under two different conditions referred as native and full RNA duplex
806 conditions. Native *CD0977.1*:RCd11 complexes were formed at 37 °C for 5 min in TMN
807 buffer, and full duplexes were obtained after a denaturation-annealing treatment in TE Buffer
808 (2 min 90°C, 30 min 37°C). The complexes were immediately loaded on native
809 polyacrylamide gels to control for hybridization efficiency. RNA levels were quantified by
810 phosphoimager and complex proportion is indicated.

Figure S5. Northern blots showing the stability of *CD0977.1/CD2889* toxin (A) and RCd11/RCd12 antitoxin (B) transcripts in strains depleted for RNase III, RNase Y, RNase J and Hfq. To determine half-lives, samples were taken at the indicated time points after the addition of 200 µg/mL rifampicin. RNAs were extracted from strains CDIP369 (630/p), CDIP230 expressing an antisense RNA for the *rncS* gene encoding RNase III (AS *rncS*), CDIP53 strain expressing an antisense RNA for the *hfq* gene (AS *hfq*), CDIP55 strain expressing an antisense RNA for the *rnJ* gene encoding RNase J (AS *rnJ*) and CDIP57 strain expressing an antisense RNA for the *rny* gene encoding RNase Y (AS *rny*). All Northern blots were probed with a radiolabelled oligonucleotide specific to the toxin (T *CD0977.1/CD2889*) or the antitoxin (AT RCd11/RCd12) transcript and 5S RNA at the bottom serves as loading control. The relative intensities of the bands were quantified using ImageJ software. **The half-lives for toxin and antitoxin transcripts have been estimated from three independent experiments.**

Figure S6. Impact of toxin-antitoxin co-expression on growth. The effect on the toxicity of *CD0977.1* of long and short antitoxin transcripts expressed *in cis* (A) and *in trans* (C) was assessed. **(A) and (C)** Growth of *C. difficile* 630 Δ *erm* strains harbouring the pRPF185-based plasmids on BHI agar plates supplemented with Tm and the indicated concentration of ATc inducer after 24 hrs of incubation at 37°C. Schematic representations of the constructs are shown. **(B) Alkaline phosphatase activity of the RCd11 promoter P_1 + disrupted $P_2::phoZ$ reporter fusions and the promoterless *phoZ* measured after 4 h (stationary) of growth in TY broth. Values represent means \pm standard deviations ($N = 3$).**

Figure S7. Nucleotide alignment of antitoxins. (A) Nucleotide alignment of RCd11 and RCd12 using LALIGN. (B) Nucleotide alignment of RCd11 and RCd10 using LALIGN.

834 **Figure S8. Secondary structure prediction of *CD0977.1* mRNAs and corresponding**
835 **antitoxin RCd11 RNAs.** The RNA secondary structure predictions were performed by
836 Mfold software. **The predictions for long and short forms of RCd11 antitoxins are shown.**
837 Ribosome binding site (SD), translation initiation codon and stop codon positions are
838 highlighted. The positions of mismatches in RCd12 AT sequence are indicated.

839 **Figure S9. New vector for efficient gene editing in *C. difficile*.** Features of the pMSR (A)
840 and pMSR0 (B) vectors used for allele exchange in *C. difficile* 630 Δ erm and *C. difficile* 027
841 ribotype strains, respectively. The toxin gene *CD2517.1* is under the control of the ATc
842 inducible promoter P_{tet} and the RCd8 antitoxin present in pMSR0 is under control of its own
843 promoter. Schematic overview of the allele exchange protocol (C) and of the inducible
844 counterselection method used to isolate double cross-over clones (D). Isolated single cross-
845 over integrants were restreaked on ATc-containing agar plates to induce synthesis of toxin
846 CD2517.1. Cells that kept the pMSR plasmid (either integrated or excised) produced
847 CD2517.1 and were selectively killed.

848 **Figure S10. Deletion of the phiCD630-2 prophage from *C. difficile* 630 Δ erm using the**
849 **newly developed allele exchange method.** (A) Schematic representation of phiCD630-2 in
850 *C. difficile* 630 Δ erm. The location of primers used to screen for mutants is represented. (B)
851 PCR products amplified using the indicated primers from the parental strain 630 Δ erm (WT)
852 and the Δ phiCD630-2 strain (Δ). A product of 1,348 bp could be amplified with primers
853 JP528-JP527 if phiCD630-2 had been deleted, whereas a product of 1,339 bp could be
854 amplified with primers JP570-JP527 if phiCD630-2 was still present.

855 **Figure S11. Maintenance and site-specific excision of phiCD630-1 from genomic DNA of**
856 ***C. difficile* 630.** (A) Schematic representation of phiCD630-1 DNA excision from genomic
857 DNA of *C. difficile* 630, and circularization. The location of primers used to demonstrate

858 prophage excision is represented. (B) PCR products amplified using the indicated primers
859 from *C. difficile* 630 genomic DNA. (C) DNA sequences within *attP*, *attB*, *attL* and *attR* as
860 determined by Sanger sequencing. The central identical sequences where recombination
861 occurs are shown in bold. Short segments of sequence surrounding the central identity region
862 are shown in blue (bacterial sequences) and in red (phage sequences). (D) The maintenance
863 of prophage in strains Δ phiCD630-2 phiCD630-1::*erm* and Δ phiCD630-2 phiCD630-1-
864 Δ T4::*erm* was determined after four passages in TY broth by plating serial dilutions on agar
865 plates supplemented or not with 2.5 μ g/ml Erm. Values represent means \pm standard
866 deviations ($N = 3$).

867 **Figure S12. Impact of excisionase (CD0912) overproduction on excision of phiCD630-1**
868 **and phiCD630-1- Δ T4. The frequency of prophage excision was estimated by quantitative**
869 **PCR as described in Materials and Methods section.** Excision rate of phiCD630-1 was higher
870 in *C. difficile* carrying pDIA6867 (inducible expression of *CD0912*) in presence of 10 ng/ml
871 of the inducer ATc when compared to the Δ phiCD630-2 strain without plasmid and was not
872 impacted by the deletion of toxin genes. Values represent means \pm standard deviations
873 ($N = 3$).

874 **Figure S13. Heatmap and phylogenetic tree showing *C. difficile* relatedness for 27**
875 **sequenced *C. difficile* phage genomes available in the NCBI database.** The Gegenees
876 software (v2.2.1) was used to produce the heatmap of genome similarity. Similarity scores
877 are based on a fragmented all-against-all pairwise alignment using BLASTn and the accurate
878 alignment option (fragment size, 200; step size, 100). The colours reflect the similarity,
879 ranging from low (red) to high (green). Phages were assigned to a genus if they clustered
880 closely to another phage previously described as a member of that genus. The phylogenetic
881 tree is based on the sequence similarity scores from the same whole-genome comparison and
882 was constructed using the neighbour joining method with the SplitsTree4 software (v 4.13.1).

Figure S14. Identification of toxin homologs outside *C. difficile* phages. Toxin homologs found outside *C. difficile* phages were aligned. The protein sequence consensus is shown at the bottom. Phylogenetic analysis of toxins is also represented. Red dot with tag “small variant 1” indicates CD0904.1. Red dot with tag “small variant 2” indicates CD0956.3.

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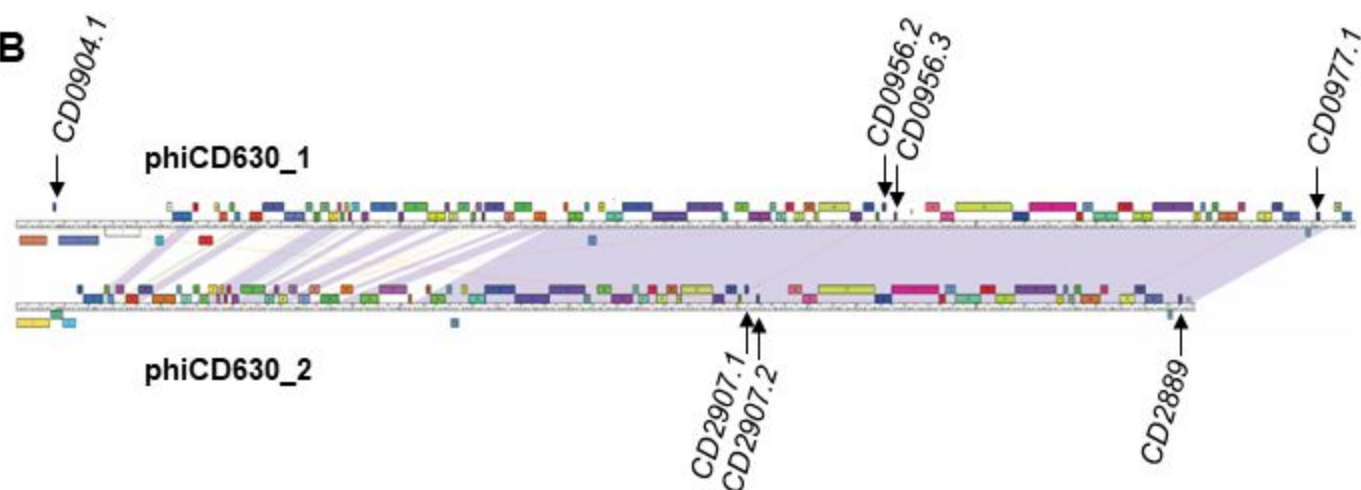
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Fig. 1

A

CD0956.3 -MIGFLLSILAGVISAYTYD-----KINIPDANNG-----DLAG
 CD0904.1 -MLINFLISIVAGIVSAYTYE-----KINIPDANNG-----DLAG
 CD0956.2 -MSEFLIGVLASITASFITYIIS---KVESIS---GSDFEIDVIRININQ
 CD0977.1 -MNNFLINVIAGVIASLIFCLICVFLVISTGSGSGWEEDFIRKPRPT-

B



C

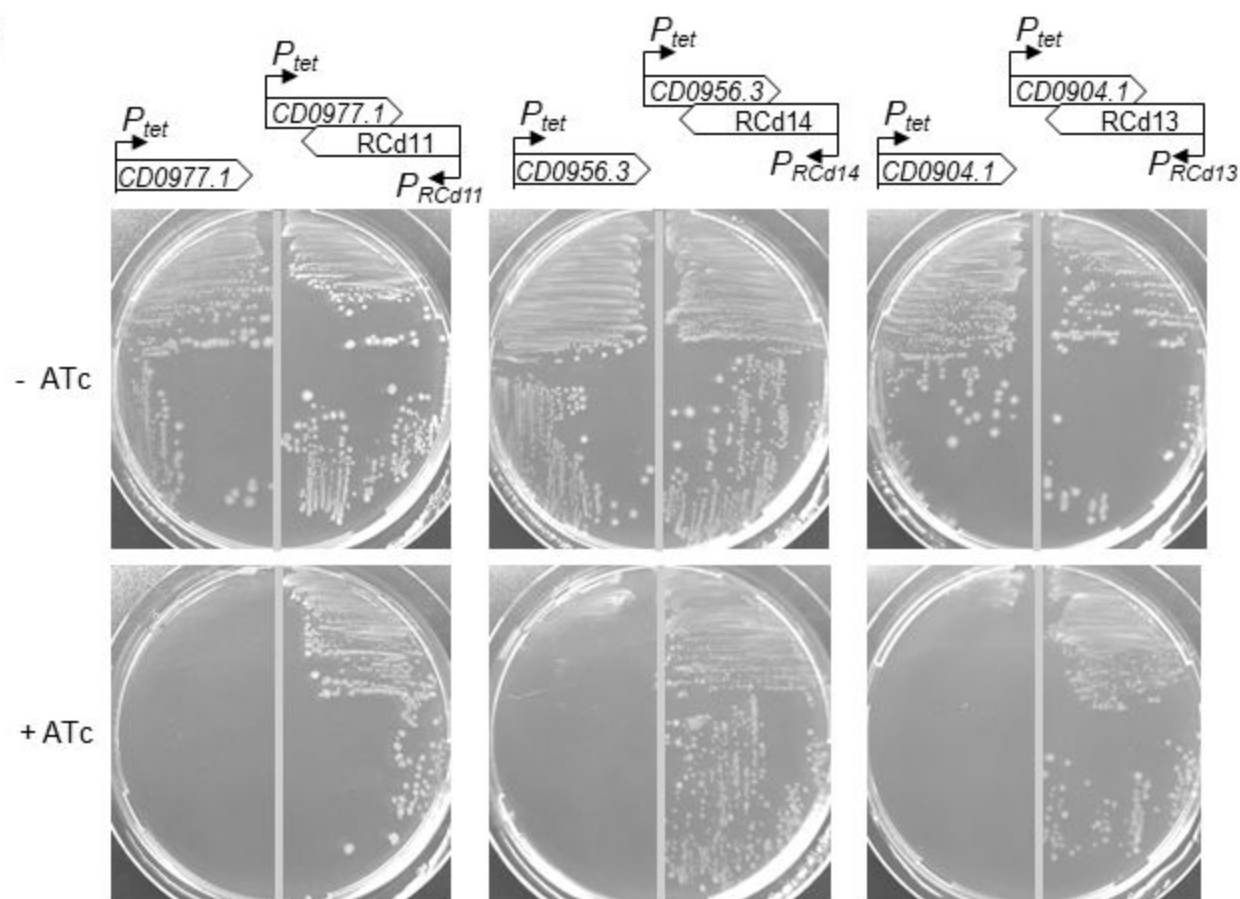


Fig. 2

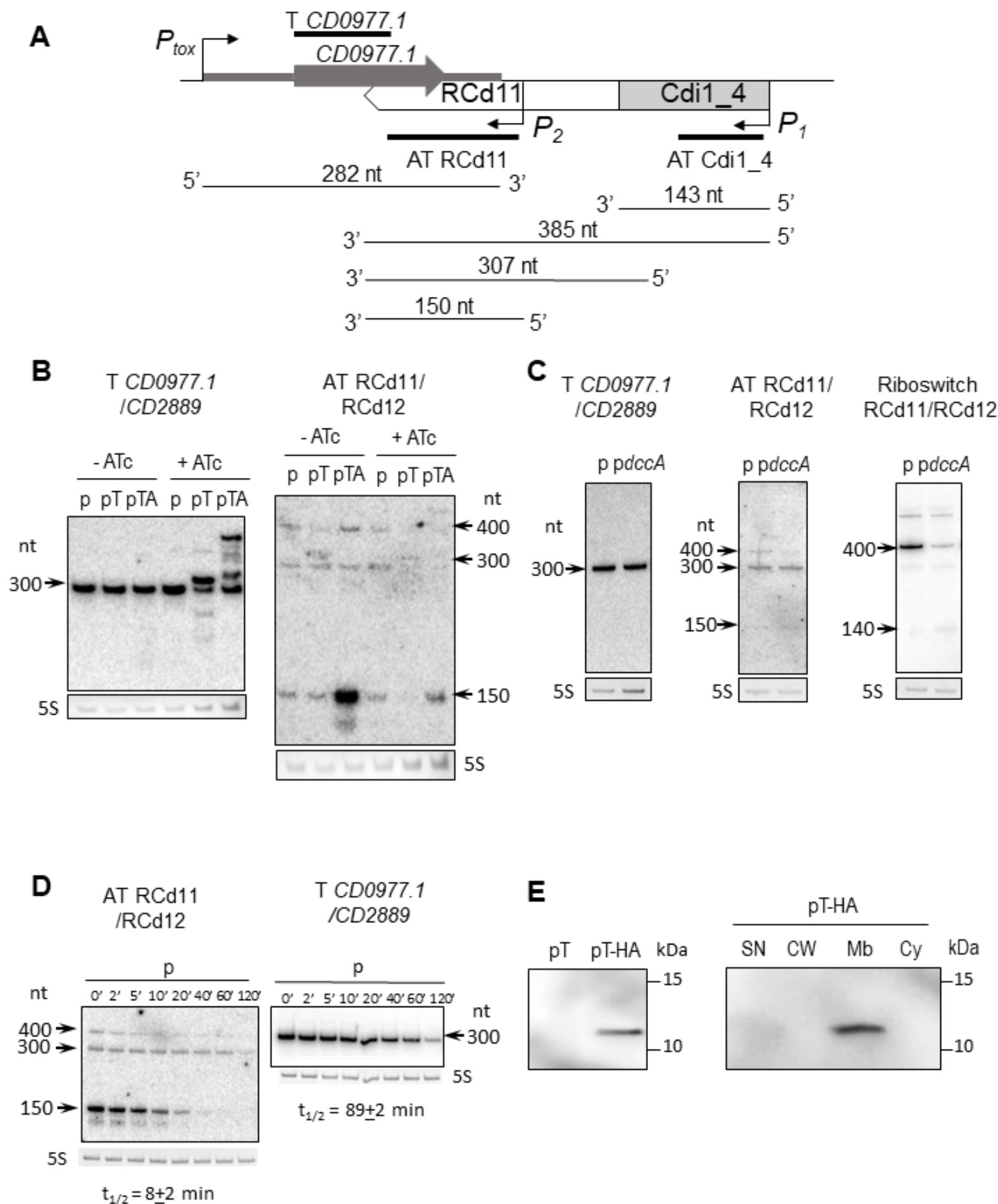


Fig. 3

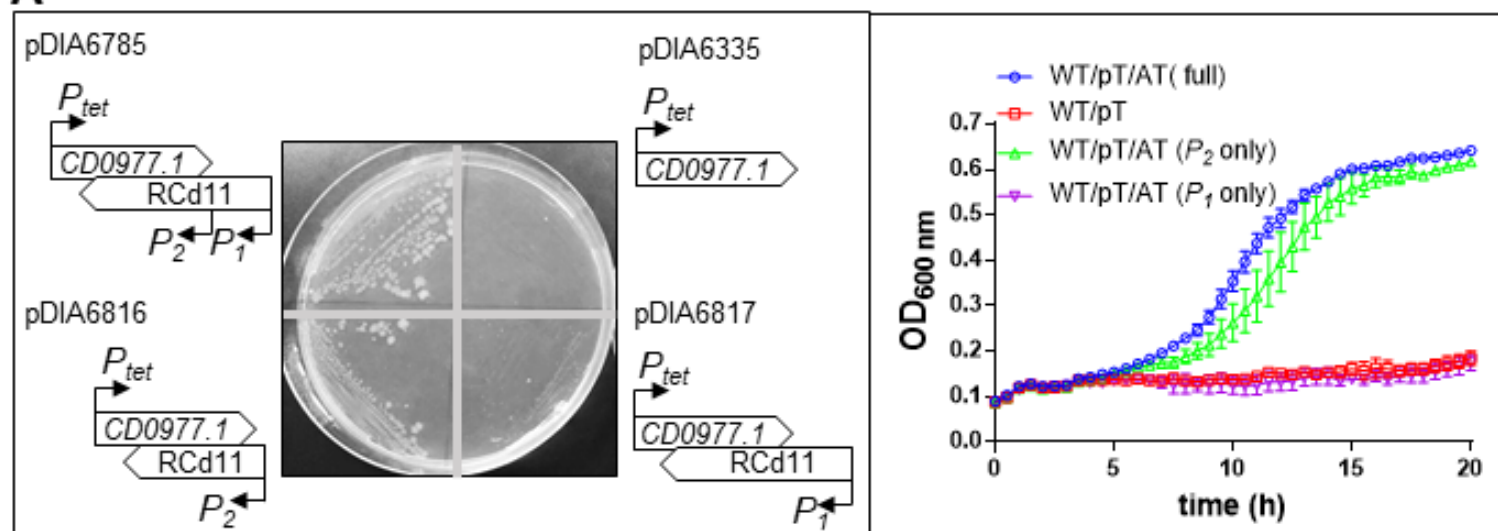
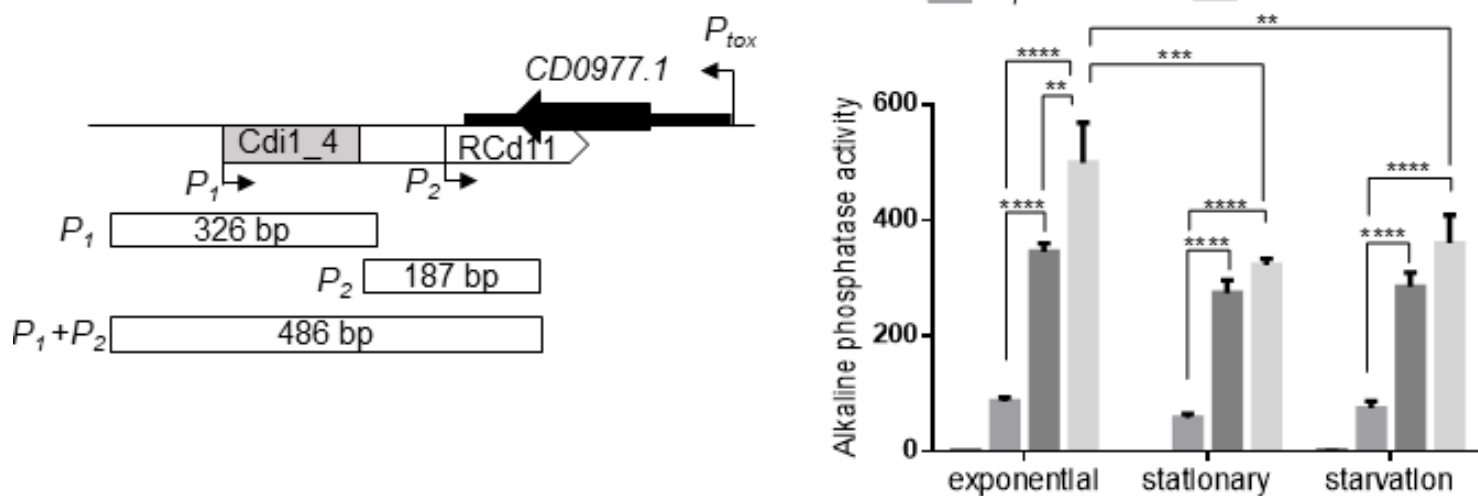
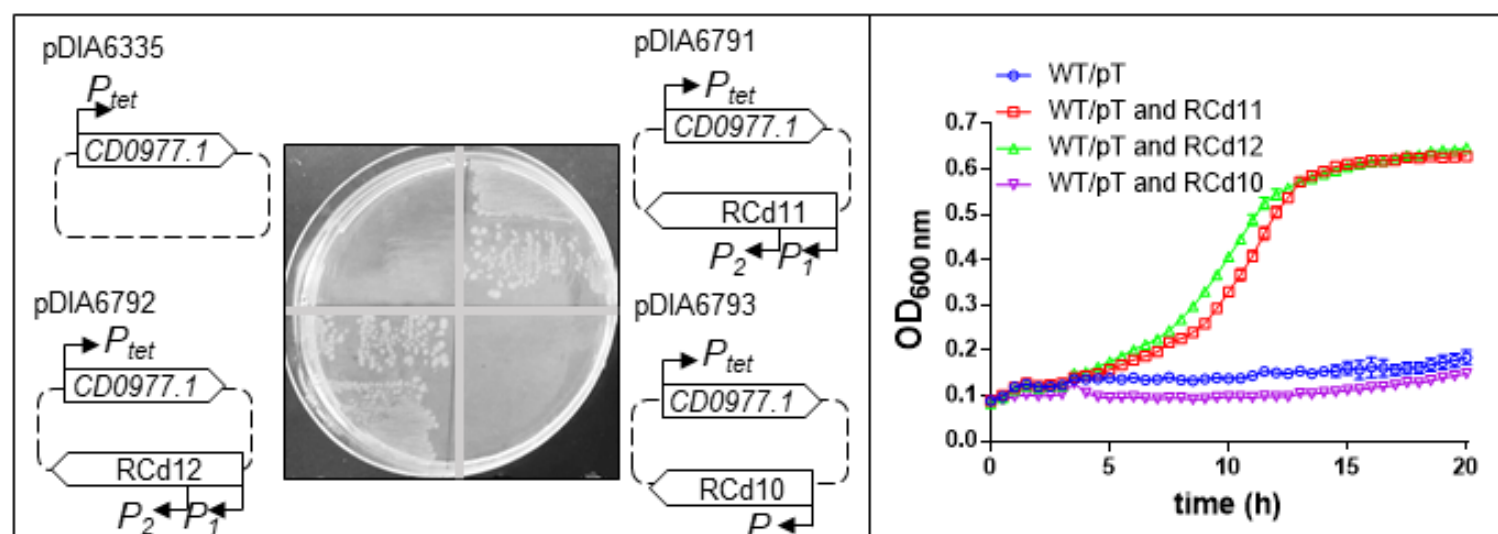
A**B****C**

Fig. 4

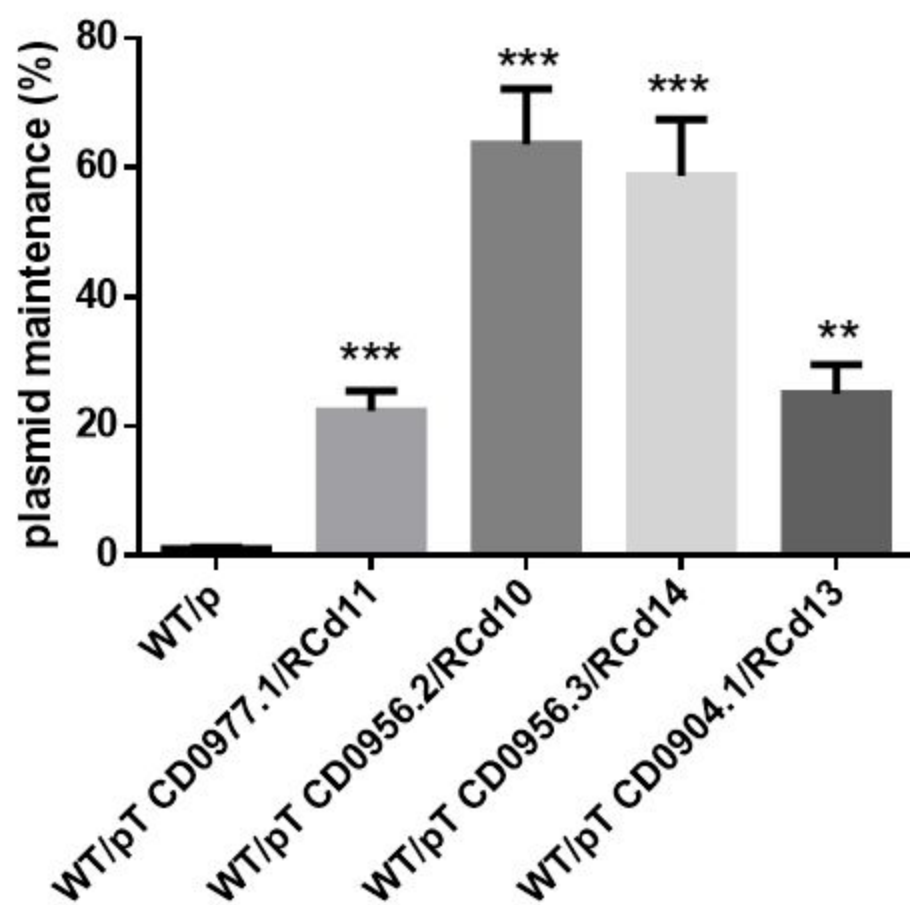
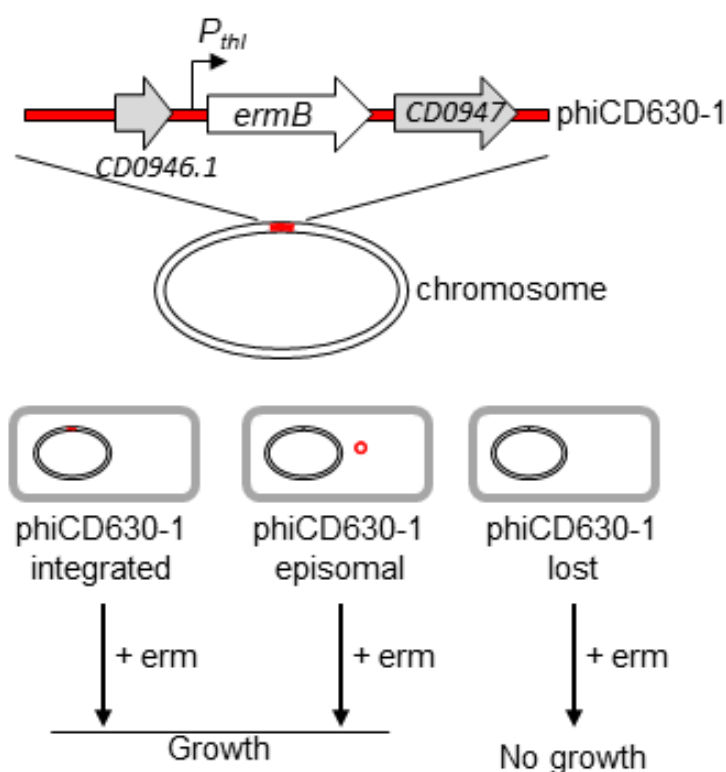
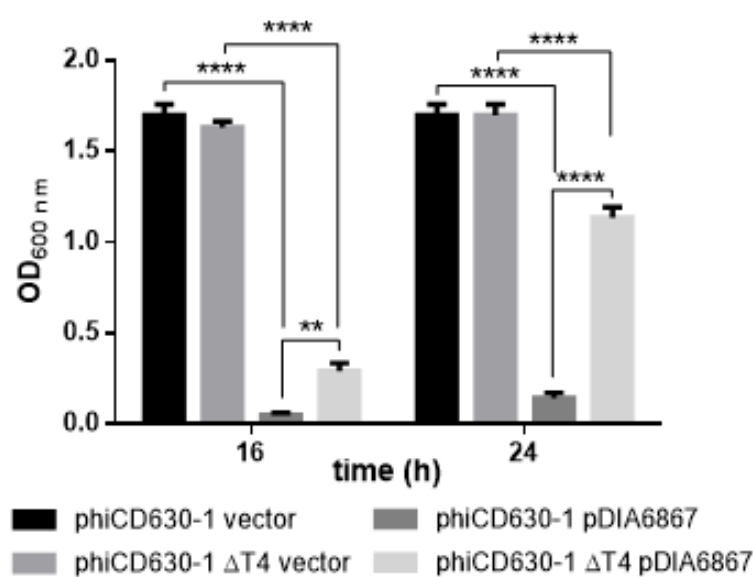


Fig. 5

A



B



C

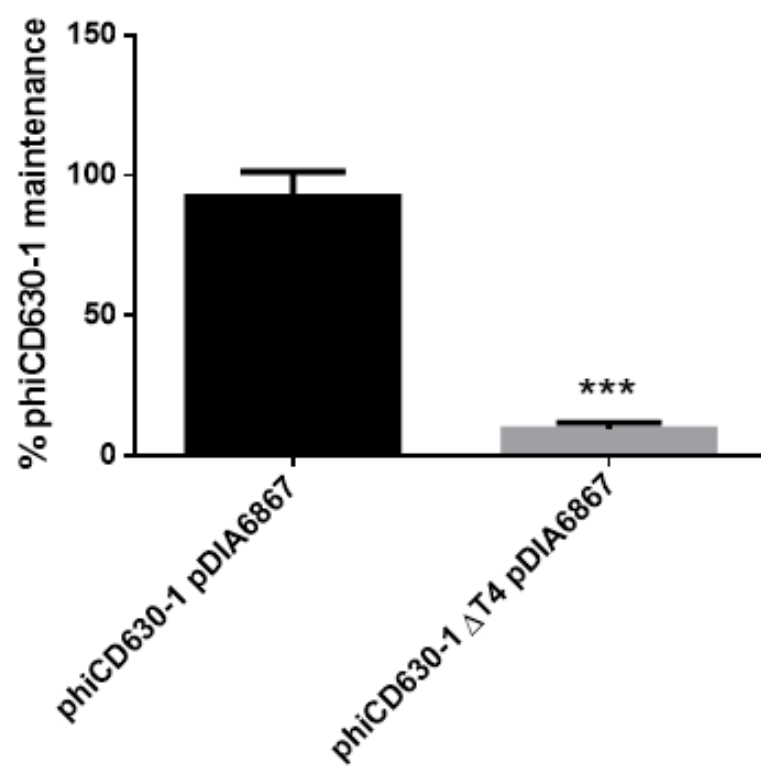
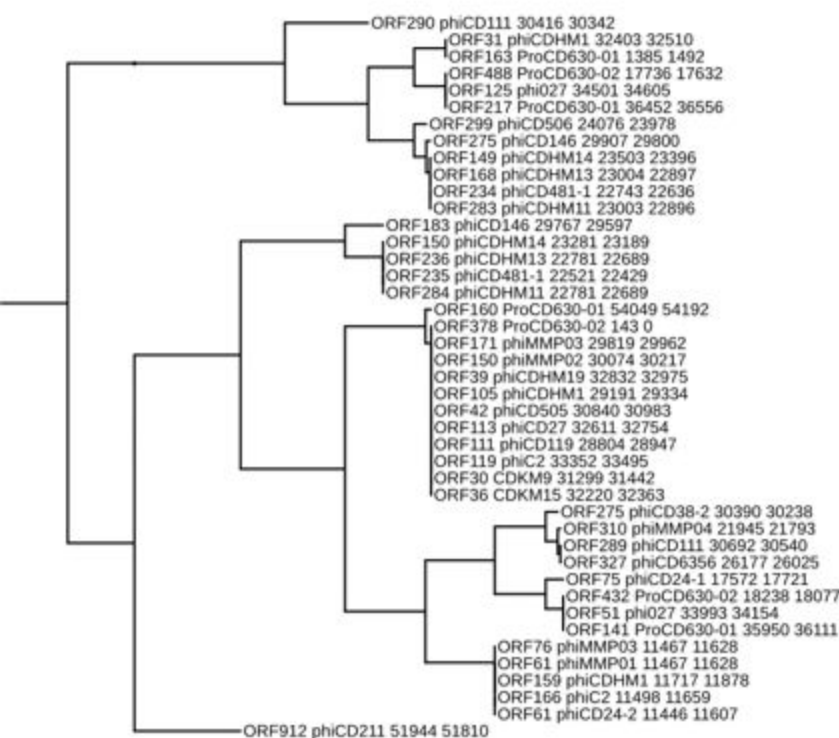


Fig. 6

- phiCD119likevirus
- phiCD38-2likevirus
- phiCDMMP04likevirus
- Unclassified phages



Tree scale: 0.1

Consensus (50%)

