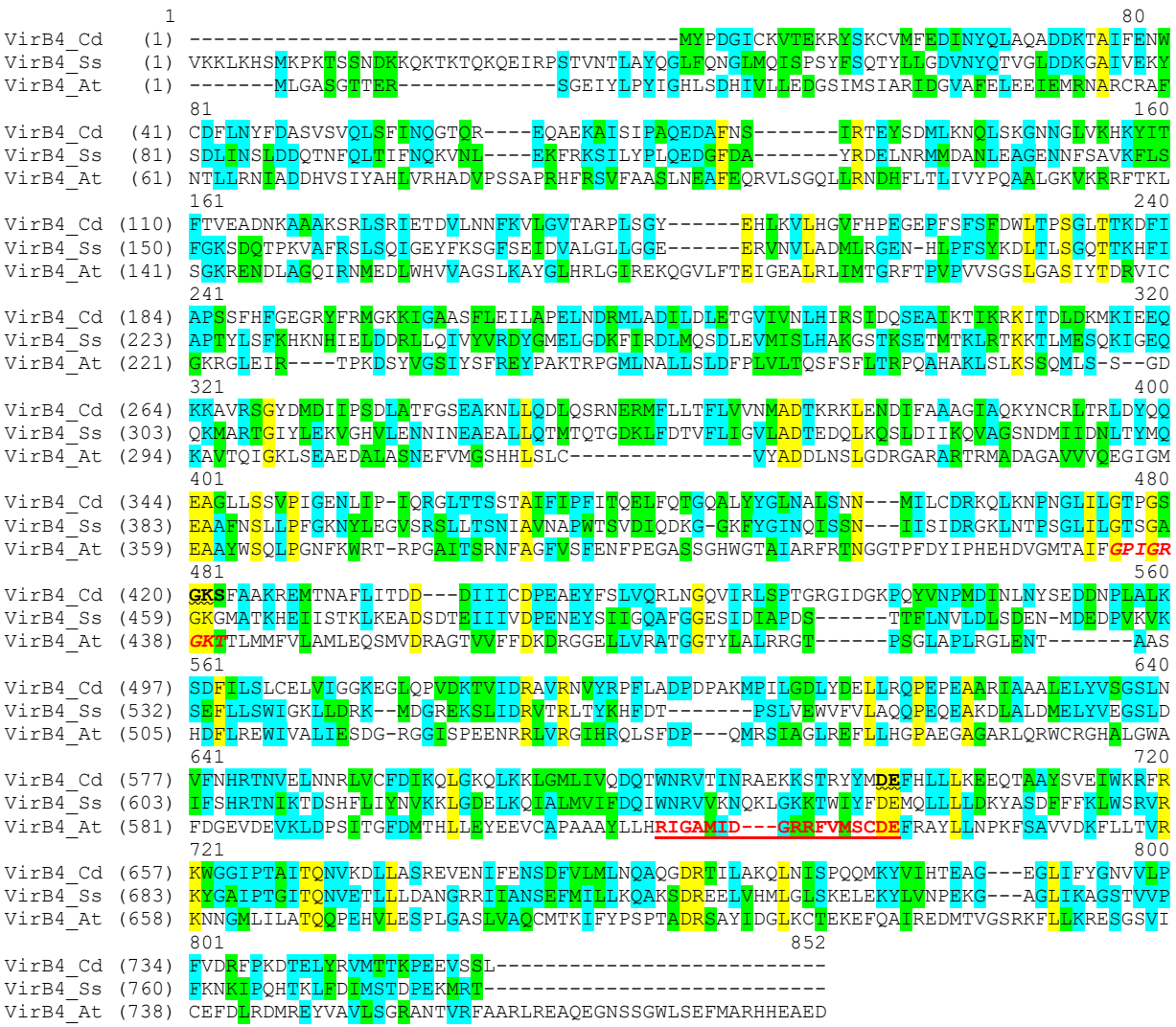
Figure S1. Amino acid sequence alignments of VirB4 (A) and VirD4 (B) from *C. difficile* (Cd, WP_011861114 for VirB4 and WP_011861117 for VirD4), *S. suis* (Ss, ABP89935 for VirB4 and ABP89939 for VirD4), and *A. tumefaciens* (At, NSY78098 for VirB4 and WP_010974920 for VirD4). Walker A and Walker B motif sequences in *A. tumefaciens* are shown in ***bold+italics*** (panel A, pos. 476-483; panel B, pos. 158-181) and **bold+underlined** (panel A, 679-698; panel B, 428-445) fonts, respectively. Amino acid residues in Walker A and Walker B motifs of VirB4_CTn4 and VirD4_CTn4, substituted during the current study, are shown in **bold+curved-underline** font.

A



B

Figure S2. Grouping of VirB4- and VirD4-like proteins within T4SS of Gram-positive and Gram-negative microorganisms. The dendrogram was created using AlignX module of Vector NTI ver.11 (Thermo Scientific). Species names, NCBI database protein tags and relatedness scores (in brackets) are shown.

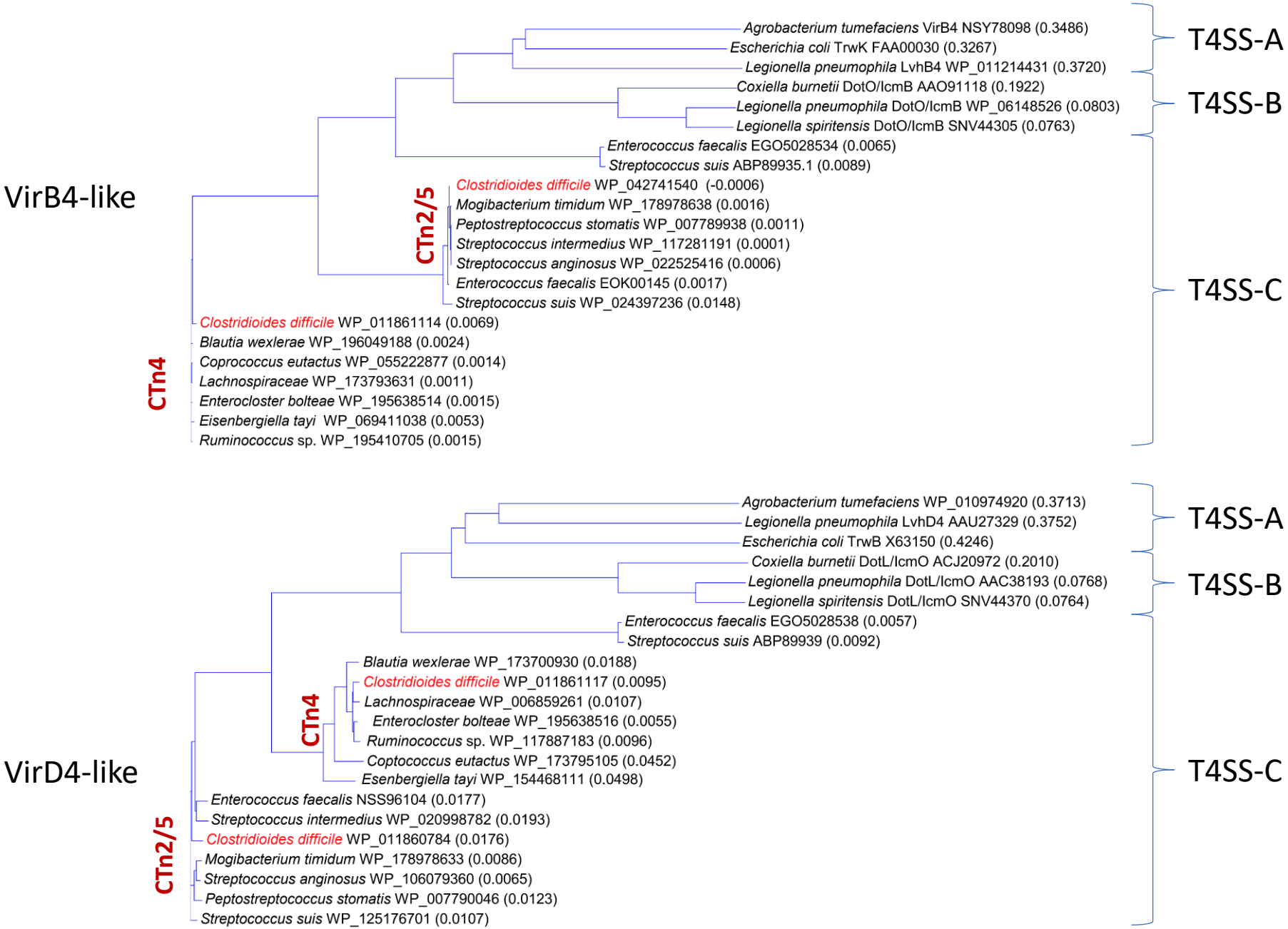


Figure S3. SDS-PAGE analysis of VirD4 and VirB4 of *C. difficile*. A. Full size VirD4_CTn4 (D4) and VirB4_CTn4 (B4) were purified from water soluble cytosolic (Cyt) and 6M urea-soluble membrane (Mem) fractions of *E. coli* extracts as 6His-tagged proteins. Expected positions of target proteins are shown by asterisks on the right. A gel with silver stain is shown B. NH_2-terminally truncated delVirB4_CTn4 (B4) and delVirD4_CTn4 (D4) were purified as cytosolic MBP-tagged proteins (10 µg each), isolated from Lysogeny broth (LB) or Terrific broth (TB) liquid cultures. A gel with Coomassie stain is shown. M, molecular mass markers in kilodaltons (kD) are shown on the left.

Figure S4. Analysis of full size VirB4_CTn4 and VirD4_CTn4 produced in *B. megaterium* as 6His-tagged proteins. A. The purified protein (B4, 10 µg) was run on 10% polyacrylamide gel and stained with PageBlue. M, molecular mass markers in kilodaltons (kD) are shown on the left. B. ATPase activity of VirB4 variants was estimated with malachite green assay as described in Materials and Methods. MBP and MBP-tagged delVirB4_CTn4 were used as negative and positive controls, respectively. C, Western blot analysis of crude full size VirD4_CTn4 (D4, 72 kD) and VirB4_CTn4 (B4, 90 kD) proteins in water-soluble cytosolic (Cyt) and 6M Urea-soluble membrane (Mem) fractions of *B. megaterium* extracts. Purified VirB4_CTn4 on the right lane (B4, as shown on panel A) served as a positive control. Reaction of the blotting membrane with Ponceau S stain and anti-HisTag reagent is shown on the left and right respectively. Expected positions of target proteins are shown by asterisks on the right. Size of molecular mass markers is shown in the middle in kilodaltons (kD).

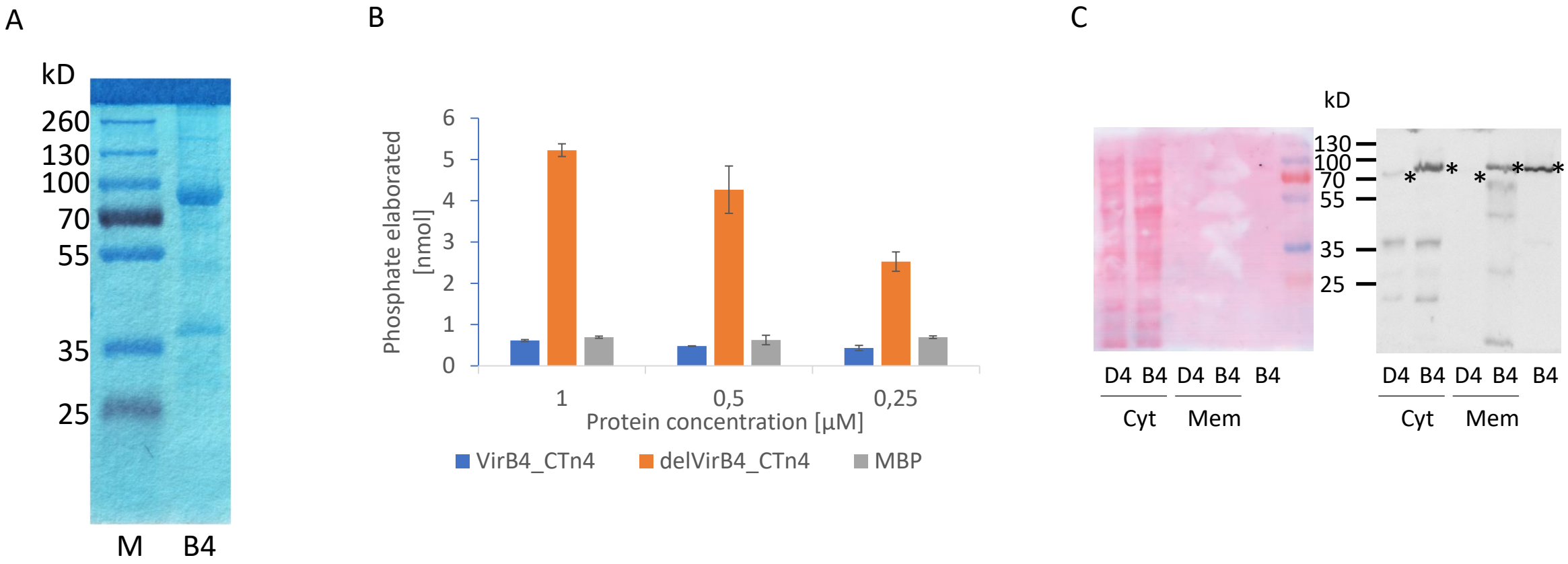


Figure S5. Influence of protein concentrations (A) and temperature of incubation (B) on ATPase activity of delVirD4_CTn4 (D4) and delVirB4_CTn4 (B4) as estimated by Enliten assay.

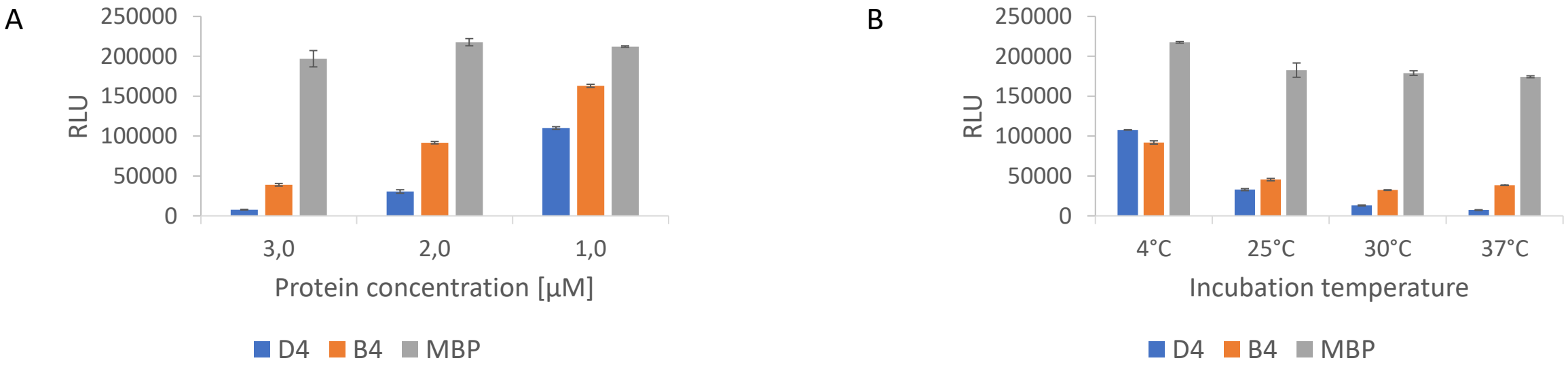


Figure S6. Oligomer stability of the wild type and site-mutated delVirB4/D4_CTn4 variants. Purified by MBP-tag chromatography delVirB4_CTn4 (Panel A) and delVirD4_CTn4 (Panel B) variants were subjected by Superdex200 chromatography in 20mM Tris-HCl, pH=7,4 plus 75 mM KCl.

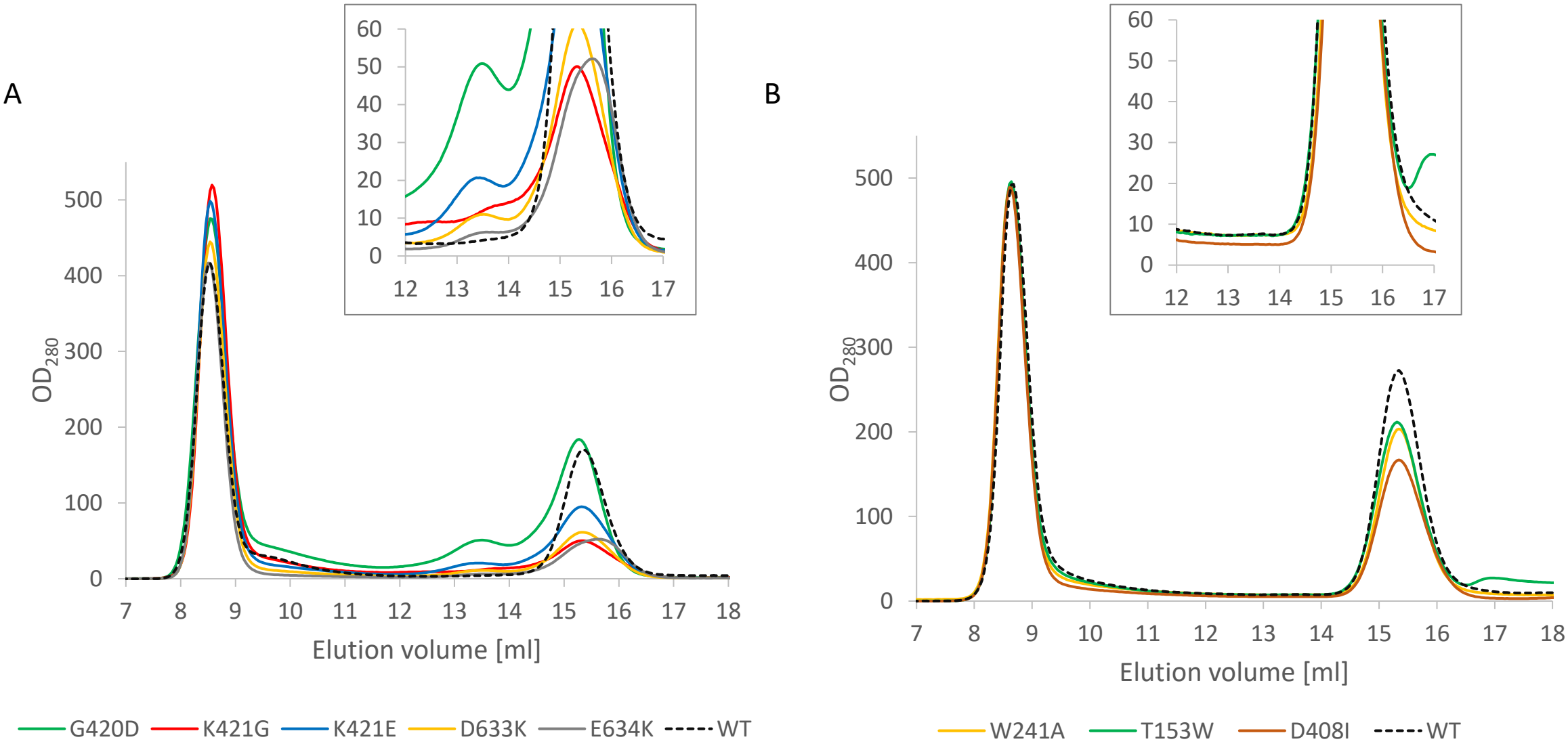


Figure S7. Influence of EDTA and salts of divalent metals on plasmid dsDNA migration in agarose gel electrophoresis. Plasmid dsDNA pRS313 (3,3 nM) was mixed with EDTA or divalent cations (2 mM each) with or without delVirB4_CTn4 or delVirD4_CTn4 *C. difficile* proteins (3 µM), incubated for 30 min on ice and analyzed by 0,5% agarose gel electrophoresis. M, nucleic acid marker, size of major fragments is shown on the left.

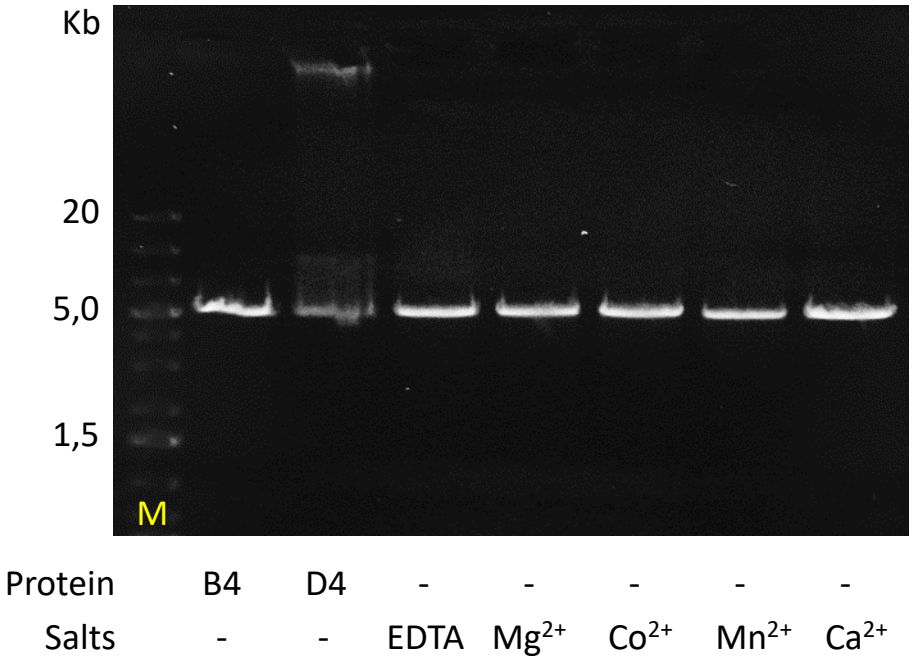


Figure S8. ATPase activity of the delVirD4_CTn4 wild type and W241A variants. The enzymatic activity was studied by malachite green ATPase assay with 100 µM ATP for 1h at 35°C.

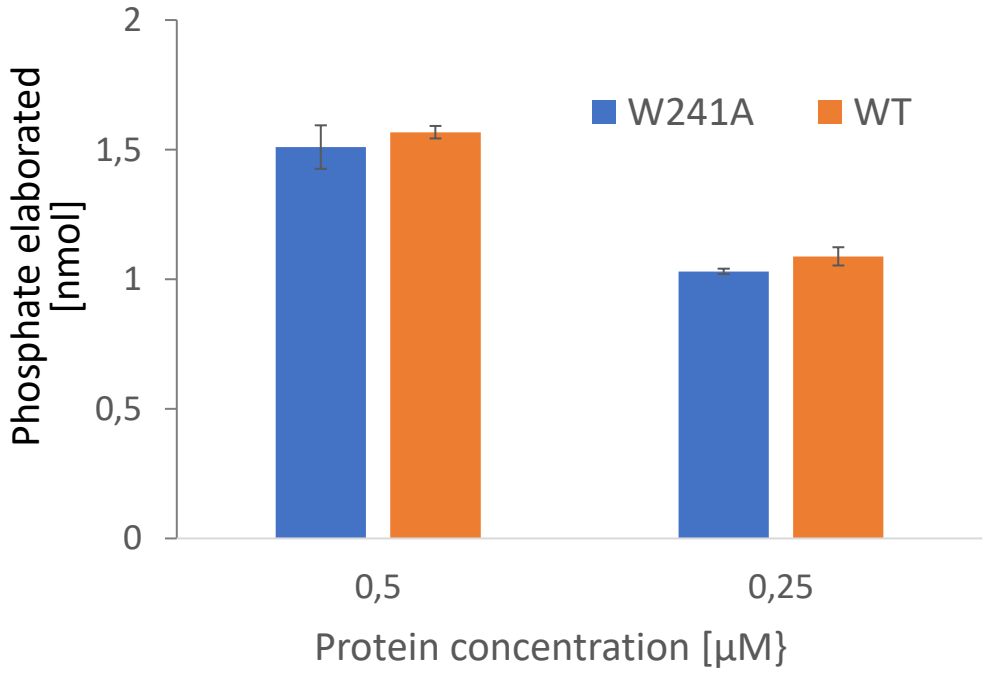


Figure S9. Influence of dsDNA (pRS313, 5µg/ml) on ATPase activities of delVirB4_CTn4 (1 µM) and delVirD4_CTn4 (0,5 µM) was studied by malachite green ATPase assay with 100 µM ATP for 1h at 35°C.

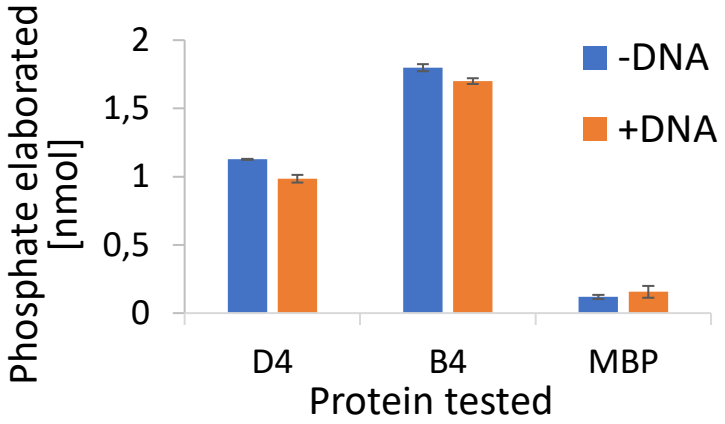


Table S1. Components of T4SS coded by *C. difficile* 630 chromosome.

Protein / Transposon	VirB4	VirB6	DNA-mt	CHAP	VirD4
CTn2	Orf12, CD630-04180	Orf9, CD630_04150	–	Orf13, CD630_04190	ORF5, CD630-04120
CTn4	ORF9, CD630-11100	Orf7, CD630_11120	Orf10, CD630_11090	Orf11, CD630_11080	ORF4, CD630-11150
CTn5	Orf12, CD630-018560	Orf9, CD630_18530	–	Orf13, CD630_18570	ORF5, CD630-018490

Table S2. Oligonucleotide primers (f-forward, r-reverse) used for PCR amplification. Engineered *restriction endonuclease sites* (if any) are shown in **bold**.

Primer ID	Nucleotide sequence, 5'-3' direction	Description
# 16f	TAATACGACTCACTATAGG	pET28, universal
# 17r	GCTAGTTATTGCTCAGCGG	pET28, universal
# 474f	GCA**GAGCTC**ATCATCATCATCATCACAG, *SacI*	pET28, universal
#475r	CTCA**GGTACC**TTTCGGGCTTTGTTAG, *KpnI*	pET28, universal
# 808f	GAGGTATC**CATATG**AAGCCGGAAC, *NdeI*	full size VirD4_CTn4
# 809r	GCGT**GAATTC**CTCCTTTCTTTTCG, EcoRI	full size VirD4_CTn4
# 812f	CTACCTT**CATATG**TACCCGGACG, *NdeI*	full size VirB4_CTn4
# 813r	GCCAGG**GAATTC**CGATTAAGTCC, *EcoRI*	full size VirB4_CTn4
# 1014f	CCCTACAC**GGATCC**GGTATTTC, *BamHI*	truncated VirD4_CTn4
# 1038f	AGACGGGG**GGATCC**CTTTACTAT, *BamHI*	truncated VirB4_CTn4
# 1613r	CGGGAAGCGGAGAATCCTTTGCGGC	VirB4 K421E
# 1614f	GCCGCAAAGGATTCTCCGCTTCCCG	VirB4 K421E
# 1615r	CGGGAAGCGGAGGTTCCTTTGCGGC	VirB4 K421G
# 1616f	GCCGCAAAGGAACCTCCGCTTCCCG	VirB4 K421G
# 1617r	CGGGAAGCGATAAATCCTTTGCGG	VirB4 G420D
# 1618f	CCGCAAAGGATTTATCGCTTCCCG	VirB4 G420D
# 1619r	GCTACTATATGCGCGAGTTTCACTTGC	VirB4 D633K
# 1620f	GCAAGTGAAACTCGCGCATATAGTAGC	VirB4 D633K
# 1621r	CGCTACTATATGGACAAGTTTCACTTGCTC	VirB4 E634K
# 1622f	GAGCAAGTGAAACTTGTCCATATAGTAGCG	VirB4 E634K
# 1639r	AAGCGGCAGCGGCGACACAAGATTT	VirD4 K152D
# 1640f	AAATCTTGTGTCGCCGCTGCCGCTT	VirD4 K152D
# 1641r	AAGCGGCAGCGGCAAGAAGAGATTT	VirD4 T153K
# 1642f	AAATCTCTTCTTGCCGCTGCCGCTT	VirD4 T153K
# 1643r	AAGCGGCAGCGGCAAGTGGAGATTT	VirD4 T153W
# 1644f	AAATCTCCACTTGCCGCTGCCGCTT	VirD4 T153W
# 1645r	GCTGTCTGTTAAAGGAGTTTGCGAAT	VirD4 D408K
# 1646f	ATTCGCAAACTCCTTTAACAGACAGC	VirD4 D408K
# 1647r	GCTGTCTGTTAATCGAGTTTGCGAATA	VirD4 D408I
# 1648f	TATTCGCAAACTCGATTAACAGACAGC	VirD4 D408I
# 1649r	GCTGTCTGTTAGACAAGTTTGCGAATAT	VirD4 E409K
# 1650f	ATATTCGCAAACTTGTCTAACAGACAGC	VirD4 E409K
# 1651r	TTTGCGGTAAAATCGGAACGGCTCT	VirD4 W241A
# 1652f	AGAGCCGTTCCGATTTTACCGCAAA	VirD4 W241A
# 1502	CAAATCACCATTGAACCTGGTGTGGA TGTCGAAGTTGTTGTTGCTTCCAACAG CAGCGGTCACCATCACCATCACCATTAG	ssDNA