Supplementary information

Rhs toxin complexes secreted via the type IX secretion system mediate kin discrimination in environmental Bacteroidota

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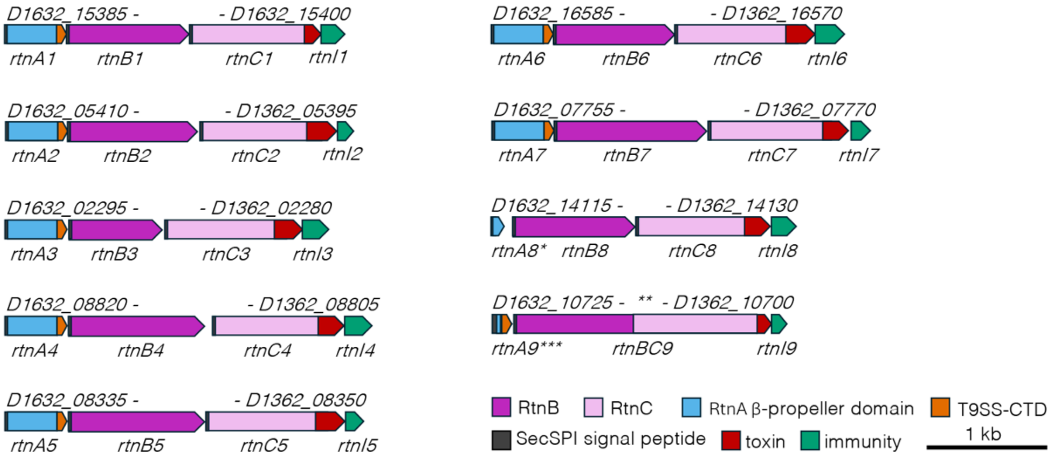

Figure S1 | *Rtn* loci in *C. nematophagum* JUb275 genome. Representation of genetic organisation of all *rtn* loci in the genome of *C. nematophagum* JUb275 (accession number: SAMN09925764). Genes coding for RtnA are shown in blue, RtnB in dark magenta, RtnC in light magenta. Toxic domains of RtnCs are shown in red and genes coding putative immunity proteins are shown in green. Scale, 1 kb. * gene of rtnA8 is C-terminally truncated and lacks most of the β-propeller and the T9SS-CTD domain. ** in the deposited genome, *rtnBC9* is truncated by nonsense mutations, the scheme corresponds to full length ORF as confirmed by re-sequencing. *** *rtnA9* does not have complete β-propeller domain, only a short fragment of two strands and T9SS-CTD-like domain.

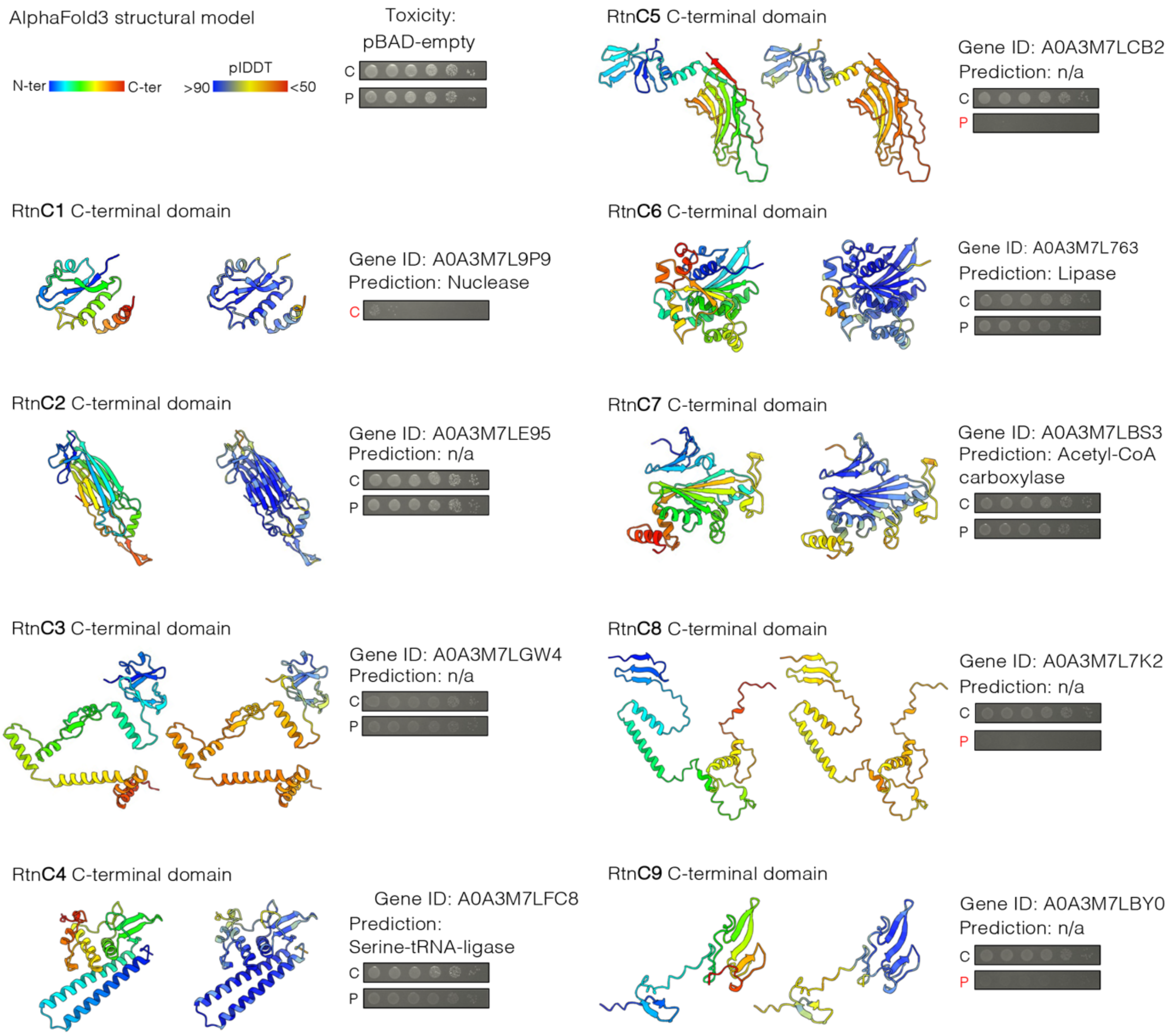

Figure S2 | C-termini of *rtnC* code for variety of toxic domains. AlphaFold3 structural models of RtnC C-terminal toxic domains coloured from N- to C-terminus (left model), and by pIDDT values (right model). GeneBank accession numbers of corresponding *rtnC* are provided on the right if each panel alongside functional prediction based on structural comparisons using DALI server and AlphaFold3 models as inquiry. Toxicity assays correspond to heterologous expression of toxic domains in *E. coli*. *E. coli* cells were transformed with pBAD33 vectors encoding C-terminal variable toxic domains for expression in cytoplasm (C) or in the periplasm (P) by cloning in fusion with tat secretion signal peptide from torA gene. Overnight cultures were serially diluted (10^{-1} to 10^{-6}, from left to right) and spotted on LB agar plates supplemented with 1% of L-arabinose.

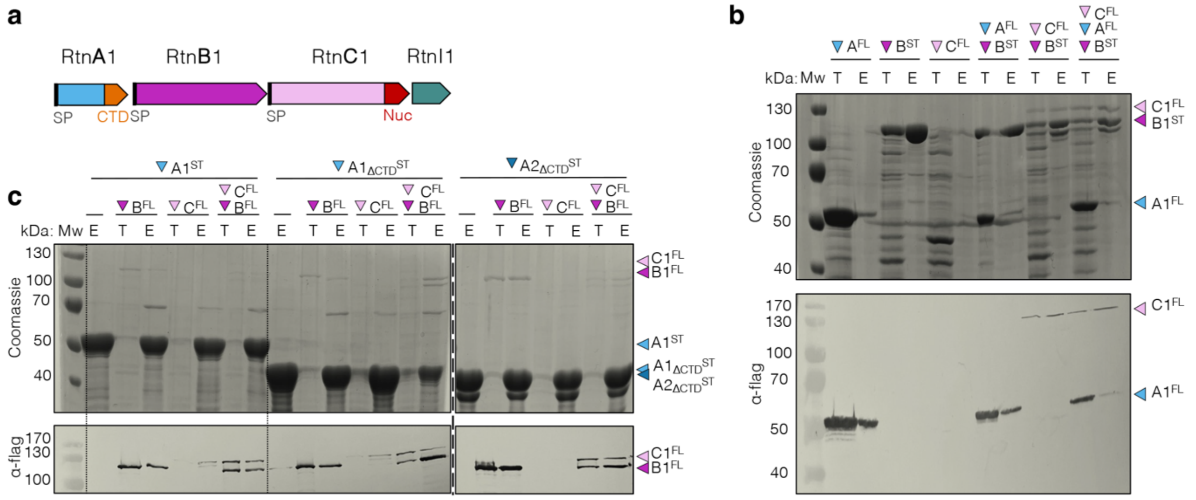

Figure S3 | RtnA interaction with RtnB and RtnC. **a** schematic representation of the *C. nematophagum rtn1* locus coloured by the indicative scheme as in following panels. **b** pull-down assays using strep-tagged RtnB1 (B^{ST}) as a bait. RtnA1^{FL}, B1ST and C1^{FL} proteins were co-expressed in periplasm of *E. coli* BL21 and purified on streptactin-sepharose resin. Note non-specific binding of RtnA^{FL} to streptactin resin. **c** Pull-down assays using RtnA1ST (A^{ST}), C-terminal domain lacking derivative ($A1_{\Delta CTD}{}^{ST}$), or RtnA2 lacking C-terminal domain ($A1_{\Delta CTD}{}^{ST}$) as a bait co-expressed with flag-tagged RtnB1 and C1 as indicated. Total periplasmic extracts (T) and strep-elution fraction (E) were analysed by SDS-PAGE, co-purified flagged proteins were detected by western-blot with anti-flag antibodies. Representative replicate of at least three independent experiments is shown.

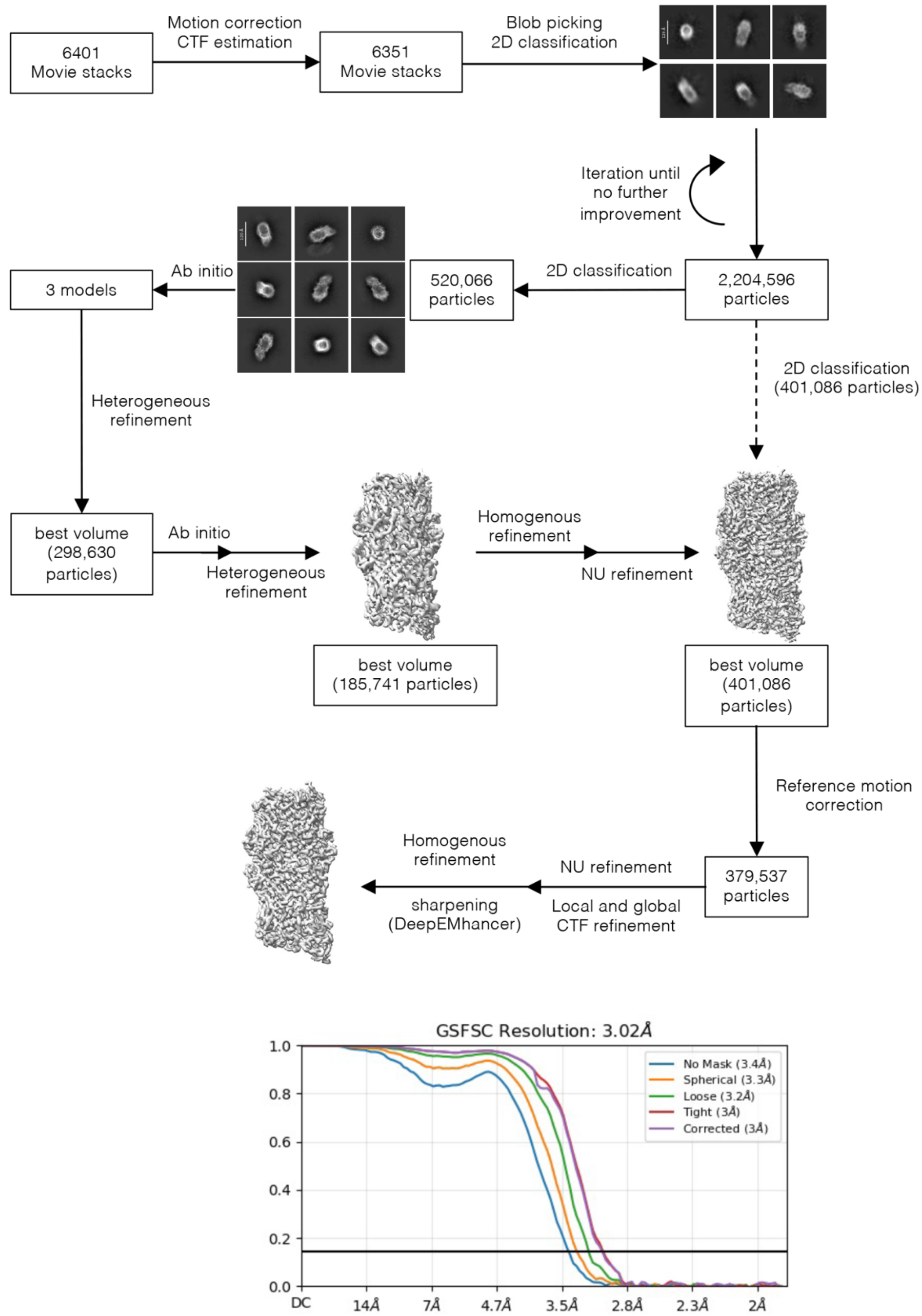

Figure S4 | Workflow of cryo-EM data-processing. Workflow depicting procedures of data analysis used to generate final cryo-EM density map of RtnB1-RtnC1 at 3.4 Å (unmasked) resolution. Numbers of movie stacks, particles and volumes used at all essential steps are provided in boxes and procedures are indicated above arrows. Fsc curve of last refinement is provided below.

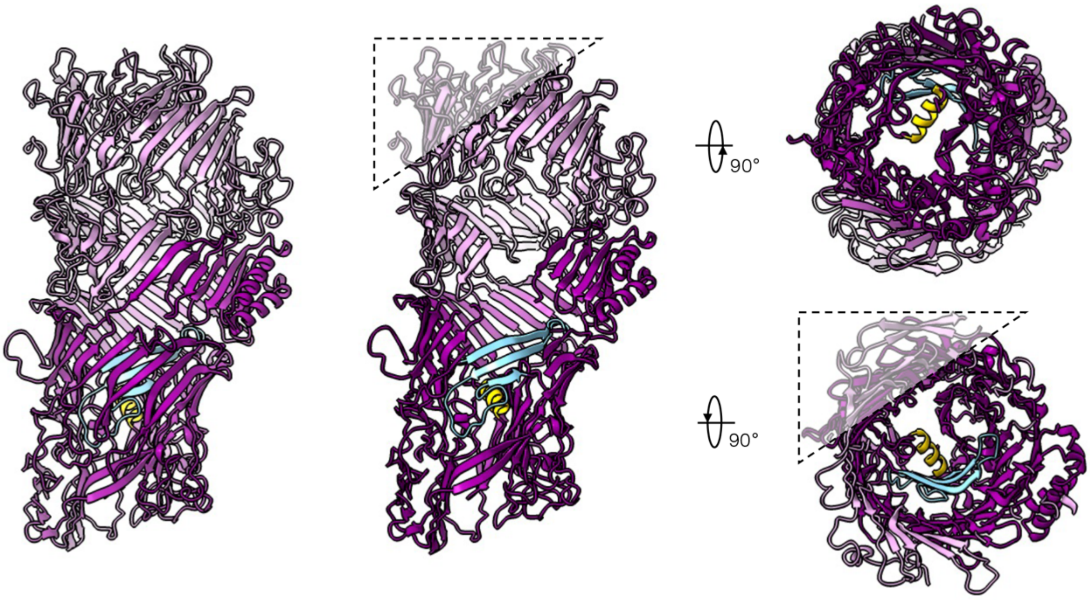

Figure S5 | N-terminal RtnB1 features involved in closing the bottom of the cocoon. Cryo-EM structure of the RtnBC1 cocoon (left) or its cut-through view (middle), as well as bottom view (right, top) and cut-through view from the top (bottom, right). N-terminal helix corresponding to residues 33-43 of full length RtnB1 (or 9-19 without the signal peptide) is shown in yellow and following inner β-sheet layer corresponding to residues 54-98 of full length RtnB1 (or 30-74 without the signal peptide) is shown in blue.

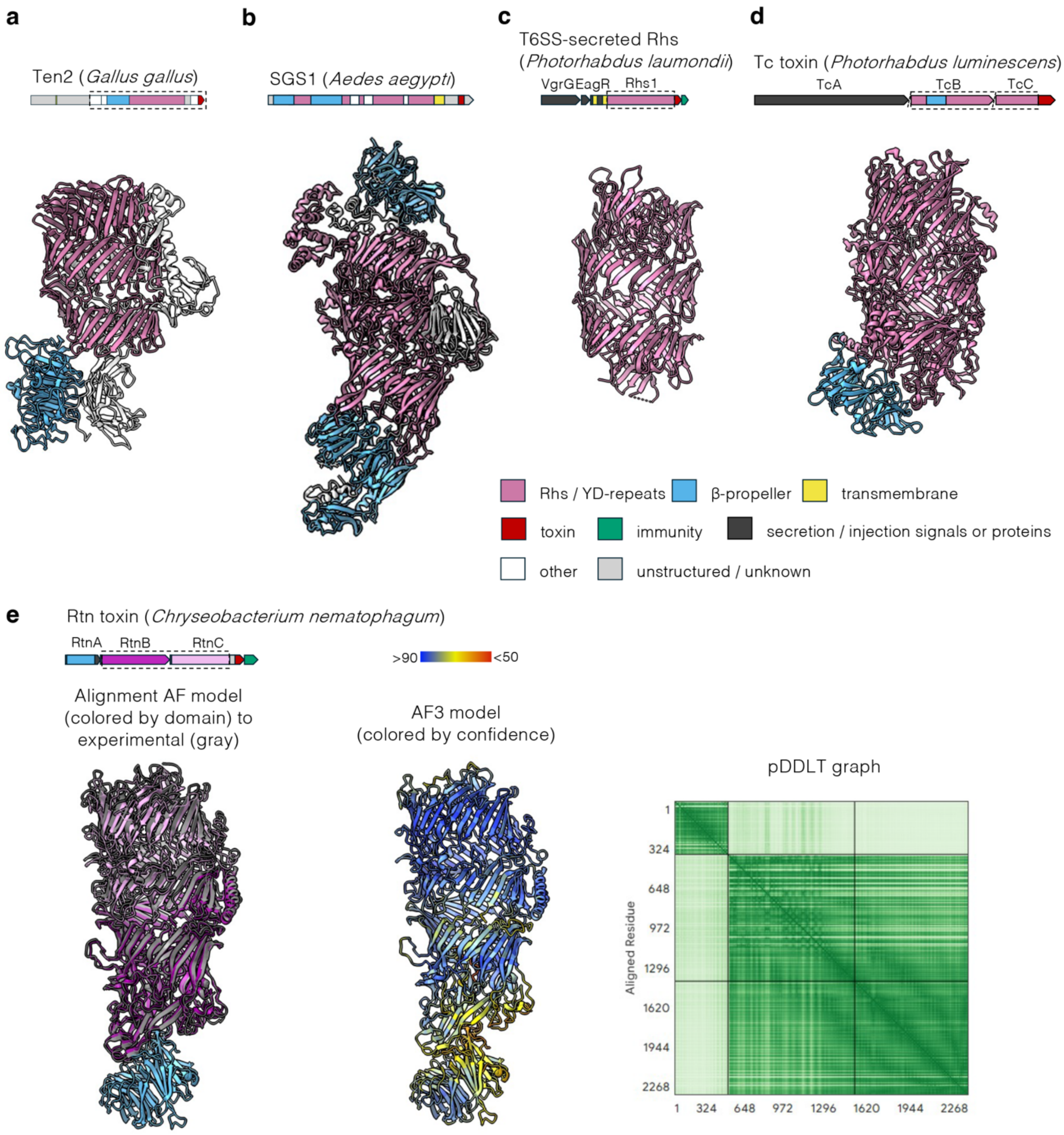

Figure S6 | Structural comparison of Rhs repeat-comprising proteins. a Chicken teneurin Ten2 (PDB: 6FB3); **b** Mosquito salivary gland surface protein 1 (PDB: 8FJP); **c** *Photorhabdus laumondii* T6SS-secreted Rhs1 toxin (PDB: 7PQ5); **d** *Photorhabdus luminescens* insecticidal Tc toxin (PDB: 6H6F). **e** *Chryseobacterium nematophagum* Rtn toxin (this study), colored in gray overlayed with AlphaFold3 model of complete secreted RtnABC1 complex colored by chain as shown in scheme (left) and by confidence (middle). Predicted Local Distance Difference Test graph for the AF3 model of RtnABC1 is provided on the right.

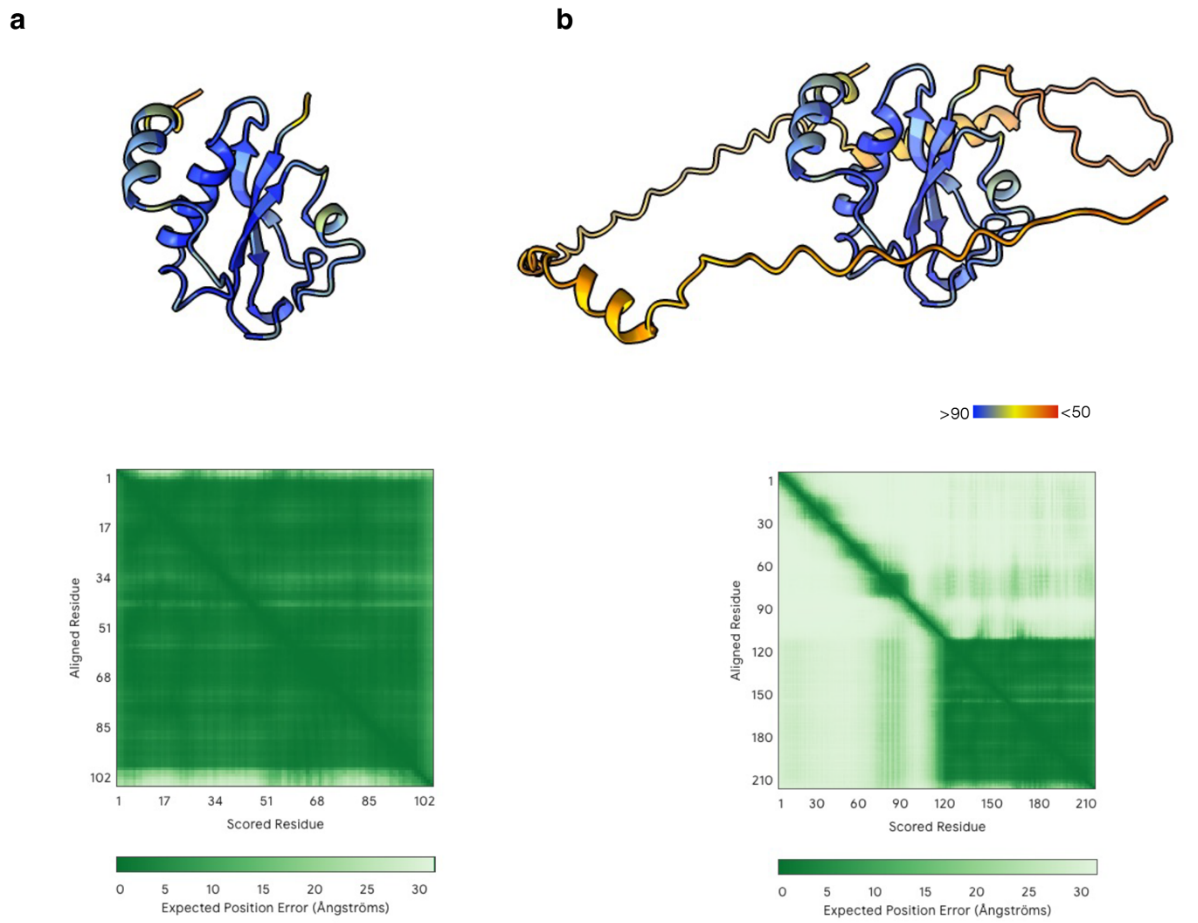

Figure S7 | Structural prediction of RtnC1 C-terminal domain. a Prediction of RtnC1-C-terminal toxin domain coloured by confidence. **b** Prediction of entire autoproteolytically cleaved C-terminus coloured by confidence. Predicted Local Distance Difference Test graphs for the AF3 models are provided on the right.

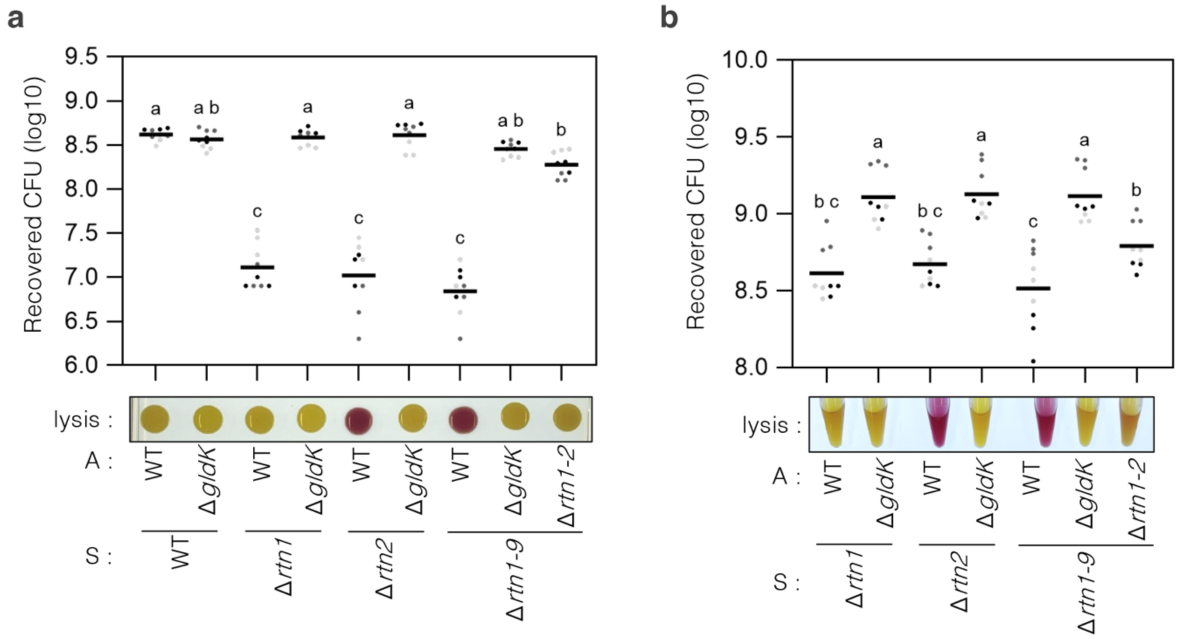

Figure S8 | T9-dependant killing by Rtn1 and Rtn2 toxins on solid agar media and in liquid co-cultures with sensitive strains. a *C. nematophagum* WT or *rtn* loci mutant strains sensitive (S) to specific toxin attacks due to missing immunity protein were challenged by WT attacker (A) strain, or T9SS-deficient mutant (Δ*gldK*) or mutant lacking two first *rtn* loci (Δ*rtn1-2*) on solid media or **b**, in liquid culture. CmR marker carrying sensitive strains were recovered and their CFUs were counted by plating on chloramphenicol cultures. Three biological replicates are represented in different shades of grey, with each point in same colour is a technical replicate. Bar represents means of all replicates. Comparison of means was assessed with one-way ordinary ANOVA. No difference in survival was detected between experiments, which is denoted by a same letter group. Difference of survival between groups p < 0,0001; except for difference between groups ab versus a, or b in panel a and between group bc versus b, or c in panel b (p = 0,01). For lysis experiments, *C. nematophagum* sensitive strains were *lacZ*+ and their lysis is assessed using CPRG (yellow), which is converted to (purple) by liberated β-Gal.

Tree scale: 0.1
Class
Cytophagia
Bacteroidia
Flavobacteriia
Sphingobacteriia
Genus
Chryseobacterium
Rtn
0
1
2
3
4
5
6
7
8
9
10
T9SS
absent
present
T6SSiii
absent
present
Class
Rtn
T9SS
T6SSiii
Cytophagia (166 genomes)
Saprospiria+2 others (11 genomes)
Chitinophagia (55 genomes)
Flavobacteriia (16 genomes)
Bacteroidia (182 genomes)
Chryseobacterium

Figure S9 | Distribution of *rtn l*oci across Bacteroidota genomes. Phylogenetic tree was built using PhyloPhlAn for the 1087 analysed genomes from phylum Bacteroidota. Genome identifiers are provided on the right side. Heatmap representing number of *rtn* loci detected in genomes and presence of T9SS and T6SSiii are indicated along the species names. Clades from taxa without *rtn* loci were collapsed (number of genomes in collapsed subtrees is indicated), with exception of the class of Bacteroidia which was collapsed despite containing one genome with a Rtn locus. The tree was rooted with the Cytophagia clade. Red circles indicate branches with a UF-boot support above 95%. The length of branches is expressed in substitutions per site.

Table S1 | T9SS-dependant Rtn proteins identified by label-free mass spectrometry in culture supernatant of *C. nematophagum* JUb275. Tsc indicates total spectrum count, which is the total number of tandem mass (MS/MS) spectra identified for a specific protein.

GenBank accession number	Name	TSC	WT/Δ*gldK*		Mol. Weight (kDa)	Sequence coverage (%)
			Ratio	p-value		
A0A3M7L8L5	RtnA1	28	2,74	0,017888	57,5	12,2
A0A3M7L862	RtnB1	347	16,68	4,6E-07	114,8	78
A0A3M7L9P9	RtnC1	642	15,34	2,03E-05	124	78,33
A0A3M7LFP1	RtnA2	/	/	/	57,2	/
A0A3M7LE61	RtnB2	10	1024	0,000736	121,7	6,63
A0A3M7LE95	RtnC2	181	13,18	9,65E-06	127,6	41
A0A3M7LDK1	RtnA3	8	5,07	0,028737	56,9	3,1
A0A3M7LE58	RtnB3	3	1,54	0,725515	90,4	3,26
A0A3M7LGW4	RtnC3	53	1024	0,000775	130,5	14
A0A3M7LCA9	RtnA4	/	/	/	56,9	/
A0A3M7LCD3	RtnB4	23	1024	0,012609	127,5	21,33
A0A3M7LFC8	RtnC4	2	1024	2,71E-07	130,5	2,7
A0A3M7LA42	RtnA5	/	/	/	57,1	/
A0A3M7LBP5	RtnB5	24	1024	3,18E-05	126,4	6,9
A0A3M7LCB2	RtnC5	99	16,75	0,000168	129,6	20,33
A0A3M7L8Y9	RtnA6	2	1024	0,188761	57,1	5,1
A0A3M7L7E0	RtnB6	161	15,93	1,2E-05	114,4	78
A0A3M7L763	RtnC6	170	11,95	3,94E-06	127,9	30
A0A3M7LC12	RtnA7	12	1024	7,94E-05	57,9	8,73
A0A3M7L9Q1	RtnB7	/	/	/	142,8	/
A0A3M7LBS3	RtnC7	/	/	/	135,7	/
A0A3M7L7I6	RtnA8	/	/	/	12,1	/
A0A3M7LB16	RtnB8	17	1024	0,00014	115	9,87
A0A3M7L7K2	RtnC8	94	15,99	4,74E-06	125,1	17,33
A0A3M7LDD8	RtnA9	7	0,81	0,128602	17,4	18
A0A3M7LBY0	RtnBC9	115	3,02	0,000399	237,1	39,33

Table S2 | Bacterial strains used in this study.

Strain	**Description**	**Culture**	**Reference**
E. coli BL21	For production of recombinant proteins	LB, 37°C	NEB #C2530H
E. coli DH5α	For toxicity assays	LB, 37°C	NEB #C2987H
E. coli NEB10β	For cloning	LB, 37°C	NEB #C3019H
E. coli S17	For conjugation of pYT313	LB, 37°C	Fisher Scientific #NC1526716
E. coli MG1655	WT strain, for killing assays	LB, 37°C	Laboratory collection
C. nematophagum JUb275	WT strain	CYE, 30°C	Page et al. [1]
C. nematophagum JUb129	WT strain, for killing assays	CYE, 30°C	Page et al. [1]
C. indologenes ATCC29897	WT strain, for killing assays	CYE, 37°C	BCCM/LMG Bacteria Collection LMG8337
F. johnsioniae CJ1827	Streptomycin-resistant rpsL2 derivative of ATCC 17061 (UW101) for killing assays	CYE, 30°C	Rhodes et al [2]
JUb275 Δ*gldK*	*C. nematophagum* JUb275 deleted for the T9SS protein GldK	CYE, 30°C	This study
JUb275 Δ*rtn1*	*C. nematophagum* JUb275 deleted for Rtn1 locus (Rtn1 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn2*	*C. nematophagum* JUb275 deleted for Rtn2 locus (Rtn2 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn3*	*C. nematophagum* JUb275 deleted for Rtn3 locus (Rtn3 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn4*	*C. nematophagum* JUb275 deleted for Rtn4 locus (Rtn4 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn5*	*C. nematophagum* JUb275 deleted for Rtn5 locus (Rtn5 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn6*	*C. nematophagum* JUb275 deleted for Rtn6 locus (Rtn6 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn7*	*C. nematophagum* JUb275 deleted for Rtn7 locus (Rtn7 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn8*	*C. nematophagum* JUb275 deleted for Rtn8 locus (Rtn8 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn9*	*C. nematophagum* JUb275 deleted for Rtn9 locus (Rtn9 sensitive)	CYE, 30°C	This study
JUb275 Δ*rtn1-2*	*C. nematophagum* JUb275 deleted for Rtn1 and Rtn2 loci (Rtn1 and 2 sensitive)	CYE, 30°C	This study

JUb275 Δ*rtn1-9*	*C. nematophagum* JUb275 deleted for all the 9 *rtn* loci (Rtn1 to 9 sensitive)	CYE, 30°C	This study
JUb275 *site1:lacZ*	JUb275 with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn1 site1:lacZ*	JUb275 Δ*rtn1* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn2 site1:lacZ*	JUb275 Δ*rtn2* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn3 site1:lacZ*	JUb275 Δ*rtn3* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn4 site1:lacZ*	JUb275 Δ*rtn4* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn5 site1:lacZ*	JUb275 Δ*rtn5* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn6 site1:lacZ*	JUb275 Δ*rtn6* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn7 site1:lacZ*	JUb275 Δ*rtn7* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn8 site1:lacZ*	JUb275 Δ*rtn8* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn9 site1:lacZ*	JUb275 Δ*rtn9* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn1-9 site1:lacZ*	JUb275 Δ*rtn1-9* with *lacZ* inserted at site 1	CYE, 30°C	This study
JUb275 *site1:cmR*	JUb275 with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn1 site1:cmR*	JUb275 Δ*rtn1* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn2 site1:cmR*	JUb275 Δ*rtn2* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn3 site1:cmR*	JUb275 Δ*rtn3* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn4 site1:cmR*	JUb275 Δ*rtn4* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn5 site1:cmR*	JUb275 Δ*rtn5* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn6 site1:cmR*	JUb275 Δ*rtn6* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn7 site1:cmR*	JUb275 Δ*rtn7* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn8 site1:cmR*	JUb275 Δ*rtn8* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn9 site1:cmR*	JUb275 Δ*rtn9* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn1-9 site1:cmR*	JUb275 Δ*rtn1-9* with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb275 Δ*rtn1 site1:lacZ , site2:rtnI1*	JUb275 Δ*rtn1 site1:lacZ* complemented with the immunity protein RtnI1 sequence at site 2	CYE, 30°C	This study
JUb275 Δ*rtnA1*	JUb275 deleted for *rtnA1* sequence	CYE, 30°C	This study
JUb275 Δ*rtnA1-rtnA2*	JUb275 deleted for *rtnA1* and *rtnA2* sequences	CYE, 30°C	This study
JUb275 $rtnA1^{-CTD}$	JUb275 with *rtnA1* deleted for the CTD sequence	CYE, 30°C	This study
JUb275 *rtnC1ΔCt1*	JUb275 with RtnC1 deleted for the C-terminal nuclease domain sequence	CYE, 30°C	This study
JUb275 Δ*tssBC*	JUb275 deleted for *tssB* and *tssC* T6SS genes	CYE, 30°C	This study
JUb275 Δ*porZ*	JUb275 deleted for *porZ* coding sequence	CYE, 30°C	This study
JUb275 $rtnC1_{D879N}$	JUb275 with a point mutation on RtnC1 D879N	CYE, 30°C	This study

JUb275 *site1:mLytchee*	JUb275 with *mLytchee* inserted at site 1	CYE, 30°C	This study
JUb275 *site1:mChartreuse*	JUb275 with *mChartreuse* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn1* *site1:mChartreuse*	JUb275 Δ*rtn1* with *mChartreuse* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn2* *site1:mChartreuse*	JUb275 Δ*rtn2* with *mChartreuse* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*rtn1-9* *site1:mChartreuse*	JUb275 Δ*rtn1-9* with *mChartreuse* inserted at site 1	CYE, 30°C	This study
JUb275 Δ*gldK* *site1:mChartreuse*	JUb275 Δ*gldK* with *mChartreuse* inserted at site 1	CYE, 30°C	This study
JUb129 *site1:cmR*	JUb129 with *cmR* inserted at site 1	CYE, 30°C, Cml	This study
JUb129 *site1:mlytchee*	JUb129 with *mLytchee* inserted at site 1	CYE, 30°C	This study

Table S3 | Plasmids used in this study

Plasmid	**Application**	**Resistance**	**Reference**
pBAD33	Arabinose-inducible vector	Cml	Guzman et al [3]
pCDF-Duet	IPTG-inducible vector	Sm	Novagen
pRSF-Duet	IPTG-inducible vector	Km	Novagen
pACYC-Duet	IPTG-inducible vector	Cml	Novagen
pYT313	Suicide vector for chromosome mutagenesis in *C. nematophagum*	Ap (*E. coli*), Ery (*C. nematophagum*)	Zhu et al [4]
pCP23	Confers tetracycline resistance to *F. johnsoniae*	Tc	Agarwal et al [5]
pYT313 *site1:lacZ*	Insertion of *lacZ* gene in *C. nematophagum* JUb275 chromosome at site 1	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 *site1:cmR*	Insertion of *cmR* gene for chloramphenicol resistance in *C. nematophagum* JUb275 chromosome at site 1	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 *site1:mChartreuse*	For insertion of *mLytchee* in *C. nematophagum* JUb275 at site 1	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 *site1:mLytchee*	For insertion of *mLytchee* in *C. nematophagum* JUb275 at site 1	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*gldK*	Deletion of *gldK* sequence	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtn1*	Deletion of *rtn1* locus	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtn2*	Deletion of *rtn2* locus	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtn3*	Deletion of *rtn3* locus	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtn4*	Deletion of *rtn4* locus	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtn5*	Deletion of *rtn5* locus	Ap (*E. c*), Ery (*C. n*)	This study

pYT313 Δ*rtn6*	Deletion of *rtn6* locus	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtn7*	Deletion of *rtn7* locus	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtn8*	Deletion of *rtn8* locus	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtn9*	Deletion of *rtn9* locus	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 *site2:rtnI*	Complementation of immunity *rtnI1* gene in *C. nematophagum* chromosome at site 2	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtnA1*	Deletion of *rtnA1* sequence	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 $rtnA1^{-CTD}$	Deletion of *rtnA1* CTD sequence	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 *rtnC1ΔCt1*	Deletion of *rtnC1* C-terminal nuclease domain sequence	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*rtnA2*	Deletion of *rtnA2* sequence	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*tssB-C*	Deletion of *tssB* ans *tssC* sequences	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 Δ*porZ*	Deletion of *porZ* sequence	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 $rtnC1_{D879N}$	For point mutation on *rtnC1* D879N	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 JUb129-*site1:cmR*	For insertion of *cmR* in JUb129 at site 1	Ap (*E. c*), Ery (*C. n*)	This study
pYT313 JUb129-*site1:mLytchee*	For insertion of *mLytchee* in JUb129 at site 1	Ap (*E. c*), Ery (*C. n*)	This study
pRSF-Duet pelB-RtnB1-Flag	IPTG-inducible expression vector for Flag-tagged RtnB1 protein expression in *E. coli* periplasm with a *pelB* signal peptide from *E. coli*	Km	This study
pRSF-Duet pelB-RtnB1-Strep	IPTG-inducible expression vector for Strep-tagged RtnB1 protein expression in *E. coli* periplasm with a *pelB* signal peptide from *E. coli*	Km	This study
pCDF-Duet pelB-RtnC1-Flag	IPTG-inducible expression vector for Flag-tagged RtnC1 protein expression in *E. coli* periplasm with a *pelB* signal peptide from *E. coli*	Sm	This study
pCDF-Duet pelB-$RtnC1^{D879N}$-Flag	IPTG-inducible expression vector for Flag-tagged RtnC1 protein expression with a point mutation on aspartate 879 in *E. coli* periplasm with a *pelB* signal peptide from *E. coli*	Sm	This study
pACYC-Duet pelB-RtnA1-Flag	IPTG-inducible expression vector for Flag-tagged RtnA1 protein expression in *E. coli* periplasm with a *pelB* signal peptide from *E. coli*	Cml	This study
pACYC-Duet pelB-RtnA1-Strep	IPTG-inducible expression vector for Strep-tagged RtnA1 protein expression in *E. coli* periplasm with a *pelB* signal peptide from *E. coli*	Cml	This study
pACYC-Duet pelB-RtnA1ΔCTD-Strep	IPTG-inducible expression vector for Strep-tagged RtnA1 protein expression with no CTD	Cml	This study

	sequence in *E. coli* periplasm with a *pelB* signal peptide from *E. coli*		
pACYC-Duet pelB-RtnA2ΔCTD-Strep	IPTG-inducible expression vector for Strep-tagged RtnA2 protein expression with no CTD sequence in *E. coli* periplasm with a *pelB* signal peptide from *E. coli*	Cml	This study
pBAD33 Cyto-Ct1	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn1 (Cyto-Ct1)	Cml	This study
pBAD33 Cyto-Ct1$_{Y7A}$	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn1 with a catalytic point mutation (Cyto-Ct1$_{Y7A}$)	Cml	This study
pBAD33 Cyto-Ct1$_{Y20A}$	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn1 with a catalytic point mutation (Cyto-Ct1$_{Y20A}$)	Cml	This study
pBAD33 Cyto-Ct2	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn2 (Cyto-Ct2)	Cml	This study
pBAD33 Peri-Ct2	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn2 with a *torA*-signal peptide for periplasmic translocation (Peri-Ct2)	Cml	This study
pBAD33 Cyto-Ct3	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn3 (Cyto-Ct3)	Cml	This study
pBAD33 Peri-Ct3	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn3 with a *torA*-signal peptide for periplasmic translocation (Peri-Ct3)	Cml	This study
pBAD33 Cyto-Ct4	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn4 (Cyto-Ct4)	Cml	This study
pBAD33 Peri-Ct4	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn4 with a *torA*-signal peptide for periplasmic translocation (Peri-Ct4)	Cml	This study
pBAD33 Cyto-Ct5	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn5 (Cyto-Ct5)	Cml	This study
pBAD33 Peri-Ct5	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn5 with a *torA*-signal peptide for periplasmic translocation (Peri-Ct5)	Cml	This study
pBAD33 Cyto-Ct6	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn6 (Cyto-Ct6)	Cml	This study
pBAD33 Peri-Ct6	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn6 with a *torA*-signal peptide for periplasmic translocation (Peri-Ct6)	Cml	This study
pBAD33 Cyto-Ct7	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn7 (Cyto-Ct7)	Cml	This study

pBAD33 Peri-Ct7	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn7 with a *torA*-signal peptide for periplasmic translocation (Peri-Ct7)	Cml	This study
pBAD33 Cyto-Ct8	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn8 (Cyto-Ct8)	Cml	This study
pBAD33 Peri-Ct8	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn8 with a *torA*-signal peptide for periplasmic translocation (Peri-Ct8)	Cml	This study
pBAD33 Cyto-Ct9	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn9 (Cyto-Ct9)	Cml	This study
pBAD33 Peri-Ct9	pBAD33 with arabinose-inducible expression of toxic C-terminal domain of Rtn9 with a *torA*-signal peptide for periplasmic translocation (Peri-Ct9)	Cml	This study

Table S4 | DNA oligos used in this study. In the column describing the purpose, RL stands for oligos used for restriction-ligation cloning, GA – Gibson assembly and SLIC - sequence and ligation independent cloning.

Primer	**Sequence**	**Purpose**
F-pYT-open	GCATGCAAGCTTGGCGTAAT	(GA) pYT313 mutagenesis plasmids
R-pYT-open	TCTAGAGGATCCCCCGAATT	(GA)pYT313 mutagenesis plasmids
F-up-d1-rtn	GGAAAAATTCGGGGGATCCTCTAGAATACAAAATTGATCTTGAAAGAAGG	(GA) pYT313-Δrtn1
R-up-d1-rtn	CTGACATTCAAATATTATAAAGATTATTGTTTTATTTTTTTAGTTTGGAACTTAA	(GA) pYT313-Δrtn1
F-down-d1-rtn	TAAGTTCCAAACTAAAAAAATAAAACAATAATCTTTATAATATTTGAATGTCAGT	(GA) pYT313-Δrtn1
R-down-d1-rtn	CCATGATTACGCCAAGCTTGCATGCCCTTATTTATCTACAGATACAGCAA	(GA) pYT313-Δrtn1
F-verif1-rtn	AATCTCATGTGCAGGAATTTTTAAC	verification of Δrtn1
R-verif1-rtn	AGTTGCTTTTAGGGTAAACTGAA	verification of Δrtn1
F-up-d2-rtn	GGAAAAATTCGGGGGATCCTCTAGAACTAAATTTTATTTAGTTAAATTTATTACGG	(GA) pYT313-Δrtn2
R-up-d2-rtn	TAGGAGATTCTCAGAGCATTAGTTTACCTTGTAATGCTTTTTATTAGATGGTCG	(GA) pYT313-Δrtn2
F-down-d2-rtn	TTTTTGCGACCATCTAATAAAAAGCATTACAAGGTAAACTAATGCTCTGAGAA	(GA) pYT313-Δrtn2

R-down-d2-rtn	CCATGATTACGCCAAGCTTGCATGCATGCTTAGAACTCCATGAGAAATTA	(GA) pYT313-Δrtn2
F-verif2-rtn	TTCCATAAATGAGGCCGAACTA	verification of Δrtn2
R-verif2-rtn	CTCCAAAATCCTCGACATCCAA	verification of Δrtn2
F-up-d3-rtn	GGAAAAATTCGGGGGATCCTCTAGAAATATCTTCCAGCTCTTCCAAT	(GA) pYT313-Δrtn3
R-up-d3-rtn	ATAGGAGACAATTTTTTCTAGGAAGTGGATTCAATTGTGTTTTAATTTAAAAGCG	(GA) pYT313-Δrtn3
F-down-d3-rtn	GCACGCTTTTAAATTAAAACACAATTGAATCCACTTCCTAGAAAAAATTGTC	(GA) pYT313-Δrtn3
R-down-d3-rtn	CCATGATTACGCCAAGCTTGCATGCACTCCACCATCTGTTATAGTGAT	(GA) pYT313-Δrtn3
F-verif3-rtn	AAATTATGCCTTCCGAAAGGT	verification of Δrtn3
R-verif3-rtn	AGCGTACATTTCCTAAATGGTC	verification of Δrtn3
F-up-d4-rtn	GGAAAAATTCGGGGGATCCTCTAGATATTAGCTAAGTTAAGTGTTAATTTTTT	(GA) pYT313-Δrtn4
R-up-d4-rtn	AAGTTTTTTCAAAAAATAAGTTTTAATTCTTAGTGTATAAAATGGTTTTAGTATT	(GA) pYT313-Δrtn4
F-down-d4-rtn	AATACTAAAACCATTTTATACACTAAGAATTAAAACTTATTTTTTGAAAAAACTT	(GA) pYT313-Δrtn4
R-down-d4-rtn	CCATGATTACGCCAAGCTTGCATGCCGCTCCATAATCATACATCCCT	(GA) pYT313-Δrtn4
F-verif-d4-rtn	CCCAACACAAAAAGCAAATTAC	verification of Δrtn4
R-verif-d4-rtn	TCGGATTATGAGCCATACTGTAAA	verification of Δrtn4
F-up-d5-rtn	GGAAAAATTCGGGGGATCCTCTAGATATTTATGTACTAGGAAATCATGAGTATTA	(GA) pYT313-Δrtn5
R-up-d5-rtn	AATGGTGACTTTTTTATTAGGTTAATCAATTGTGTTTTAATTTTAAATTTTGTG	(GA) pYT313-Δrtn5
F-down-d5-rtn	AAATTTAAAATTAAAACACAATTGATTAACCTAATAAAAAAGTCACCATTTC	(GA) pYT313-Δrtn5
R-down-d5-rtn	CCATGATTACGCCAAGCTTGCATGCTTAAGTATTGGTGTAGCTCCTTT	(GA) pYT313-Δrtn5
F-verif-d5-rtn	GAAGTTGGAGGAATTCAGTATTTTG	verification of Δrtn5
R-verif-d5-rtn	GGACAATCAACATTCCGAAAAA	verification of Δrtn5
F-up-d6-rtn	GGAAAAATTCGGGGGATCCTCTAGAAACTAAGCTTCAACAATTTGAG	(GA) pYT313-Δrtn6
R-up-d6-rtn	CTGCCAAGGGTAGGAGTTTTAATTGTGTTTTATTTTTTAGTTGTATTTAGAAATT	(GA) pYT313-Δrtn6
F-down-d6-rtn	CTAAATACAACTAAAAAATAAAACACAATTAAAACTCCTACCCTTGG	(GA) pYT313-Δrtn6

R-down-d6-rtn	CCATGATTACGCCAAGCTTGCATGCCGTAGAAGTAATTTATTTTTTTACAGAAT	(GA) pYT313-Δrtn6
F-verif-d6-rtn	ATATACGTCAAGTTTTGACGCT	verification of Δrtn6
R-verif-d6-rtn	CCTGAAACTTGTATTGATAACTCG	verification of Δrtn6
F-up-d7-rtn	GGAAAAAATTCGGGGGATCCTCTAGATTTAGGAATTTATTTTCTCCTTCCT	(GA) pYT313-Δrtn7
R-up-d7-rtn	GGTAACTTCTTCTACAATTAATAAATCTTAATGTTTATTTCGAGTATTGATTATT	(GA) pYT313-Δrtn7
F-down-d7-rtn	TCAATACTCGAAATAAACATTAAGATTTATTAATTGTAGAAGAAGTTACCTCA	(GA) pYT313-Δrtn7
R-down-d7-rtn	CCATGATTACGCCAAGCTTGCATGCTACAGTTGTTTTGGGTAAAGCT	(GA) pYT313-Δrtn7
F-verif-d7-rtn	GCGACATCATAATAATTGAATTACT	verification of Δrtn7
R-verif-d7-rtn	TGTTTCAAAAAGGGCACTATTTT	verification of Δrtn7
F-up-d8-rtn	GGAAAAAATTCGGGGGATCCTCTAGATATTATTGGACGTTCCCAAAATAT	(GA) pYT313-Δrtn8
R-up-d8-rtn	CCTTCTTCAAAGTATTTCTTATTTTTCAAGTGTGTTTTAATTTAAAATTGTG	(GA) pYT313-Δrtn8
F-down-d8-rtn	CAATTTTAAATTAAAACACACTTGAAAAATAAGAAATACTTTGAAGAAGGAT	(GA) pYT313-Δrtn8
R-down-d8-rtn	CCATGATTACGCCAAGCTTGCATGCTACATCCAACCGGAGAAATAAA	(GA) pYT313-Δrtn8
F-verif-d8-rtn	GATCGCAAAAGTAATAATTAACTCAC	verification of Δrtn8
R-verif-d8-rtn	GTAGCTCAATCAAGATACTTGAC	verification of Δrtn8
F-up-d9-rtn	GGAAAAAATTCGGGGGATCCTCTAGACTGATGCTTATTTTTGACAAAGAAG	(GA) pYT313-Δrtn9
R-up-d9-rtn	TTAATGTTTTTTTAAATAACAATTACCTCTTGTTAAGTAATTTTATTTAATAATTTGC	(GA) pYT313-Δrtn9
F-down-d9-rtn	CGCAAATTATTAAATAAAATTACTTAACAAGAGGTAATTGTTATTTAAAAAAACATT	(GA) pYT313-Δrtn9
R-down-d9-rtn	CCATGATTACGCCAAGCTTGCATGCTGATGTTATTTTTTGTCTTTTCATAATC	(GA) pYT313-Δrtn9
F-verif-d9-rtn	CTCATGTGCAGGAATTTTTGTAAAC	verification of Δrtn9
R-verif-d9-rtn	CTGCAAAGGGATTAACAAACTCT	verification of Δrtn9
F-up-pYT-d-gldK	TGGTACTCCATGAGTTGCCGGATCCTCTAGAGTCGACCTG	(SLIC) pYT313 ΔgldK
R-up-pYT-d-gldK	GGCTGGTAATATTACATTAACGGATCCCCCGAATTTTTCCG	(SLIC) pYT313 ΔgldK
F-up-d-gldK	AGGTCGACTCTAGAGGATCCGGCAACTCATGGAGTACCA	(SLIC) pYT313 ΔgldK
R-up-d-gldK	AAAAATTGTCTAGTTGATATTATCTTTTCATGTTAATTTTTATAATTAAG	(SLIC) pYT313 ΔgldK

F-down-d-gldK	TAAAAATTAACATGAAAAGATAATATCAACTAGACAATTTTTC	(SLIC) pYT313 ΔgldK
R-down-d-gldK	CGGAAAAATTCGGGGGATCCGTTAATGTAATATTACCAGCC	(SLIC) pYT313 ΔgldK
F-up-pYT-site1:lacZ	TATATAACTCACATGAAGATTCGGATCCTCTAGAGTCGACC	(SLIC) pYT313 *site1:lacZ*
R-up-pYT-site1:lacZ	ATATAATTCAAGTATATTTGCTGCTGGATCCCCCGAATTTTTC	(SLIC) pYT313 *site1:lacZ*
F-up-site1:lacZ	TGCAGCGGAAAAATTCGGGGGATCCAGCAGCAAATATACTTG	(SLIC) pYT313 *site1:lacZ*
R-up-site1:lacZ	TTCTTTGGTATGCGCTGCCGAATCAAATAAGGTTTAGCTCAC	(SLIC) pYT313 *site1:lacZ*
F-down-site1:lacZ	GGTCTGGTGTCAAAAATAAAAGATTACATATTGAATATTATTTTTG	(SLIC) pYT313 *site1:lacZ*
R-down-site1:lacZ	CTGCAGGTCGACTCTAGAGGATCCGAATCTTCATGTGAGTTATATATTG	(SLIC) pYT313 *site1:lacZ*
F-down-lacZ	CGTGAGCTAAACCTTATTTGATTCGGCAGCGCATACCAAAG	(SLIC) pYT313 *site1:lacZ*
R-down-lacZ	ATAATATTCAATATGTAATCTTTTATTTTTGACACCAGACC	(SLIC) pYT313 *site1:lacZ*
F-up-pYT-site1:cmR	AGTGGCAGGGCGGGGCGTAAAAGATTACATATTGAATAT	(SLIC) pYT313 *site1:cmR*
R-up-pYT-site1:cmR	TCCAGTGATTTTTTTCTCCATTTTTTAATTACAATTTAG	(SLIC) pYT313 *site1:cmR*
F-down-cmR	AACTAAATTGTAATTAAAAAATGGAGAAAAAAATCACTGG	(SLIC) pYT313 *site1:cmR*
R-down-cmR	TAATATTCAATATGTAATCTTTTACGCCCCGCCCTGCCAC	(SLIC) pYT313 *site1:cmR*
F-up-pYT-site1:mChartreuse	CATGGATGAGCTCTACAAATAAAAGATTACATATTGAATATTATTTTTG	(SLIC) pYT313 *site1:mChartreuse*
R-up-pYT-site1:mChartreuse	ACAGTTCTTCACCTTTAGACATTTTTTAATTACAATTTAGTTAATTAC	(SLIC) pYT313 *site1:mChartreuse*
F-down-mChartreuse	TTAACTAAATTGTAATTAAAAAATGTCTAAAGGTGAAGAACTG	(SLIC) pYT313 *site1:mChartreuse*
R-down-mChartreuse	ATAATATTCAATATGTAATCTTTTATTTGTAGAGCTCATCCATG	(SLIC) pYT313 *site1:mChartreuse*
F-up-pYT-d-rtnA1	TTTCATTAAGGTCAACTTTGGATCCTCTAGAGTCGACCTGC	(SLIC) pYT313 ΔrtnA1
R-up-pYT-d-rtnA1	CAGAGATTCAAATCCTGCATTGGATCCCCCGAATTTTTCCGC	(SLIC) pYT313 ΔrtnA1
F-up-d-rtnA1	TGAATATGTTCATACTTATATTGTTTTATTTTTTTAGTTTGG	(SLIC) pYT313 ΔrtnA1
R-up-d-rtnA1	TGCAGGTCGACTCTAGAGGATCCAAAGTTGACCTTAATGAAAGAAGAC	(SLIC) pYT313 ΔrtnA1
F-down-d-rtnA1	AGCGGAAAAATTCGGGGGATCCAATGCAGGATTTGAATCTCTGG	(SLIC) pYT313 ΔrtnA1
R-down-d-rtnA1	TCCAAACTAAAAAAATAAAACAATATAAGTATGAACATATTCAAG	(SLIC) pYT313 ΔrtnA1
F-up-pYT-rtnA1-CTD	GATAAAAACTATTACGTATCCTGGATCCCCCGAATTTTTCCGCTG	(SLIC) pYT313 rtnA1^{-CTD}

R-up-pYT-rtnA1-CTD	TATTATAATTCACAGGAGATGTGGATCCTCTAGAGTCGACCTGC	(SLIC) pYT313 rtnA1^{-CTD}
F-up-rtnA1-CTD	TGCAGGTCGACTCTAGAGGATCCACATCTCCTGTGAATTATAATAATAG	(SLIC) pYT313 rtnA1^{-CTD}
R-up-rtnA1-CTD	GAATATGTTCATACTTATATTTATATTAATTGATCAATTTGTTTATC	(SLIC) pYT313 rtnA1^{-CTD}
F-down-rtnA1-CTD	GATAAACAAATTGATCAATTAATATAAATATAAGTATGAACATATTC	(SLIC) pYT313 rtnA1^{-CTD}
R-down-rtnA1-CTD	AGCGGAAAAATTCGGGGGATCCAGGATACGTAATAGTTTTTATC	(SLIC) pYT313 rtnA1^{-CTD}
F-up-pYT-rtnC1ΔCt1	TTTTCAGTATCCGTATTCAAGGATCCTCTAGAGTCGACCTG	(SLIC) pYT313 rtnC1ΔCt1
R-up-pYT-rtnC1ΔCt1	TTTATTTCATATCGATTACCCGGATCCCCCGAATTTTTCCGC	(SLIC) pYT313 rtnC1ΔCt1
F-up-rtnC1ΔCt1	ATATTCTATATGTGTTCATAGTATTTTCAACAACACTTTC	(SLIC) pYT313 rtnC1ΔCt1
R-up-rtnC1ΔCt1	AGGTCGACTCTAGAGGATCCTTGAATACGGATACTGAAAAAG	(SLIC) pYT313 rtnC1ΔCt1
F-down-rtnC1ΔCt1	CGGAAAAATTCGGGGGATCCGGGTAATCGATATGAAATAAAATTTAAG	(SLIC) pYT313 rtnC1ΔCt1
R-down-rtnC1ΔCt1	AAAGTGTTGTTGAAAATACTATGAACACATATAGAATATC	(SLIC) pYT313 rtnC1ΔCt1
F-up-pYT-d-rtnA2	AACCTACCATCACGTAATGCTGGATCCTCTAGAGTCGACCTG	(SLIC) pYT313 ΔrtnA2
R-up-pYT-d-rtnA2	CTTTATTGATCTTGGTATCTGGATCCCCCGAATTTTTCCG	(SLIC) pYT313 ΔrtnA2
F-up-d-rtnA2	CGGAAAAATTCGGGGGATCCAGATACCAAGATCAATAAAG	(SLIC) pYT313 ΔrtnA2
R-up-d-rtnA2	GTAACTTTATTCATTTGTATGTAATGCTTTTTATTAGATG	(SLIC) pYT313 ΔrtnA2
F-down-d-rtnA2	CATCTAATAAAAAGCATTACATACAAATGAATAAAGTTAC	(SLIC) pYT313 ΔrtnA2
R-down-d-rtnA2	CAGGTCGACTCTAGAGGATCCAGCATTACGTGATGGTAGGTTTTC	(SLIC) pYT313 ΔrtnA2
F-up-d-tssB-C	AGGTCGACTCTAGAGGATCCCACTATTTGCTAGAAGCCAC	(SLIC) pYT313 ΔtssB-C
R-up-d-tssB-C	AATGTTGTTATTCTAACATATAAAATTTTGTTTAATTTTTTATTAAATA	(SLIC) pYT313 ΔtssB-C
F-down-d-tssB-C	TTATATGTTAGAATAACAACATTAATTTTAATG	(SLIC) pYT313 ΔtssB-C
R-down-d-tssB-C	CGGAAAAATTCGGGGGATCCGAAGATTAGAAGCAGTCTGCC	(SLIC) pYT313 ΔtssB-C
F-up-d-porZ	GGAAAAATTCGGGGGATCCTCTAGATAGATCTTACCAAACAAGGTAATAATAGTAATC	(SLIC) pYT313 ΔporZ
R-up-d-porZ	GTTCATCAATTAACTACTGCTATTTAATTATGCGATTATACTGCTATCTATTG	(SLIC) pYT313 ΔporZ
F-down-d-porZ	TAGATAGCAGTATAATCGCATAATTAAATAGCAGTAGTTAATTGATGAACTCA	(SLIC) pYT313 ΔporZ
R-down-d-porZ	CCATGATTACGCCAAGCTTGCATGCTATAAAAATTATTTATTTTTACCTTTTCGTGATGC	(SLIC) pYT313 ΔporZ

F-up-pYT-rtnC1D879N	TTACTCCTCGATTTCGTATGGATCCTCTAGAGTCGACCTG	(SLIC) pYT313 rtnC1^{D879N}
R-up-pYT-rtnC1D879N	GATGGATTATCTGTTAGGACGGATCCCCCGAATTTTTCCG	(SLIC) pYT313 rtnC1^{D879N}
F-up-rtnC1D879N	AATTGAGCCTATAACAATAACTGTTTCTAGAGCGACACC	(SLIC) pYT313 rtnC1^{D879N}
R-up-rtnC1D879N	AGGTCGACTCTAGAGGATCCATACGAAATCGAGGAGTAAAG	(SLIC) pYT313 rtnC1^{D879N}
F-down-rtnC1D879N	CGGAAAAATTCGGGGGATCCGTCCTAACAGATAATCCATC	(SLIC) pYT313 rtnC1^{D879N}
R-down-rtnC1D879N	GGTGTCGCTCTAGAAACAGTTATTGTTATAGGCTCAATTG	(SLIC) pYT313 rtnC1^{D879N}
F-up-pYT-site2:rtnI1	ACTTATTAGAACAAATTTAACCTAAAACTTTGTCTAAATCTG	(SLIC) pYT313 *site2:rtnI1*
R-up-pYT-site2:rtnI1	TGATATTCTATATGTGTTCATAAATATTAATTATTAATTAC	(SLIC) pYT313 *site2:rtnI1*
F-up-site2:rtnI1	AGTAATTAATAATTAATATTTATGAACACATATAGAATATCAG	(SLIC) pYT313 *site2:rtnI1*
R-up-site2:rtnI1	AGATTTAGACAAAGTTTTAGGTTAAATTTGTTCTAATAAGTG	(SLIC) pYT313 *site2:rtnI1*
F-up-CNins2	GGAAAAATTCGGGGGATCCTCTAGACACCATAACCCAAATTTCTCATAAA	(GA) pYT313 site2:mLytchee
R-up-CNins2	CATCTGAATAGCTTTAAAACAAAGGGCTTTTGTTATATCTGGAAAAAGTCATGCA	(GA) pYT313 site2:mLytchee
F-down-CNins2	ACGCACGGCATGGATGAGCTCTACAAATAACCTAAAACTTTGTCTAAATCTGAAAAA	(GA) pYT313 site2:mLytchee
R-down-CNins2	CCATGATTACGCCAAGCTTGCATGCCCGTACATTGTAACAAAAGAATTAAAT	(GA) pYT313 site2:mLytchee
F-ins2-verif	GTAGTAGTCGCAGATAGTAAAACTA	(GA) pYT313 site2:mLytchee
R-ins2-verif	GTACAACGTATGTCAAGTACGGAGC	(GA) pYT313 site2:mLytchee
F-mLychee	ATGGATTCAACAGAAGCAATTATT	(GA) pYT313 site2:mLytchee
R-mLychee	TTACTTGTACAAACTTCCGCCA	(GA) pYT313 site2:mLytchee
R-CNins2-mLychee	TAATAATTGCTTCTGTTGAATCCATAAATATTAATTATTAATTACTAAAATTCCCTTCA	(GA) pYT313 site2:mLytchee
F-CNins2-mLychee	AAGGCGGCTCTGGCGGAAGTTTGTACAAGTAACCTAAAACTTTGTCTAAATCTGAAAAA	(GA) pYT313 site2:mLytchee
F-up-pYT-JUb129 site1:cmR	AGCAGCTAATGGTATTTTGGAGGATCCTCTAGAGTCGACCTG	(SLIC) pYT313 JUb129 *site1:cmR*
R-up-pYT-JUb129 site1:cmR	AGATTTTAGGAAAAGTTGAGGGATCCCCCGAATTTTTCCG	(SLIC) pYT313 JUb129 *site1:cmR*
F-up-JUb129 site1:cmR	CGGAAAAATTCGGGGGATCCCTCAACTTTTCCTAAAATCTG	(SLIC) pYT313 JUb129 *site1:cmR*
R-up-JUb129 site1:cmR	CTTTGGTATGCGCTGCCGAAAAAATGCGAGAACTTTATTAAG	(SLIC) pYT313 JUb129 *site1:cmR*

F-down-JUb129 site1:cmR	GAGTGGCAGGGCGGGGCGTAATGGCTTCTAATATAAGCTT	(SLIC) pYT313 JUb129 *site1:cmR*
R-down-JUb129 site1:cmR	AGGTCGACTCTAGAGGATCCTCCAAAATACCATTAGCTGC	(SLIC) pYT313 JUb129 *site1:cmR*
F-down-cmR	TTAATAAAGTTCTCGCATTTTTTCGGCAGCGCATACCAAAG	(SLIC) pYT313 JUb129 *site1:cmR*
R-down-cmR	AAGCTTATATTAGAAGCCATTACGCCCCGCCCTGCCACTC	(SLIC) pYT313 JUb129 *site1:cmR*
F-up-pYT-JUb129 site1:mLytchee	GCGGAAGTTTGTACAAGTAATGGCTTCTAATATAAGCTTAC	(SLIC) pYT313 JUb129 *site1:mLytchee*
R-up-pYT-JUb129 site1:mLytchee	ATTGCTTCTGTTGAATCCATTTTTTAATTACAATTTAGTTAATTA	(SLIC) pYT313 JUb129 *site1:mLytchee*
F-up-JUb129 site1:mLytchee	TTAACTAAATTGTAATTAAAAAATGGATTCAACAGAAGCAATT	(SLIC) pYT313 JUb129 *site1:mLytchee*
R-up-JUb129 site1:mLytcheesite2:rtnI1	TAAGCTTATATTAGAAGCCATTACTTGTACAAACTTCCGC	(SLIC) pYT313 JUb129 *site1:mLytchee*
F-pBAD-MCS	CTGCAGGCATGCAAGCTTGGC	(RL) pBAD33-plasmids
R-pBAD-SD-Sal	GATCAGTCGACCCTCCTTCTAGAGGATCCCCGGGTACCG	(RL) pBAD33-Cyto plasmids
R-torA-ss-Sal	GACTGTCGACCGCCGCAGTCGCACGTCGC	(RL) pBAD33-Peri plasmids
F-C1-CT-Sal	GACTGTCGACATGAATGAAACTGGAAAAATTTATAGAGT	(RL) pBAD33-Ct1
R-C1-CT-Hind	GACTAAGCTTCTATATGTGTTCATTGTTCCCAT	(RL) pBAD33-Ct1
F-C2-CT-Sal	GACTGTCGACATGGGAACTGAAGATTTTGTACAGA	(RL) pBAD33-Ct2
R-C2-CT-Sph	CAGTGCATGCTTAATTTCTTTTATAAAAACGTGATGA	(RL) pBAD33-Ct2
F-C3-CT-Sal	GACTGTCGACATGAGAGAAGGCTTAGGATGGGG	(RL) pBAD33-Ct3
R-C3-CT-Sph	CAGTGCATGCTCATTTCTTTTTACCATAATTTTTTATTG	(RL) pBAD33-Ct3
F-C4-CT-Sal	GACTGTCGACATGAGAACCTGGGGGGACCCGAAA	(RL) pBAD33-Ct4
R-C4-CT-Sph	CAGTGCATGCTTATTTGTCTTTAACATCTAGTGCTTTATA	(RL) pBAD33-Ct4
F-C5-CT-Sal	GACTGTCGACATGAGACAAGGTACAGACTGGGT	(RL) pBAD33-Ct5
R-C5-CT-Hind	GACTAAGCTTTTATCTGATTCCAAATTTTAAATTAAGGTC	(RL) pBAD33-Ct5
F-C6-CT-Sal	GACTGTCGACATGCAGGCTATGAAGCCTACACC	(RL) pBAD33-Ct6
R-C6-CT-Hind	GACTAAGCTTTTATTTAGTATATTTTTTAGGATTAATTCCG	(RL) pBAD33-Ct6
F-C7-CT-Sal	GACTGTCGACATGAGAGCTCCTATGGATGAATATG	(RL) pBAD33-Ct7
R-C7-CT-Hind	GACTAAGCTTTTAAGGTGTACCATTTTTAATATAAT	(RL) pBAD33-Ct7
F-C8-CT-Sal	GACTGTCGACATGGAGGGAAAAGAGGGAGTA	(RL) pBAD33-Ct8
R-C8-CT-Hind	GACTAAGCTTCTACTTATATTTTTTAAAACCGGGT	(RL) pBAD33-Ct8
F-C1-CT-Y7A	GCGAGAGTCCCTGGAGATCAGAC	(MUT) pBAD33 Cyto-Ct1Y7A
R-C1-CT-Y7A	AATTTTTCCAGTTTCATTCATGTCG	(MUT) pBAD33 Cyto-Ct1Y7A
F-C1-CT-Y20A	GCGATTGGAAGAACTAAACAACGTAG	(MUT) pBAD33 Cyto-Ct1Y20A

R-C1-CT-Y20A	CGGTTTGCCACTTTCTGTCT	(MUT) pBAD33 Cyto-Ct1Y20A
F-pBAD-C9-CT	TTACCGAATGTTCCATCCATGTCGACCCTCCTTCTAGAGG	(SLIC) pBAD33 Cyto-Ct9
R-pBAD-C9-CT	TGGCCGTTTTATAAAGAATAAGCATGCAAGCTTGGCTGTTTTG	(SLIC) pBAD33 Cyto-Ct9
F-C9-CT	CCTCTAGAAGGAGGGTCGACATGGATGGAACATTCGGTAA	(SLIC) pBAD33 Cyto-Ct9
R-C9-CT	AAAACAGCCAAGCTTGCATGCTTATTCTTTATAAAACGGCCAT	(SLIC) pBAD33 Cyto-Ct9
F-pBAD-C9-P	TGTTTTACCGAATGTTCCATCCATGTCGACCGCCGCAGTCG	(SLIC) pBAD33 Peri-Ct9
R-pBAD-C9-P	TGGCCGTTTTATAAAGAATAAGCATGCAAGCTTGGCTGTTTTG	(SLIC) pBAD33 Peri-Ct9
F-C9-P	GACTGCGGCGGTCGACATGGATGGAACATTCGGTAAAACA	(SLIC) pBAD33 Peri-Ct9
R-C9-P	AAAACAGCCAAGCTTGCATGCTTATTCTTTATAAAACGGCCAT	(SLIC) pBAD33 Peri-Ct9
F-pETd-synth	GCCAGGATCCGAATTCGAGCTC	Construction of Duet plasmids with pelB
R-pelB-duet	GGCCATCGCCGGCTGGGCAGCGAGGAGCAGCAGACCAGCAGCAGCGGTCGGCAGCAGGTATTTCATGGTATATCTCCTTATTAAAGTT	Construction of Duet plasmids with pelB
R-pel-sac	GGAGGAGCTCGGCCATCGCCGGCTGGGCAG	(RL) Duet plasmids with pelB
F-duet-strep-sal	GACTGTCGACTGGAGCCACCCGCAGTTCGAAAAATAAAAGCTTGCGGCCGCATAATGC	(RL) Duet plasmids with C-ter strep
F-duet-flag-sal	GACTGTCGACTGGAGCCACCCGCAGTTCGAAAAATAAAAGCTTGCGGCCGCATAATGC	(RL) Duet plasmids with C-ter flag
F-RtnA1-Sac	GGAGGAGCTCCAGGAAGTCGTCTGGCAAAGAA	(RL) pACYC-Duet pelB-RtnA1-Strep / Flag
R-RtnA1-Sal	GATCGTCGACTTTCTTAATCAATTTTGCGTTTGC	(RL) pACYC-Duet pelB-RtnA1-Strep / Flag
F-RtnB1-Sac	GGAGGAGCTCCAAATAATACCTACCATTAAATCTCC	(RL) pRSF-Duet pelB-RtnB1-Strep / Flag
R-RtnB1-Sal	GATCGTCGACGTTCTTGTAATTGTATTTATATTCTTTCA	(RL) pRSF-Duet pelB-RtnB1-Strep / Flag
F-RtnC1-Sac	GGAGGAGCTCCAGTTAAGTCCTACTGAAAACT	(RL) pCDF-Duet pelB-RtnC1-Flag
R-RtnC1-Sal	GATCGTCGACTATGTGTTCATTGTTCCCATAT	(RL) pCDF-Duet pelB-RtnC1-Flag
F-pACYC-RtnA1ΔCTD	AAACAAATTGATCAATTAATAGTCGACTGGAGCCAC	(SLIC) pACYC-Duet pelB-RtnA1ΔCTD-Strep
R-pACYC-RtnA1ΔCTD	TATTAATTGATCAATTTGTTTATCTCCTAACTTGATAATTTTCC	(SLIC) pACYC-Duet pelB-RtnA1ΔCTD-Strep
F-pACYC-RtnA2ΔCTD	GTCGACTGGAGCCACCCGCAGTTCGAAAAATAAAAGCTTGCGGCCGCATAATG	(SLIC) pACYC-Duet pelB-RtnA2ΔCTD-Strep
R-pACYC-RtnA2ΔCTD	TTATTTTTCGAACTGCGGGTGGCTCCAGTCGACGATTAATTGATCAATTTG	(SLIC) pACYC-Duet pelB-RtnA2ΔCTD-Strep
F-pCDF-RtnC1D879N	AATCCATTAAGTGAGAAATTTC	(SLIC) pCDF-Duet pelB-RtnC1D879N-Flag
R-pCDF-RtnC1D879N	AGTTGTGGCAAATCTCCCAATATC	(SLIC) pCDF-Duet pelB-RtnC1D879N-Flag

Table S5 | cryo-EM data collection, processing and model refinement statistics

	RtnB1-RtnC1
PDB accession code	29RD
EMDB accession code	EMD-57319
Data collection	
Microscope	Talos Arctica
Camera	Gatan K2 Summit
Voltage (kV)	200
Magnification	45 000
Total Dose	49.95
Processing	
Symmetry imposed	C1
Micrographs number	6401
Initial particle images (no.)	2204596
Final particle images (no.)	379537
Map resolution (Å)–(0.143 FSC threshold model)	3.4
Model composition	
No. of chains	2
No. of residues	1852
No. of atoms	14836
No. of ligands	0
Bond lengths (Å)	0.002
Bond angles (°)	0.547
Ramachandran favored %	97.23
Ramachandran allowed %	2.77
Ramachandran outliers %	0.00
Rotamers outliers %	0.55
MolProbity score	1.17
Clashscore	2.44
CC (mask)	0.83
CC (box)	0.74
CC (peaks)	0.72
CC (volume)	0.80

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