

RES-Xre toxin-antitoxin locus KnaAT maintains the stability of virulence plasmid in *Klebsiella pneumoniae*

Supplementary data

Table S1. Comparison of TA families between plasmid types (ARG-carrying, virulence, and “other”) and their correlation.

Table S2. Sequence type of *K. pneumoniae* carrying virulence plasmid.

Table S3. Details of the TAFinder-predicted RES-Xre TA loci in *K. pneumoniae* genomes.

Table S4. The information on bacteria strains, plasmids and primers that were used in this study.

Figure S1. Inferred phylogenetic relationships of the 186 RES toxin proteins encoded by RES-Xre TA loci of *K. pneumoniae*.

Figure S2. The amino acid percentage identity matrix of RES toxins of *K. pneumoniae* and other reported species.

Figure S3. Spot plate assays showing the bacteriostatic effect of the RES toxin KnaT in *K. pneumoniae* RJF293Δ*knaAT*.

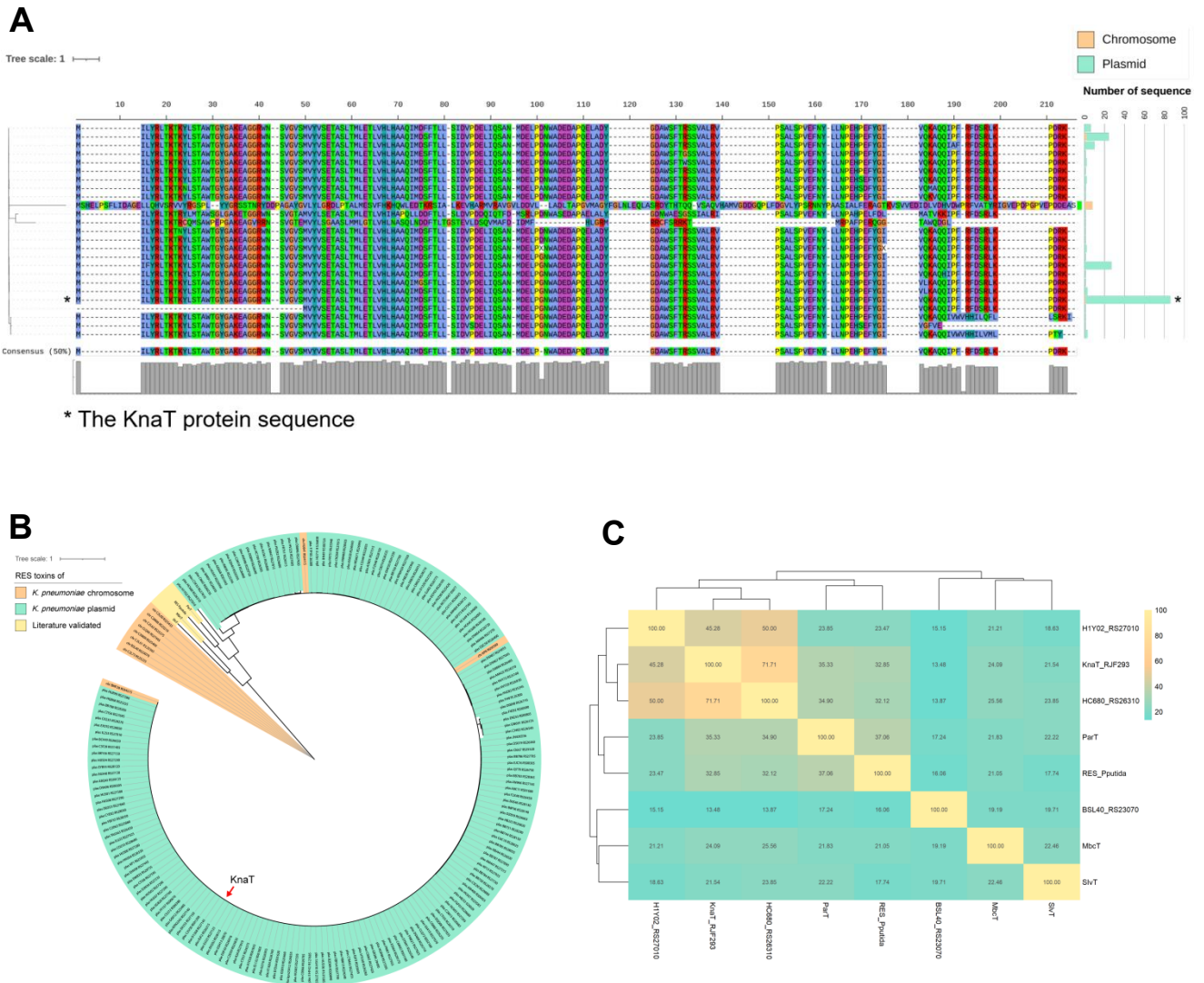


Figure S1. Inferred phylogenetic relationships of the 186 RES toxin proteins encoded by RES-Xre TA loci of *K. pneumoniae*. **(A)** Multiple sequence alignment of the 186 RES toxin proteins. Protein sequences of 187 identified RES toxins were clustered by CD-HIT (identities = 100% and coverage = 100%), resulting in 25 non-redundant sequences. The multiple sequence alignment was performed and the phylogenetic tree was constructed for these 25 unique sequences. The consensus sequence is displayed below the multiple sequence alignment. **(B)** Inferred phylogenetic relationship of 186 RES toxin proteins encoded by the RES-Xre TA loci in the chromosomes and plasmids of *K. pneumoniae* strains. The Maximum Likelihood tree was generated with IQ-TREE [27] with the default settings and displayed by iTOL [28]. The RES toxin KnaAT encoded by the virulence plasmid pRJF293 of the hypervirulent *K. pneumoniae* 293 is marked. More details of the RES toxins are available in **Table S3**. **(C)** The amino acid percentage identity matrix of RES toxins from different clades of *K. pneumoniae* (KnaT of pRJF293, H1Y02 of RS27010, HC680 of RS26310 and BSL40 of RS23070) and other reported species (MbcAT of *M. tuberculosis* H37Rv, SlvAT of *P. putida* S12, RES-Xre_Pputida of *P. putida* KT2440 and ParST of *Sphingobium* sp. YBL2).

A

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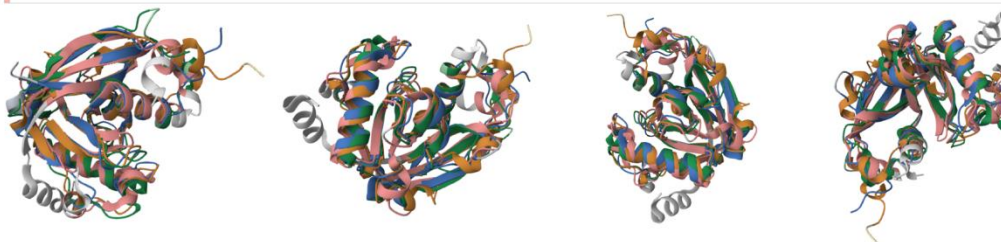
KnaT      1  ----- M I L Y R L T K T K - - Y L S T A W T G Y C A K E A G G R N S V G V - S M V Y V S E T A S L T M L E T L V H L H - - A A Q - - - - - I M D S F T L L S D V P D E 72
RES_Pputida 1  ----- M I L W R I S A Y A - - - - - D L S G T G L R V S G A H Q A G R - P V Y A A T S P P G A M L E V L V H L E I D P E D - - - - - F P T T M R L R I E L P D T 70
ParT      1  ----- P V T T S F W R I A T D A R T Y E A D D L S G A C A K I T G G R N E V G V - A I V Y A A S S R A L A C L E T V V H L N - - S G G - - - - - L P L N R Y V E I E V P D E 77
MbcT      1  V S D A L D E G L V Q R I D A R G T I E W S E T C Y R Y T G A H R - - - - - D A L S G E C A R R F G G R N P L L F P A I L A D S A Q A C M V E R A A Q - - A A S T T A E K M L E A A Y R H T I D V T D L 99

KnaT      73 L I Q S A N M - - - - - D E L P G N W A D E D A P Q E L A D - Y G D A W S F T R - - - - - S S V A L R V P S A L S P V E - - - - - F N Y L L N P E H P E F Y G I V Q K A - Q Q I P F R F - - - - - D S R L K P D R K 156
RES_Pputida 71 V S Q - A Q L - - - - - P A L Q P G W S A Q P - - - - - E L T R T L G N R F L D D C - - - - - S A L L P V P S A I M P S T - - - - - T N Y L F N P R H P Q A Q S A - K I - Q V E D F T P - - - - - D S R L F - - - - - 145
ParT      78 V L A S A E V A T P G N L P V G W D A E P A G R V S I S - F S Q W A Q S Q - - - - - R T A L L L V P S V I V P E E - - - - - T N L L I N P A H P D A K G I - - K A R K V R K W L Y - - - - - D P R M I - - - - - R 160
MbcT      100 A V L - - - - - D L T T P Q A R E A V G L E N D D I Y G D W S G C Q A V G H A A W F L H M Q G V L V P A A G G V G L V V T A V E Q R T R P G Q L Q L R - - - - - Q S V D L T P A L Y Q E L R A T - - - - - 186

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B

	Entry	Chain	RMSD	TM-score	Identity	Equivalent Residues	Sequence Length	Modelled Residues
KnaT	AF-A0A377ZUI9-F1-model_v4.pdb	A	-	-	-	-	156	156
ParT	6D0H	A	2.46	0.8	33%	152	160	160
RES	6GW6	A	2.09	0.78	31%	140	145	145
MbcT	6FKG	A	2.63	0.68	21%	146	186	182



C

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KnaA      1  M K T F S L S S T - - - - - P A R P Q R L W Q V A G L N N A D G V A L L G Q I N E G L D G K V A N R I T D W A R I T Q N D L R K M S G I P S T T F S R S V K A R F N P E 79
ParS      1  ----- G A M G V N V L A S T V S G A I E R L G L T Y E E V G D I V D A S P R S V A R W - - - T A G Q V V P Q R L N - - 51
MbcA      1  M L A E V L R D N G Y H E Y R A R L Q A L L D I P E L - - A S D F E I H T R I T D G F A A T W L V K L T E R G V L T P V E R D Q I I P L - - - - - R T L K S R I E R D 76
Xre_Pputida

KnaA      80 Q - - - - - S E R L V R I I R V I D R A V D L F E G D K E A - - - - - A Q K V L N E P N R A L S W K V P A D L M A S E T G A Y E I K L I T R L E H G V Y S 147
ParS      1  ----- V L G L A K L V G Q L E D M V E E S G E T D G F D A P E W L S S L R Q L P A L G G V N F I D L D T M E G Q A V Y S R A L A Q I Q S G A F A 72
MbcA      52 - - - - - K Q R L I E L A Y V A D A L A E V L P R D Q - - - - - A N V M F S P N R L L E H R K P A D L V R D G E Y Q R V L A L I D A M A E G V F V 115
Xre_Pputida 77 Q P L T V D E S D R L F R S A H I T A M A E A V F G E A G K - - - - - A K R L S K P K E R F S G L T M Q M L T T Q Q G T T Q E E M L L Q I A E C Y G L 149

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D

	Entry	Chain	RMSD	TM-score	Identity	Equivalent Residues	Sequence Length	Modelled Residues
KnaA	antitoxin-AF-A0A377ZTX7-F1-model_v4.pdb	A	-	-	-	-	147	147
ParS	6D0H	B	2.39	0.36	22%	64	72	72
Xre	6GW6	B	2.37	0.73	19%	131	149	149
MbcA	6FKG	C	3.07	0.55	20%	107	115	111

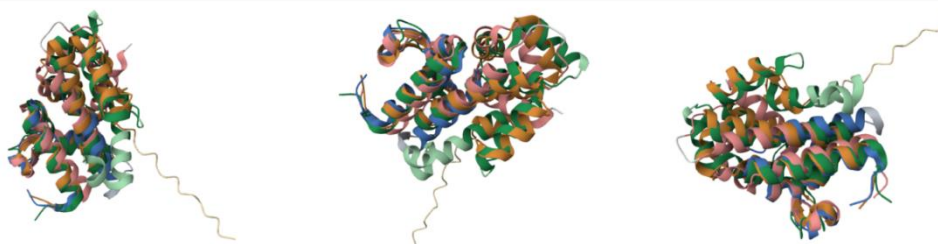


Figure S2. Sequence alignment of the RES toxins and the Xre antitoxin encoded by the RES-Xre TA loci of *K. pneumoniae* and other reported species. **(A)** The multiple sequence alignment of RES toxin proteins. Residues with deeper colors indicate higher identities. **(B)** The protein structure comparison of RES toxins. The protein tertiary structure of the RES toxin KnaT of *K. pneumoniae* 293 archived in the AlphaFold Protein Structure Database (Accession: A0A377ZUI9, chain A) was compared to the experimentally determined structures of 3 RES domain-containing proteins, including ParT of *Spingobium* sp. YBL2 (PDB ID: 6D0H, chain A), PpRES of *P. putida* KT2440 (PDB ID: 6GW6, chain A) and MbcT of *M. tuberculosis* H37Rv (PDB ID: 6FKG, chain A), by using jFATCAT algorithm in the PDB pairwise structure alignment server (<https://www.rcsb.org/alignment>). **(C)** The multiple sequence alignment of Xre antitoxin proteins. **(D)** The protein tertiary structure of KnaA archived in AlphaFold Protein Structure Database (Accession: A0A377ZTX7, chain A) was compared to the experimentally determined structures of 3 Xre-like proteins ParS (PDB ID: 6D0H, chain B), Xre (PDB ID: 6GW6, chain B) and MbcA (PDB ID: 6FKG, chain C).

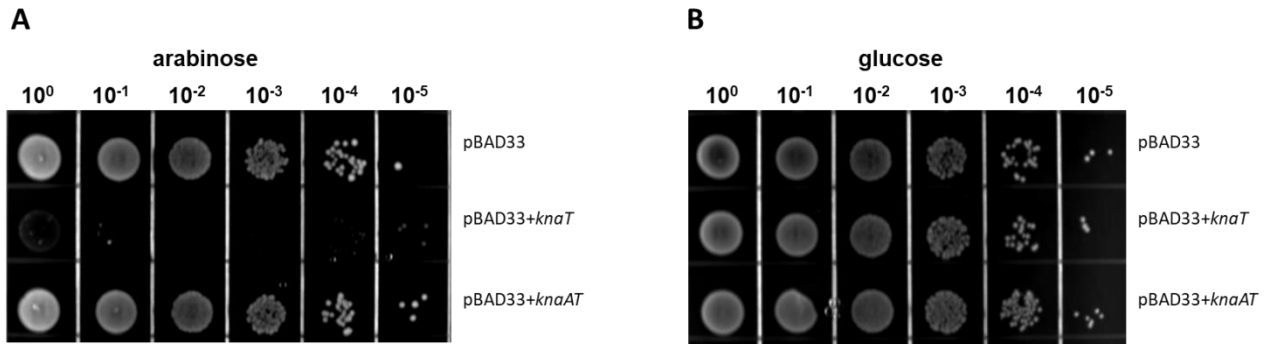


Figure S3. Spot plate assay showing the bacteriostatic effect of the RES toxin KnaT in *K. pneumoniae*

RJF293Δ*knaAT*. The serially diluted *K. pneumoniae* RJF293Δ*knaAT* with different plasmid combinations

were plated on LB agar supplemented with 30 µg/ml of chloramphenicol, complement with **(A)** 0.2 %

arabinose (w/v) or **(B)** 0.2 % glucose (w/v). The plasmids for phenotypic assays in *K. pneumoniae*

RJF293Δ*knaAT* were constructed by cloning the RES toxin gene (*knaT*) and its complete RES-Xre TA

operon (*knaAT*) into the pBAD33 plasmid. The *knaT* gene and the *knaAT* operon were separately amplified

from the genomic DNA of RJF293 using the primers shown in Table S4. Then, the PCR products were

digested with restriction enzymes (*SacI* and *HindIII*) and cloned into pBAD33 using T4 DNA ligase, producing

pBAD33-*knaT* and pBAD33-*knaAT* plasmids. The generated plasmids were then transformed into *K.*

pneumoniae RJF293Δ*knaAT*.