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POINT OF VIEW

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Introducing the new bacterial branch of the RNase A superfamily

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ABSTRACT

Bovine pancreatic ribonuclease (RNase A) is the founding member of the RNase A superfamily. Members of this superfamily of ribonucleases have high sequence diversity, but possess a similar structural fold, together with a conserved His-Lys-His catalytic triad and structural disulfide bonds. Until recently, RNase A proteins had exclusively been identified in eukaryotes within vertebrae. Here, we discuss the discovery by Batot *et al.* of a bacterial RNase A superfamily member, CdiA-CT^{Ykris}: a toxin that belongs to an inter-bacterial competition system from *Yersinia kristensenii*. CdiA-CT^{Ykris} exhibits the same structural fold as conventional RNase A family members and displays *in vitro* and *in vivo* ribonuclease activity. However, CdiA-CT^{Ykris} shares little to no sequence similarity with RNase A, and lacks the conserved disulfide bonds and catalytic triad of RNase A. Interestingly, the CdiA-CT^{Ykris} active site more closely resembles the active site composition of various eukaryotic endonucleases. Despite lacking sequence similarity to eukaryotic RNase A family members, CdiA-CT^{Ykris} does share high sequence similarity with numerous Gram-negative and Gram-positive bacterial proteins/domains. Nearly all of these bacterial homologs are toxins associated with virulence and bacterial competition, suggesting that the RNase A superfamily has a distinct bacterial subfamily branch, which likely arose by way of convergent evolution. Finally, RNase A interacts directly with the immunity protein of CdiA-CT^{Ykris}, thus the cognate immunity protein for the bacterial RNase A member could be engineered as a new eukaryotic RNase A inhibitor.

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Review

To date all identified RNase A superfamily members stem from vertebrates.[1] These RNase A family members possess highly divergent sequences and play a variety of roles in many distinct biological pathways.[1] As a result of their heterogeneous sequences, it is especially challenging to recognize and classify RNase A proteins in the absence of biochemical characterization. All previously identified RNase A proteins contain three to four conserved, structural disulfide bonds (Fig. 1). RNase A proteins act on RNA to catalyze a phosphotransferase bond cleavage to produce a cyclic 2′–3′-phosphodiester fragment and a 5′-hydroxyl terminated fragment.[2,3] While some RNase A members can degrade both single and double-stranded RNAs, the preferred substrate for catalysis is single-stranded RNA.[1] Typically, poly-pyrimidine strands are more rapidly cleaved than poly-purine RNAs, and a conserved His-Lys-His catalytic triad is required for the hydrolytic activity to occur.[2]

Bacteria possess many pathways to compete and communicate with their bacterial neighbors. One such system is contact-dependent growth inhibition (CDI), which is found in Gram-negative bacteria that exchange toxins upon direct cell-to-cell contact.[4,5] CDI relies on a two-partner (Type V) secretion system comprised of CdiA and CdiB. CdiB, an Omp85 β-barrel, is responsible for the export and display of CdiA on the cell surface. CdiA proteins are large (180–630 kDa). They recognize specific surface receptors of neighboring bacteria, and upon contact, initiate transfer of the CdiA C-terminal toxin domain (CdiA-CT) into the target cell.[5-7] A single gene cluster encodes CdiA and CdiB, as well as CdiI, the cognate immunity protein.[5,8] CdiI proteins specifically bind and restrict the activity of cognate CdiA-CT toxin domains, providing species-specific protection.[5,8]

To date, nearly all characterized CDI toxins are nucleases, each with its own specificity. For example, the CdiA-CT of *Enterobacter cloacae* cleaves 16S ribosomal RNA,[9] while uropathogenic *E. coli* 536 bears a unique ribonuclease fold with no sequence similarity to other RNase families and only cleaves tRNA in the presence of endogenous *O*-acetylserine sulfhydrylase (CysK).[10,11] Additionally, several CdiA-CT toxins belong to the PD-(D/E)XK phosphodiesterase superfamily. These include a Zn^{2+}-dependent DNase from *E. coli* TA271,[12,13] as well as CdiA-CTs from *Burkholderia pseudomallei* 1026b and E479, both of which are tRNases that specifically recognize a unique subset of tRNAs and cleave at different tRNA sites.[12,14]

Often the structure of an uncharacterized protein provides insight into its precise function. Recently, the crystal structure of *Yersinia kristensenii* ATCC 33638 CdiA-CT toxin (CdiA-CT^{Ykris}) in complex with its immunity protein (CdiI^{Ykris}) was determined (PDB ID 5E3E).[15] The toxin structure adopts a kidney shape that is formed by two curved β-sheet domains, which strongly resembles several RNase A family members: human angiogenin (PDB ID 4B36[16]), *Rana pipiens* protein P-30 or

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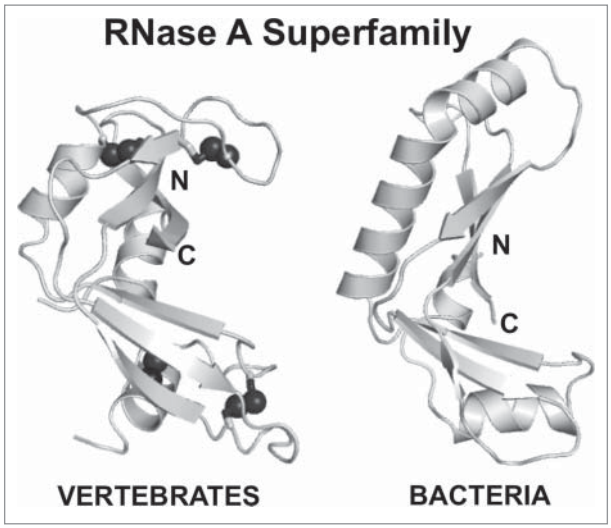

Figure 1. The RNase A superfamily has two divergent branches: one in vertebrates and a recently identified bacterial branch. Notably the disulfide bonds (black spheres) present in all vertebrate RNase A family members (RNase 1 – PDB ID 3TSR[18]) are absent in bacterial RNase A proteins (CdiA-CT^{Yrkis} – PDB ID 5E3E[15]). The N- and C- termini are indicated. Figures were generated in PyMOL.

onconase (PDB ID 3SNF[17]), and mouse pancreatic RNase A (RNase 1, PDB ID 3TSR[18]). Strikingly, while eukaryotic RNase A family members contain 3 to 4 conserved disulfide bonds, CdiA-CT^{Ykris} contains none at all (Fig. 1).

In addition to lacking some structural elements that are highly conserved among eukaryotic RNase A homologs, the composition of the catalytic core of CdiA-CT^{Ykris} is distinct from the typical RNase A His-Lys-His catalytic triad. In angiogenin, the reaction is initiated when His13, acting as a general base, deprotonates the 2′ hydroxyl of substrate RNA.[2] Lys40 stabilizes the resulting transition state intermediate, and, acting as a general acid, His116 terminates the reaction by donating a proton to the 5′ leaving group.[2] From structural alignments with RNase A proteins, the active site of CdiA-CT^{Ykris} was predicted to comprise of His175 (aligns to His13), Val192 (aligns to Lys40), and Thr276 or Tyr278 (aligns to His116). As Val192 is unlikely to participate in catalysis, Batot *et al.* identified the neighboring residue Arg186, which appears to be conserved as shown by multiple sequence alignments of putative bacterial homologs,[15] as a potential transition state stabilizer.

Further, experiments show that CdiA-CT^{Ykris} has metal-independent RNase activity. However, in the presence of the immunity protein, CdiI^{Ykris}, a toxin-immunity complex is formed that effectively neutralizes CdiA-CT^{Ykris} ribonuclease activity. Notably, the CdiA-CT^{Ykris} variants H175A and Y278A display no RNase activity both *in vitro* and *in vivo*, whereas R186A and T276A variants retain partial RNase activity. Like RNase A, CdiA-CT^{Ykris} can also hydrolyze cCMP.[3] All four of the H175A, R186A, T276A and Y278A CdiA-CT^{Ykris} variants exhibited reduced cCMP hydrolytic activity. These results suggest that His175, Arg186, Thr276 and Tyr278 residues are required for full CdiA-CT^{Ykris} ribonuclease activity. The RNase and cCMP hydrolytic activities of CdiA-CT^{Ykris}, in addition to its structural similarity to the RNase A family members, support its classification as a novel bacterial member of the RNase A superfamily.

The distinct set of catalytic/active site residues for CdiA-CT^{Ykris} suggest an alternate mechanism of ribonuclease action. The reaction likely begins via His175, which may behave as a general base to initiate the reaction. Subsequently, Arg186 could stabilize the transition state while Thr276 or Tyr278, acting as a general acid, terminate the reaction. Though distinct from other RNase A family members, aspects of this unique catalytic core have been observed in other endonuclease families. For example, the RNase T1 family of nucleases employ a highly conserved arginine residue to stabilize the reaction intermediate.[19] Further, tRNA-splicing endonucleases use a tyrosine residue as a general base to initiate the reaction, and a histidine residue to terminate the reaction by acting as a general acid.[20] Similarly, tyrosine appears to be involved in the reaction mechanism of other eukaryotic endonucleases, RNase L and Ire1, acting by an analogous mechanism to tRNA-splicing.[21] While the proposed catalytic residues of bacterial CdiA-CT^{Ykris} have been observed in other nucleases, they signify a dramatic shift from the conserved His-Lys-His core of eukaryotic RNase A family members. Further biochemical analyses are required to understand the RNase mechanism of CdiA-CT^{Ykris}; notably characterizing the pKa of key residues could aid in understanding specific residue roles in general acid/base chemistry. Further, little is known about the substrate specificity of CdiA-CT^{Ykris} and whether its unique catalytic core, as compared with eukaryotic RNAse A, affects its RNA substrate specificity.

Before the characterization of this CdiA-CT^{Ykris} toxin,[15] RNase A family members had only been discovered in vertebrates.[1] Placing CdiA-CT^{Ykris} as a member of the RNase A family allows for the integration of a novel and populous bacterial branch into this superfamily. Based on sequence homology to CdiA-CT^{Ykris}, this subfamily includes a multitude of bacterial proteins/domains from both Gram-negative and Gram-positive bacteria, many of which play a role in bacterial virulence or competition and all of which have predicted immunity proteins and associated secretion systems.[15] CdiA proteins from *Serratia proteamaculans, Photorhabdus luminescens, Pseudomonas citronellolis,* and *Bordetella* species contain RNase A-like C-terminal toxin domains. Many of the CdiA-CT^{Ykris} homologs are associated with type VI secretion systems (T6SS); the RNase domain is found in T6SS effectors in *Burkholderia* and *Enterobacteria* species as well as in Rhs (Rearrangement hotspot) proteins – which are exported by T6SS – in *Pseudomonas* and other species.[22,23] The RNase domain is also present in Gram-positive *Bacillus* lipoproteins and at the C-terminus of putative type VII secretion system (T7SS) effectors.[24-26]

Because members of this branch of bacterial RNase A proteins are frequently association with a secretion system, these toxins are presumably unfolded and refolded during the secretion/delivery process. As such, the lack of disulfide bonds in bacterial RNase A proteins offers a practical advantage; the proteins can be readily unfolded and refolded without the need to break or reform disulfide bonds. Regarding catalysis, the predicted CdiA-CT^{Ykris} His-Arg-Tyr-Thr active site residues are well conserved across these homologs: His175 and Thr276 are completely conserved, while a phenylalanine residue is frequently substituted for Tyr278. Thus, it seems likely that there is a common active site amongst these bacterial RNase A family members, though Thr276 is perhaps less likely to play a role in

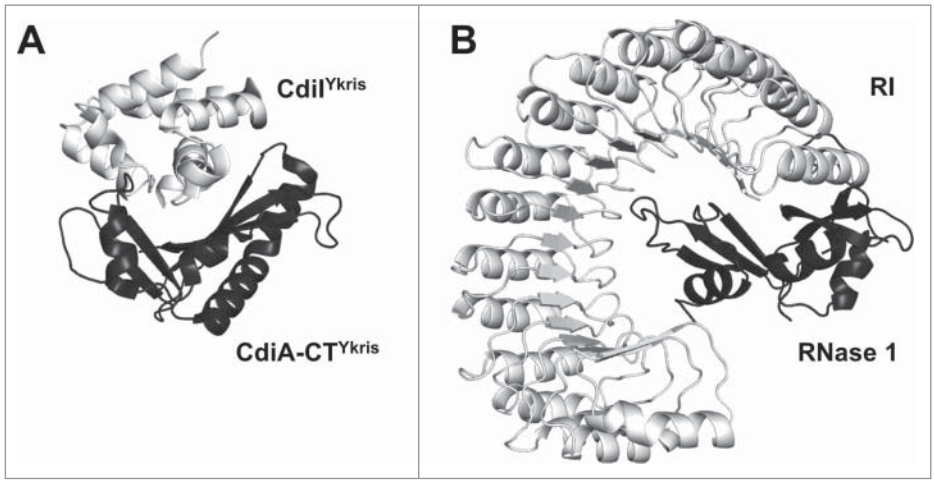

Figure 2. Comparison of the interaction between (A) CdiA-CT^{Ykris} and its immunity protein CdiI (PDB ID 5E3E[15]) with the interaction of (B) mouse pancreatic RNase 1 with mouse RNase inhibitor (RI) (PDB ID 3TSR[18]). CdiI^{Ykris} interacts directly with the active site residues of CdiA-CT^{Ykris}. Alternatively, the RI, composed entirely of leucine-rich repeats, essentially wraps itself around RNase 1, effectively encapsulating the active site and much of the RNase A protein. Differences between the CdiA-CT/CdiI^{Ykris} and RNase 1-RI interactions are reflected in quite different buried surface areas of 12,500 and 23,700 Å^2, respectively.[28]

catalysis than Tyr278. In conclusion, further mechanistic analysis of CdiA-CT^{Ykris} residues involved in ribonuclease activity is required.

Most CdiA-CT^{Ykris} bacterial homologs are encoded near putative immunity proteins. While eukaryotic RNase A family members do not encode a neighboring immunity protein, the RNase A inhibitor (RI) is abundant in the eukaryotic cytoplasm to prevent RNase cytotoxicity.[27] Interestingly, there is no sequence or structural similarity between CdiI^{Ykris} and RI (Fig. 2). While CdiI^{Ykris} is nearly spherical, composed of eight densely packed α-helices,[15] RI resembles an horseshoe, with a structural repeat of alternating α-helices and β-strands that form a curved, right-handed solenoid consisting of 15 leucine-rich repeats.[18] Unlike RI, which surrounds mouse pancreatic RNase A (RNase 1, Fig. 2B), CdiI^{Ykris} interacts directly with CdiA-CT^{Ykris} active site residues (Fig. 2A); hydrogen bonds and electrostatic interactions are formed between CdiI^{Ykris} Gln20 and CdiA-CT^{Ykris} Tyr278, and CdiI^{Ykris} Asp21 and CdiA-CT^{Ykris} His175 and Thr276. Thus, it seems likely that CdiI^{Ykris} protects against CdiA-CT^{Ykris} action by restricting the access of substrates to the toxin's active site. Although CdiI^{Ykris} has no sequence or structural similarity to RI (Fig. 2), preliminary experiments demonstrate that CdiI^{Ykris} co-purifies with and partially inactivates eukaryotic RNase A (unpublished data, personal communication). CdiA-CT^{Ykris} and eukaryotic RNase A are dramatically different proteins with low sequence similarity and key differences in their active site composition and geometry. Thus, the ability of CdiI to inhibit eukaryotic RNase A is remarkable and could have significant implications. As CdiI^{Ykris} interacts specifically with CdiA-CT^{Ykris} active site residues, CdiI^{Ykris} could be engineered to bind specific RNase A proteins with high affinity. This could be advantageous as the CdiI^{Ykris} protein is small (~11 kDa) and can be recombinantly produced and purified from bacteria, while RI, a larger protein (~50 kDa) with numerous cysteine residues, is typically extracted from animal tissue. Engineering CdiI^{Ykris} to have both high affinity and neutralizing power for eukaryotic RNase A proteins, together with its ease in expression and purification from bacterial systems, would significantly reduce the cost of a potential RNase A inhibitor for laboratory usage.

Significantly, Batot *et al.* have discovered a diverse new branch of the RNase A superfamily in bacteria.[15] As the RNase A superfamily has no sequence homologs in lower eukaryotes or invertebrates, and this unique bacterial subset of RNase A toxins has no sequence similarity to the vertebrate RNase A proteins, the bacterial and eukaryotic RNase A branches seem indicative of convergent evolution. Thus, bacteria appear to have independently evolved an analogous ribonuclease that – while structurally somewhat similar to eukaryotic RNase A family members – operates via a novel mechanism. While vertebrate RNase A family members cleave RNA using a His-Lys-His catalytic triad, bacterial RNase A family members appear to utilize a His-Arg-(Tyr/Thr) triad or quartet. Further, the structure of CdiA-CT^{Ykris} was solved in complex with its immunity protein CdiI^{Ykris}, which incidentally can also partially neutralize RNase A activity (unpublished). It is remarkable that CdiI^{Ykris}, a highly specific protein partner for CdiA-CT^{Ykris}, may also neutralize a protein from another species with a similar function to CdiA-CT^{Ykris}, but with no detectable sequence similarity and different active site architecture. The broadly neutralizing ability of CdiI^{Ykris} could be harnessed to generate effective RNase A inhibitors at low cost for laboratory or industrial settings.

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