An RNA surprise in bacterial effector mechanisms

Milan Gerovac[1], Jörg Vogel[1,2,*]

[1] Institute of Molecular Infection Biology (IMIB), University of Würzburg, 97080 Würzburg, Germany

[2] Helmholtz Institute for RNA-based Infection Research (HIRI), 97080 Würzburg, Germany

* Corresponding author: joerg.vogel@uni-wuerzburg.de

Summary

Bacterial pathogens secrete effector proteins to manipulate host signaling proteins and cellular structures. In this issue of Cell Host & Microbe, Pagliuso *et al.* (2019) propose an effector mechanism in *Listeria monocytogenes* whereby an RNA-binding protein associates with bacterial RNA that stimulate RIG-I based innate immunity in the host cytosol.

Text

Pathogenic bacteria possess stunning abilities to manipulate eukaryotic cells to their own benefit. To this end, they often secrete specialized virulence factors, so-called effector proteins, which directly interfere with host cell functions during infection. Activities of effectors ran the gamut of manipulation of the eukaryotic cytoskeleton, evasion of immune reactions, reduction of apoptosis signaling, and redirection of vesicle trafficking to prevent lysosomal degradation. These proteins are attractive to study thanks to often unique molecular mechanisms and structures, but also because their cellular targets indicate host functions a pathogen must usurp for infection to be successful (Jimenez et al., 2016).

Almost every of the dozens of effectors characterized thus far acts on host proteins. By contrast, effectors interacting with nucleic acids have been rare, being represented only by TALE proteins of some plant pathogens, which act as transcription factors in the host nucleus. While TALEs bind to DNA, effectors binding RNA have remained unknown, despite much systematic effort to predict them via conserved RNA-binding domains (Tawk et al., 2017). This paucity of RNA-centric mechanisms amongst bacterial effectors

has been puzzling given that another major class of intracellular pathogens—viruses—deploy RNA-binding proteins (RBPs) to manipulate host gene expression. Now, a study published in this issue of *Cell Host & Microbe* reports the discovery of an RBP that is secreted by the pathogenic bacterium *Listeria monocytogenes* and whose RNA clients stimulate a particular innate immune sensor in the host cell (Pagliuso et al., 2019) (Fig. 1A).

L. monocytogenes is a model species to dissect basic aspects of cell biology, pathogenesis, and innate and acquired immunity (Radoshevich and Cossart, 2018). Upon escape from host vacuoles, *L. monocytogenes* replicates in the host cytoplasm and spreads to neighboring cells avoiding the humoral immune response in the bloodstream. However, this bacterium is also known to release RNA species that activate a type I interferon response through cytosolic antiviral surveillance pathways (Abdullah et al., 2012), an observation that will become important further below.

Seeking to better understand the virulence factor arsenal of this pathogen, Pagliuso *et al.* (2019) focused on the Zea protein of previously unknown function whose gene is present in *L. monocytogenes* but not in related non-pathogenic *Listeria* species. Indeed, mice inoculated with a Zea-deficient strain of *L. monocytogenes* displayed higher bacterial load in liver and spleen, as compared to wild-type infection (Pagliuso et al., 2019), suggesting that Zea helps to prevent hypervirulence. Analysis of its cellular localization showed Zea to be a secreted protein, bolstering the hypothesis that it is involved in host-pathogen interactions. But how?

While Zea lacked a recognizable protein domain of known function, a deposited X-ray crystallography structure of the protein suggested a hexameric configuration that was reminiscent of well-studied bacterial RBPs such as Hfq or Rho (Fig. 1B). To test

potential RNA binding, the authors purified Zea from bacterial supernatant and indeed recovered with it several different *L. monocytogenes* transcripts; their binding to Zea was independently validated *in vitro*. Similarly, overexpression of Zea caused several of these RNA ligands to strongly accumulate in the extracellular space, not only when tested in *L. monocytogenes,* but also when Zea and these RNAs were co-expressed in a *Listeria* species that naturally lacks them. Using appropriate controls, the authors ruled out that the observed increase in extracellular RNA resulted from bacterial lysis. Rather, Zea appeared to selectively stabilize a distinct set of *L. monocytogenes* RNA species upon their release into the environment. But what would be the function of these RNAs?

An intracellular pathogen, the relevant "environment" of *L. monocytogenes* is the host cytoplasm which is guarded by several pathogen detectors including sensors for foreign nucleic acids (Tan et al., 2018). Thus, extracellular RNAs stabilized by Zea will not go unnoticed in the host cytosol. Indeed, testing different cytosolic RNA sensors, Pagliuso *et al.* (2019) established that overexpression of Zea (increasing the abundance of extracellular RNAs) or direct injection of selected Zea-associated RNA species stimulated RIG-I (retinoic acid inducible gene I), which is foremost known to sense 5' triphosphate viral RNA (Tan et al., 2018). Moreover, sequencing of purified RNA ligands of RIG-I in *Listeria*-infected cells identified considerable overlap with Zea-associated RNAs in the extracellular space. Lastly, Zea and RIG-I not only co-purified when pulled out individually from the cytosol of *Listeria*-infected cell, they also showed co-localization when visualized by immunofluorescence. Together, these observations suggest that Zea is a novel type of bacterial effector that delivers extracellular bacterial RNAs of *L. monocytogenes* to the crucial RIG-I sensor to stimulate innate immune sensing.

These findings are exciting for several reasons. Firstly, although *L. monocytogenes* must generally avoid immune recognition in the host, local stimulation of innate immunity provides a growth-permissive microenvironment for it (Witte et al., 2012). Others have also shown that secreted *Listeria* RNA species trigger RIG-I (Abdullah et al., 2012), with small RNA rli32 constituting a super-activator of RIG-I and the associated type I interferon response (Frantz et al., 2019). The RNA ligands of Zea described by here are different; most of them stem from an uncharacterized prophage with no linkage to the *zea* gene or those previous immunostimulatory transcripts (Pagliuso et al., 2019). While the relative contribution of Zea and its RNA cargo to RIG-I induction remains to be determined, *L. monocytogenes* clearly lends itself as a model to understand how bacteria target RNA sensing pathways to induce local inflammation. Importantly, such mechanisms also bear therapeutic potential for other diseases such as cancer, for strong RIG-I induction may help to turn an immunosuppressive tumor microenvironment into an immunosupportive tissue.

Secondly, Zea finally provides a lead to explore the molecular underpinnings of RNA secretion in bacteria. There has been much talk of "social RNA" in host-pathogen interactions, which includes the concept of bacteria delivering RNA species to manipulate gene expression in the host (Braukmann et al., 2017). As it stands, we have zero understanding of how such RNA secretion may work. Whereas type IV secretion translocates DNA, bacterial RNA transporters—export or import—are unknown. The previous work on *Listeria* secreted RNA implicated the SecA2 system in the process (Abdullah et al., 2012), but whether SecA2 could export an RNA-protein complex without unfolding it, remains unclear. Therefore, future work should determine which pathway Zea gets secreted by, and whether those *Listeria* RNA species that stimulate

RIG-I are already bound by Zea in the bacterial cytoplasm for co-export, or only meet Zea in the extracellular space (Fig. 1A).

Of note, Pagliuso et al. (2019) predict Zea-like proteins in numerous other Gram-positive bacteria, mostly non-pathogenic ones. If these were secreted RBPs as well, there might be a whole new biology of extracellular RNA functions. That would include the human digestive tract because possible Zea homologs do not only occur in the *Bacillus* genus, but are also present in different *Clostridia* and *Actinobacteria* species, thus common members of our gut microbiota.

Thirdly, the discovery of Zea reminds us how little we know about the scope and abundance of bacterial RBPs. There are ~180 annotated RBPs in *E. coli*, but RBP discovery has much lagged behind in the many more other bacteria that matter to human health and disease, also for a paucity of suitable capture methods (Holmqvist and Vogel, 2018). Not only is Zea the first RBP amongst effector proteins, it also happens to be the first secreted bacterial RBP to be described (disregarding the *Yersinia* YopD protein, which binds mRNAs in the bacterial cytosol before getting secreted to become a structural component of a translocation pore across the eukaryotic plasma membrane (Holmqvist and Vogel, 2018)). Comparison to other bacterial hexameric RBPs shows that Zea is roughly between the size of termination factor Rho and the small-RNA chaperone Hfq (Fig. 1B). However, structural similarities with Hfq (which is present in *Listeria* but has fewer functions than in Gram-negative species) are limited to a hexameric torus with a positively charged face built of beta sheets. While we do not know the *in vivo* structure of Zea, it is tempting to speculate that it changes from intra- to extracellular localization because Zea seems to show less RNA association in the *Listeria* cytosol (Pagliuso et al., 2019).

In conclusion, the concept that bacteria actively secrete RNA for use as information or effector molecules has been entertained for a while. Proving it has been much harder for lack of associated molecular machinery, e.g., secretion systems similar to those that export bacterial toxins and other types of effectors. Some of the proposed models, e.g., that bacteria secrete microRNA-like molecules to target host mRNAs, have been difficult to reconcile with the expected number of RNA molecules needed to reach an effective concentration in the host cytosol (Braukmann et al., 2017). By contrast, protein-assisted delivery of RNA molecules to a host protein as proposed by Pagliuso *et al.* (2019) may be effective at much lower concentrations. Altogether, the RIG-I interacting RBP Zea and its prophage-derived RNA ligands combined with emerging molecular details of other RIG-I stimulating bacterial RNAs (Frantz et al., 2019) offer an outstanding opportunity to unravel what might even turn out as a conserved bacterial pathway for functional extracellular RNA.

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Figure 1. The secreted RNA-binding protein Zea aids the activation of RIG-I and type I interferon response by extracellular RNA species of *L. monocytogenes*. (A) It is currently unclear whether Zea and its RNA ligands only meet after release from the bacteria or already associate and get co-exported as an RNA-protein complex. (B) For comparison of size, the secreted Zea protein is shown together with two other hexameric bacterial RBPs, Hfq and Rho, that function in the bacterial cytosol (PDB: 5JJI, 4K15, 1KQ2, RNA, ions, and nucleotides are represented in green).