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Interspecies conflict affects RNA expression.

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One-sentence summary

Regulated changes in the RNA complement of bacterial predators and prey during predation suggest the likely involvement of RNA encoding regulators, regulatory RNAs, and OMV-mediated trafficking of RNAs during predation.

Abstract

Predation is an extreme form of competition between bacteria, involving the secretion of antimicrobial substances by predators, often packaged within outer membrane vesicles (OMVs). Recent studies into the *Myxococcus xanthus*/*Escherichia coli* predator/prey relationship have illuminated transcriptional changes during predation, identifying likely targets of predatory attack in the prey and nutrient assimilation strategies of the predator. Abundant non-coding RNAs can be observed in the predator and prey transcriptomes, with evidence of predation-dependent regulation of RNA levels. Given the observed secretion of regulatory RNAs within OMVs by bacteria, it will next be exciting to test whether the intercellular trafficking of regulatory RNAs is employed by predator and/or prey in their survival struggles.

Introduction

Interactions between bacterial cells are widespread, if not ubiquitous, both within and between species. The biological outcomes of such interactions can range from cooperation to competition and at times outright warfare (Nardell et al., 2016; Tarnita, 2017). Some organisms even cooperate together to wage war on others. An example of such warrior bacteria are the myxobacteria, who when starved will congregate to build 'towering' castle-like survival structures (fruiting bodies) to shelter within. Yet when other microbes pass through their territory, will communally sally forth, attack, kill and consume them (Muñoz-Dorado *et al.*, 2016).

Many microbes across the tree of life have adopted a predatory lifestyle and usually employ specialised mechanisms to kill their prey. The myxobacteria hunt using a 'wolf-pack' strategy, so called because the members of a hunting group of myxobacteria cooperatively secrete toxic metabolites and degradative enzymes into the shared extracellular environment (Pérez *et al.*, 2016). A feature of myxobacterial predation is its broad (albeit patchy) prey range, with myxobacteria generally able to prey on both Gram-negative and positive bacteria, as well as fungi (Morgan *et al.*, 2010; Livingstone *et al.*, 2017). There is therefore considerable potential to exploit myxobacterial predation in the development of novel bioactives, but little is yet known about the molecular mechanisms at play during predation. However, it is known that myxobacteria secrete soluble proteins and outer membrane vesicles (OMVs) that are intrinsically antimicrobial (Evans *et al.*, 2012; Whitworth *et al.*, 2015). They also produce diverse secondary metabolites including antimicrobials (Xiao et al., 2011) and several of their secreted enzymes have bacteriolytic activity (Sudo and Dworkin, 1972).

Signalling during predation

Social behaviours such as those exhibited by the myxobacteria require complex signalling, both within and between cells. The myxobacteria are renowned for the large numbers of two-component system (TCS) signalling proteins they possess (Whitworth and Cock, 2008; Whitworth, 2012) and mutations of several TCS genes of the model myxobacterium *Myxococcus xanthus* are known to affect predation (Pham *et al.*, 2005). Only 12 of the 2,758 genomes in the P2CS genomic database of TCS genes (Ortet *et al.*, 2015) are from myxobacteria (0.4 %), yet they provide five of the ten replicons with the largest number of TCS genes. The myxobacterium *Stigmatella aurantiaca* DW4/3-1 has the largest TCS gene complement of any bacterium in P2CS (335 TCS genes), including 41 DNA-binding response regulators, which have likely roles in regulating transcription initiation.

Widespread reports of behavioural changes linked to predation suggest extensive regulation of predatory activity, which is consistent with the costliness of secreting predatory enzymes and metabolites. Examples include programmed responses to prey cells (Berleman *et al.*, 2006; Pérez *et al.*, 2014; Müller *et al.*, 2016), sensation of molecules released by prey (Lloyd and Whitworth, 2017), and reactivity towards environmental phenomena potentially indicative of prey presence (Fontes and Kaiser, 1999).

A few years ago, a small non-coding RNA (ncRNA) was identified that acts a regulator of fruiting body formation in *M. xanthus* (Yu *et al.*, 2010). The Pxr ncRNA is a relatively recent evolutionary innovation restricted to a sub-clade within the myxobacteria (Chen *et al.*, 2017). It acts as an inhibitor of development when nutrients are available (presumably including during predation), and Pxr function appears to be linked to the activity of an adjacently-encoded TCS (MXAN_1077, MXAN_1078), through regulation of Pxr synthesis (Yu *et al*., 2016). Though not yet demonstrated, it

is probable that Pxr will prove to play a role in predation, as other genes that respond to nutrient levels and act as developmental gatekeepers give predatory phenotypes on deletion (Pham *et al*., 2005). In addition, the discovery of Pxr raises the possibility of regulation by other as yet unidentified ncRNAs during myxobacterial predation.

Transcriptome changes during predation

We recently carried out RNA-seq to investigate transcriptional changes of a myxobacterial predator simultaneously with its prey, during active predation (Livingstone *et al.*, 2018). After culturing separately, cells of *M. xanthus* and *Escherichia coli* were mixed together and allowed to adapt to each other's presence before being harvested for RNA extraction and sequencing. In addition to feeding live *E. coli* to *M. xanthus*, the predator was also provided with pre-killed *E. coli*, in an attempt to disentangle RNA expression changes during initial prey killing, from gene expression changes associated with the subsequent assimilation of prey biomass.

Surprisingly, there was virtually no transcriptional response in *M. xanthus* to the presence of the prey, however, hundreds of genes were differentially expressed by the prey in response to the predator's attack (Livingstone *et al.*, 2018). The low number of genes induced during prey killing was particularly surprising given the aforementioned evidence of regulation during predation, and that the non-myxobacterial Proteobacterial predators Micavibrio aeruginosavorus and *Bdellovibrio bacteriovorus* exhibit widespread transcriptional changes during predation (Wang *et al*., 2011; Karunker *et al*., 2013). Even in non-predatory systems, co-culturing bacteria has been shown to affect the expression of substantial numbers of genes in each organism (Tikhomirova *et al.*, 2015; Aharonovich and Sher, 2016)

Of the few genes that did exhibit up/down-regulation, the *M. xanthus* genes most induced by *E. coli* presence belonged to the Kdp regulon, which governs cellular responses to osmotic stress (Ballal *et al*., 2007). A TCS lies at the core of the Kdp regulon, and expression of the Kdp regulon in *M. xanthus* was confirmed to respond to osmotic stress (Livingstone *et al.*, 2018). It isn't clear whether the induction of Kdp during predation is merely an innocent response to the osmotic consequences of prey cell presence, or whether osmotic changes might be sensed as an indicator of prey presence and used as cues for the regulation of downstream predatory behaviour.

Providing *M. xanthus* with pre-killed instead of live *E. coli* caused differential regulation of a set of hundreds of genes, which was enriched in genes involved in sugar and nucleotide metabolism. The absence of amino acid and lipid metabolism gene expression changes suggests that the amino acids and lipids derived from prey may be generally shunted into predator protein and membranes, while the sugars obtained tend more to be catabolised to provide energy (Livingstone et al., 2018).

Transcriptional changes in hundreds of *E. coli* genes were observed, whether the prey was exposed to *M. xanthus* cells, OMVs, or purified culture supernatant. The up-regulated gene set of *E. coli* was enriched in genes involved with ribosome function and lipopolysaccharide (LPS) biosynthesis, suggesting that myxobacterial predatory attack targets the LPS and protein synthesis (Livingstone et al., 2018).

Are regulatory ncRNAs involved in predation?

The lifecycle of the Deltaproteobacterium *B. bacteriovorus* includes an 'attack' phase (AP) in which the predator hunts prey, and a 'growth' phase (GP) in which it consumes the prey. RNA-seq experiments comparing AP and GP-expressed RNAs found a ncRNA (merRNA) which was

abundantly expressed in AP (Karunker *et al.*, 2013). It is thought that merRNA regulates the AP to GP transition through regulating the availability of cyclic-di-GMP va its internal riboswitch.

The OMVs secreted by *Vibrio cholerae* and *E. coli* contain a cargo rich in RNAs, particularly those originating from intergenic transcripts and processed structural RNAs (Sjöström *et al.*, 2015; Ghosal *et al.*, 2015; Blenkiron *et al.*, 2016;). Therefore there exists the exciting possibility that during predation myxobacterial OMVs might traffic signalling RNAs into the prey cell, interfering with the prey's ability to resist attack. OMVs could also potentially allow the transport of RNA/proteins between predator cells (Whitworth, 2011) to synchronise a population-wide attack.

In addition to information about the relative expression of protein coding genes, the transcriptomic sequence dataset of Livingstone *et al.* (2018) also provided data regarding the relative abundance of non-coding RNAs. The toRNAdo algorithm (Hermansen *et al.*, 2018) reveals more than 170 non-overlapping transcripts in the *M. xanthus* datasets. Pxr was present in all conditions tested and as expected was induced by provision of either rich medium or *E. coli* (whether alive or pre-killed) compared to starvation medium. Observing altered expression levels for a ncRNA during predation does not necessarily mean that the RNA is involved in directly regulating predation, but as predation is such a complex pleiotropic behaviour, it certainly implies involvement in predation-related phenomena. Given the large number of ncRNAs apparent in the *M. xanthus* datasets, it would not be at all surprising if it transpired that multiple ncRNAs are involved in regulating physiological changes associated with predatory activity.

When studying predation it is important not to focus exclusively on the predator, as predatory mechanisms are at least partly prey specific. The defence and/or counter-attack mechanisms of prey bacteria may well require ncRNA regulation, and abundant RNAs have been detected in the secretions of prey organisms, both within and alongside OMVs. Pathogenic bacteria can alter host cell physiology through the OMV-mediated delivery of RNA into eukaryotic cells (Dauros-Singorenko *et al.*, 2017). It is therefore plausible that prey cells might affect predator activity through the transport of interfering ncRNAs into predatory cells.

There remain huge gaps in our understanding of RNA signalling and secretion during predation. Particularly in establishing whether OMVs can traffic functional RNAs between predator and prey, which will be dependent not only on the nature of the secreted RNAs, but also on the targeting specificity and efficiency of OMV-mediated cargo delivery.

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