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The RNA cargo of *Myxococcus* outer membrane vesicles†

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The outer membrane vesicles (OMVs) secreted by some Gram-negative bacteria contain RNA cargo, which can be introduced into target cells, affecting their cellular processes. To test whether the antimicrobial OMVs secreted by predatory myxobacteria might contain cargo RNA with a role in prey killing, we purified OMVs and cells from four different strains of *Myxococcus* spp. for RNA-seq transcriptome sequencing. Myxobacterial OMVs contained distinct sets of RNA molecules. The abundance of major cellular transcripts correlated strongly with their abundance in OMVs, suggesting non-specific packaging into OMVs. However, many major cellular transcripts were absent entirely from OMVs and some transcripts were found exclusively in OMVs, suggesting OMV RNA cargo loading is not simply a consequence of sampling the cellular transcriptome. Despite considerable variation in OMV RNA cargo between biological replicates, a small number of transcripts were found consistently in replicate OMV preparations. These 'core' OMV transcripts were often found in the OMVs from multiple strains, and sometimes enriched relative to their abundance in cellular transcriptomes. In addition to providing the first transcriptomes for myxobacterial OMVs, and the first cellular transcriptomes for three strains of *Myxococcus* spp., we highlight five transcripts for further study. These transcripts are 'core' for at least two of the three strains of *M. xanthus* studied, and encode two alkyl hydroperoxidase proteins (AhpC and AhpD), two ribosome-associated inhibitors (RaiA-like) and a DO-family protease. It will be interesting to test whether the transcripts serve a biological function within OMVs, potentially being transported into prey cells for translation into toxic proteins.

1. Introduction

Bacteria seem to universally secrete vesicles into their extracellular environment. Vesicles produced by Gram-negative bacteria are formed by pinching off outer membrane blebs, and such vesicles are therefore described as outer membrane vesicles (OMVs). OMVs have been shown to be involved in a wide variety of bacterial processes, including immunomodulation, quorum signalling, nutrient digestion, and toxin delivery.[1] OMVs contain cargo molecules derived from the outer membrane and periplasm of producing cells. Cargo loading can be specific, with (*e.g.*) some proteins being selectively incorporated into OMVs, although the mechanisms responsible for cargo selection remain unclear.[2,3] In addition to lipids, proteins, and metabolites, OMVs can also contain nucleic acids.

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RNA has been shown to be associated with the OMVs of several species of bacteria.[4] Comparative RNA-seq profiling of OMVs and their producing cells has demonstrated selective packaging of RNA into OMVs. For example, *Escherichia coli* OMVs seem to particularly contain short RNAs derived from the cleavage of non-coding RNAs (ncRNAs), although they also contain full-length mRNAs.[5–7] The specific RNA cargo of *E. coli* OMVs can also change depending on growth conditions.[7] Similar findings have been reported for *Salmonella enterica* serovar Typhimurium.[8]

The RNA cargo of OMVs produced by several pathogenic bacteria can enter host cells and affect host cell processes.[9,10] For example, Blenkiron *et al.* showed that the RNA cargo of OMVs from uropathogenic *E. coli* could enter the cytoplasm and nucleus of cultured epithelial cells.[6] Similarly, the OMVs of *Pseudomonas aeruginosa* have been found to convey RNA into human airway cells, while *Helicobacter pylori* OMVs can transfer ncRNAs into human gastric cells. In both these latter two cases, short RNAs transported in this way have also been shown to specifically reduce the strength of the host immune response.[11,12]

The myxobacteria are a phylum (Myxococcota) of non-pathogenic predatory Gram-negative bacteria. They possess

contact-dependent mechanisms for killing prey microbes, and also secrete a cocktail of antimicrobial enzymes, peptides and secondary metabolites.[13–15] The OMVs produced by myxobacteria and other predators are themselves antimicrobial, being able to kill a wide range of prey organisms, and addition of myxobacterial OMVs to prey cells leads to wholesale changes in the prey transcriptome.[16–19]

It is possible that myxobacterial OMVs might transfer functional RNAs into prey cells during predation, compromising their ability to defend themselves against predator attack, in an analogous fashion to pathogens using ncRNAs in OMVs to attenuate the host immune response during infection.[20] We therefore sought to characterise the RNA cargo of myxobacterial OMVs by RNA-seq, to determine; whether RNA transcripts are present within OMVs, whether there is any selectivity in RNA packaging, and whether selectively packaged OMV RNAs are conserved within a myxobacterial genus (*Myxococcus*).

2. Experimental

2.1. Myxobacterial strains and cultivation

Myxobacteria strains (Table 1) were maintained on DCY (20 g L^{-1} casitone, 2 g L^{-1} yeast extract, 10 mM Tris pH 8.0, 8 mM MgSO$_4$) agar plates (solidified with 15 g L^{-1} agar), re-plating onto fresh plates every three or four weeks. For long term storage, agar blocks were cut from plates and stored at -80 °C. AB022 and CA010 are wild-type strains isolated from soils in Wales, with very different predatory activities against a panel of prey organisms, DK1622 is a widely used laboratory strain of *Myxococcus xanthus*, and DSM14675^T is the type strain of *Myxococcus stipitatus*.[22,23]

2.2. Preparation of OMV and cell samples

Myxobacteria were grown in duplicate 100 mL cultures in shaken flasks (180 rpm) of DCY at 30 °C, until they reached mid-late exponential phase (optical density at 600 nm of $\sim$1). Cells were removed from cultures by centrifugation as described previously by Zwarycz *et al.*, washed with 100 mL TM buffer (10 mM Tris pH 8.0, 8 mM MgSO$_4$), before resuspending in 2 mL of TM. Duplicates were pooled, and the resulting cell samples frozen at -80 °C.[18,24] Cell-free supernatants were concentrated to 250 µL by centrifugation through ultrafiltration tubes (Amicon) at 1915 $\times$ g in a Rotina 46 R centrifuge and then size exclusion chromatography used to isolate OMVs in 0.5 mL fractions using qEV columns (Izon Science) as directed by the manufacturer and described in detail by Zwarycz *et al.*[18] OMV preparations were immediately aliquoted and frozen at 80 °C for storage. OMV samples were confirmed to contain pure OMVs and to be cell-free, by

incubating on DCY plates (which generated no colonies), and by microscopy (light microscopy and transmission electron microscopy).

2.3. Transcriptome sequencing

For each strain, OMV preparations from duplicate cultures were sequenced, along with a pooled sample of cells from the same two cultures. Transcriptome sequencing was performed by the NU-OMICS sequencing facility at Northumbria University, with a target read count of 20 million for cell samples and 5 million for OMV samples. Cell samples were also subjected to ribodepletion. RNA was extracted from samples using the MagMax Pathogen DNA/RNA kit (Thermo) using 300 µl as the sample input volume. Extracts were treated with Turbo DNase (Thermo). Libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep kit for Illumina (New England Biolabs) following the FFPE/degraded RNA protocols. Ribodepletion was performed using the NEBNext rRNA Depletion kit (New England Biolabs). Libraries were quantified prior to sequencing, and had a mean yield of 183 ng µl^{-1}. Libraries were sequenced using a NextSeq 550 2 $\times$ 75 bp kit (Illumina). All kits were used according to their manufacturer's instructions.

2.4. Transcriptome mapping

Reads were cleaned using Trimmomatic: adaptor sequences were removed, as were leading/trailing bases with quality thresholds below 20, and reads less than 36 nt long. RSEM software was then used to map reads to the specified genome sequence (Table 1), using bowtie2 and the 'rsem-calculate-expression' command, with '–estimate rpsd' and '–append-names' options. Output from RSEM gave relative expression levels of genes as FPKM values (fragments per kilobase of transcript per million mapped reads) for each annotated gene. Genes encoding highly expressed ncRNAs (rRNA genes, *rnpB* (MXAN_5732) and *ssrA* (MXAN_2093)) were removed from the dataset and FPKM values normalised for each sample to allow comparison between samples. Sequence data has been added to the Short Read Archive under accession PRJNA1028607.

3. Results

3.1. Matched OMV and cell transcriptome datasets for four myxobacteria.

Cell-free OMV samples and OMV-free cell samples were prepared from cultures of four myxobacterial strains (Table 1 and Fig. 1). Three strains belonged to the same species (*M. xanthus*) and included the model myxobacterium *M. xanthus* DK1622. The other two *M. xanthus* strains were selected for their

Table 1 Myxobacteria strains used. DSMZ refers to the German collection of microorganisms and cell cultures

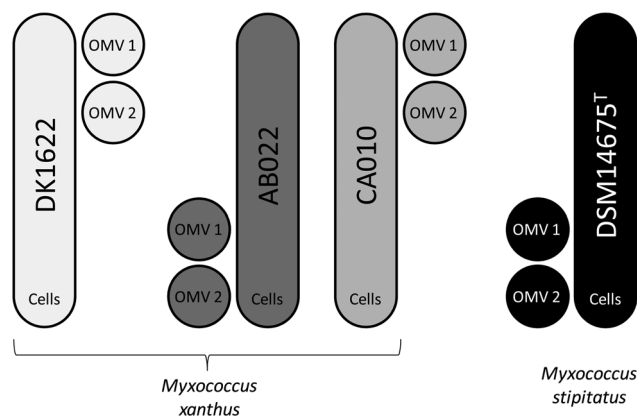| Strain	Species	Source/ref.	Genbank accession
DK1622	*Myxococcus xanthus*	Goldman *et al.*, 2006.[21]	GCA_000012685
AB022	*Myxococcus xanthus*	Zwarycz *et al.*, 2020.[24]	VHLD00000000
CA010	*Myxococcus xanthus*	Zwarycz *et al.*, 2020.[24]	VHLA00000000
DSM14675^T	*Myxococcus stipitatus*	DSMZ	GCA_002305895

Fig. 1 Myxobacterial samples for transcriptome sequencing. Strains DK1622 (reference strain), AB022 and CA010 belong to *M. xanthus*, while strain DSM14675^T is the type strain of *M. stipitatus*. For each of the four strains, duplicate cultures were grown. From each replicate, cell-free OMVs were prepared ('OMV 1' and 'OMV 2'). OMV-free cells were also prepared from each culture and replicates pooled ('Cells').

differing predatory activities against prey organisms.[21] To provide data from a second species, the fourth strain used was the type strain of *Myxococcus stipitatus* DSM14675^T. Duplicate cultures of each strain were used to prepare samples of OMVs and cells. Cells were sedimented and washed by centrifugation, and OMVs were purified from the cell-free culture supernatants using size exclusion chromatography. RNA was extracted from the resulting samples, libraries constructed and sequenced using an Illumina NextSeq 550. The resulting 40 Gb of sequence data have been added to the Short Read Archive under accession PRJNA1028607.

3.2. The DK1622 OMV transcriptome

Sequence reads from DK1622 samples were mapped to the DK1622 genome, giving FPKM values (fragments per kilobase of transcript per million mapped reads) for each protein-coding gene (CDS), which were also normalised to reflect the percentage abundance of each gene's transcript in each transcriptome (File S1, ESI†). The number of transcripts identified in each expression profile varied considerably (Table 2), with the cellular transcriptome including transcripts from 5208 of the 7396 DK1622 CDSs (70.4%), compared to just 170 and 237 CDS transcripts (2.3% and 3.2% respectively) for the OMV transcriptomes.

Each transcriptome was also characterised by calculating its 'N50' and 'N95' metrics (Table 2). The N50 metric being defined as the smallest number of the most highly expressed genes which together account for 50% of the transcripts in the transcriptome. For example, the DK1622 cell transcriptome had an N50 value of 143, indicating that the 143 genes with the largest FPKM values together made up 50% of the transcriptome. Similarly, the N95 metric is the smallest number of genes which together comprise 95% of the transcripts in the transcriptome, when ranked by decreasing abundance. The N50 and N95 values show that each transcriptome is dominated by a small number of particularly abundant transcripts, and the majority of transcripts are very low abundance.

DK1622 transcriptomes were compared by calculating the correlation coefficients (r) for pairs of expression profiles (comparing FPKM values of each gene in the DK1622 genome). There was a negligible correlation between the cellular transcriptome and each of the two OMV samples ($r = 0.277$ and 0.261) indicating that the transcripts found in OMVs are not simply a consequence of sub-sampling the cytoplasmic transcriptome.

In contrast, a weak positive correlation was found between the two OMV replicates ($r = 0.321$). In a pilot study, two OMV size-exclusion chromatography fractions from a single sample of DK1622 OMVs were sequenced as technical replicates. The resulting expression profiles correlated very strongly ($r = 0.947$), indicating that the weakness of the correlation observed between different OMV preparations is a result of considerable biological variation between replicate cultures. Nevertheless, correlation between the DK1622 OMV transcriptomes also suggests that there is at least some commonality in their cargo transcripts.

3.3. A core DK1622 OMV transcriptome?

To identify commonality between the RNA cargoes of DK1622 OMVs, the most abundant 50 transcripts of the replicate OMV transcriptomes were compared (File S2, ESI†). In both cases, the 50th most abundant transcript contributed to less than 0.5% of the sample's transcripts. Thirteen of the 50 transcripts were common to both samples (Table 3), constituting 18.4% and 24.8% of the two OMV transcriptomes. Expression of the 13 shared transcripts correlated strongly ($r = 0.799$), suggesting that the 13 transcripts in Table 3 represent 'core' components of the DK1622 OMV transcriptome. In contrast, some transcripts were abundant in one replicate but not the second, for example the MXAN_2134 transcript represented 10.2% of one OMV transcriptome, but was absent from the second.

Nine of the protein products of the 13 core OMV transcripts could be assigned to COG categories (clusters of orthologous proteins), with three belonging to category S (Function unknown), five to category J (Translation), and two mapping to category O (post-translational modification, protein turnover, chaperone functions). Functional annotation of the encoded proteins identified three as ribosomal proteins and four as hypothetical proteins of unknown function (Table 3). The remainder included two RaiA family proteins (ribosome-associated stress responsive inhibitors of translation), general stress protein GsiB, a DO family protease, and the AhpC and AhpD alkyl hydroperoxide reductases, which are encoded by tandem genes (Table 3).

If transcripts are packaged into OMVs passively, their relative abundance in OMVs would be expected to correlate with their abundance in the cellular transcriptome, giving a fold-enrichment (the % abundance in OMVs divided by % abundance in cells) of around 1. Five core OMV transcripts (*ahpC*, *ahpD*, *gsiB*, MXAN_4815 and MXAN_6282) had enrichment values between 0.1 and 10.0 (Table 3), and each represented more than 0.1% of the cellular transcriptome, suggesting that such transcripts are potentially packaged passively. Indeed, for

Table 2 Characteristics of transcriptome datasets when mapped against the DK1622 genome. The N50 and N95 metrics denote the number of protein-coding genes that cumulatively constitute 50% or 95% (respectively) of the total FPKMs of the dataset when ranked by decreasing FPKM value. Mx denotes *Myxococcus xanthus*, Ms represents *Myxococcus stipitatus*, ΣFPKM denotes the sum of all FPKM values, Mbytes and Gbases indicate the size of the sequence dataset and #Transcripts is the number of CDSs against which transcripts could be mapped

Sample	Mbytes	Gbases	ΣFPKM	N50	N95	# Transcripts	Accession
Mx DK1622 OMV#1	40	0.11	1170	25	130	170	SRX22103706
Mx DK1622 OMV#2	66	0.16	4600	33	180	237	SRX22103707
Mx DK1622 Cells	1004	2.9	6025	143	2696	5208	SRX22103700
Mx AB022 OMV 1	268	0.77	13509	171	2454	4709	SRX22103702
Mx AB022 OMV 2	296	0.86	2821	154	1648	2537	SRX22103703
Mx AB022 Cells	856	2.5	35529	183	2503	5887	SRX22103696
Mx CA010 OMV 1	345	1.0	216	46	305	415	SRX22103698
Mx CA010 OMV 2	315	0.91	25	17	74	92	SRX22103699
Mx CA010 Cells	873	2.5	3606	84	1729	3583	SRX22103701
Ms DSM14675^T OMV 1	582	1.7	0.19	1	1	1	SRX22103704
Ms DSM14675^T OMV 2	459	1.3	0.79	1	1	1	SRX22103705
Ms DSM14675^T Cells	1004	2.8	67.11	1	43	108	SRX22103697

Table 3 'Core' transcripts of DK1622 OMVs. Each transcript listed was found among the most highly expressed transcripts in replicate preparations of DK1622 OMVs. Values indicate the normalised expression of each transcript (percentage), including for the cellular transcriptome. Enrichment denotes the mean fold-increase in relative abundance in OMVs relative to cellular transcriptomes, while #OMVs denotes for how many of the AB022, CA010 and/or DK1622 OMV transcriptomes the transcripts were among the most abundant 50 transcripts (maximum 6)

Identifier	Role	OMV 1	OMV 2	Cell	Enrichment	Length (nt)	COG	#OMVs
MXAN_0457; raiA	Ribosome-associated inhibitor	3.97357	2.49484	0.01659	195	336	J	4
MXAN_0934	DO family protease	1.45526	0.61778	0.08380	12.4	1503	O	5
MXAN_1563; ahpD	Alkyl hydroperoxidase	0.65884	1.1681	1.10144	0.829	525	S	5
MXAN_1564; ahpC	Alkyl hydroperoxide reductase	1.21941	1.07645	1.67680	0.685	555	O	6
MXAN_3076; rplL	50S ribosomal protein L7/L12	2.25511	1.0438	0.06505	25.3	1350	J	3
MXAN_3323; rpsM	30S ribosomal protein S13	1.05620	0.97124	0.06488	15.6	384	J	2
MXAN_3850; gsiB	General stress protein	0.79215	0.71126	0.59627	1.26	462	—	3
MXAN_4355	Hypothetical protein	0.61868	0.54692	0.00248	235	549	S	2
MXAN_4815	RaiA family protein	3.43009	2.11921	7.56904	0.367	366	J	6
MXAN_4859	Hypothetical protein	1.48432	1.32644	0.05907	23.7	483	S	2
MXAN_5201; rpsU	30S ribosomal protein S21	4.42989	5.01230	0	Infinity	195	J	3
MXAN_5845	Hypothetical protein	2.72254	0.60474	0	Infinity	213	—	2
MXAN_6282	Hypothetical protein	0.74857	0.66974	0.11052	6.42	480	—	2

the 165 cellular transcripts which each represent at least 0.1% of the cellular transcriptome (together making 52% of the cellular transcriptome), there was a strong correlation between their abundance in cells and in OMVs (r = 0.692), indicating that many transcripts are likely to be packaged passively. Nevertheless, many highly abundant cellular transcripts are not found in OMVs (*e.g.* 54 of the 165 most abundant cellular transcripts), suggesting that some/many transcripts may be excluded from being passively loaded into OMVs.

In contrast, eight of the OMV core transcripts had enrichment values greater than 10 (Table 3), suggesting they may be specifically targeted for inclusion in OMVs. In two cases (*rpsU* and MXAN_5845) the enrichment value could not be calculated (infinite), as those transcripts were not detected at all in the cellular transcriptome. The transcripts encoding RaiA and MXAN_4355 also had particularly high enrichment values (195 and 235, respectively). Across the entire OMV transcriptome, neither the enrichment nor relative abundance of OMV transcripts correlated with their length (r = −0.176 and −0.033 respectively), although there was a weak negative correlation between abundance and length when considering just the 13 core OMV transcripts (r = −0.335). It seems that length of a

transcript is not a good predictor of whether a transcript is included/excluded from OMVs, and that enrichment in OMVs is not due to the more efficient packaging of smaller transcripts.

3.4. Conservation of the core OMV transcriptome

To see whether the same transcripts were packaged into OMVs in AB022, CA010 and DSM14675^T as they were for DK1622, sequence data for AB022, CA010 and DSM14675^T samples were mapped against the DK1622 genome. Mapping seemed to work very well for AB022, less well for CA010, and poorly for DSM14675^T, as indicated by the sum of FPKM values for each dataset (Table 2). For the DSM14675^T cell sample, only 108 transcripts were mapped to DK1622 CDSs, and for both OMV samples only 1 transcript was mapped successfully, precluding more detailed analysis. Mapping effectiveness was not a consequence of sequencing depth, as the amount of sequence data in each dataset did not correlate (r = 0.277) with the number of transcripts mapped (sum of FPKM values). The poor mapping of DSM14675^T sequence data to DK1622 is likely because the strains belong to different species. However, taxonomic distance cannot explain why AB022 samples mapped to DK1622 an

order of magnitude better than CA010 samples, as both are equally related to DK1622, with an average nucleotide identity (ANI) of 97% with DK1622, and an ANI of 100% between CA010 and AB022.

As shown in Table 4, the normalised FPKM values of the CA010 cellular transcriptome correlated moderately with those of AB022 and DK1622 ($r = 0.560$ and 0.510, respectively), which also correlated with one another, albeit not as strongly ($r = 0.418$). Weaker correlations were observed between OMV transcriptomes of different strains (r values of 0.202, 0.217 and 0.227), suggesting there is greater variability between OMV transcriptomes than for cellular transcriptomes. Comparing cellular with OMV transcriptomes typically gave weak correlations, except notably when comparing the CA010 cellular and CA010 OMV transcriptomes, which gave a moderate strength correlation ($r = 0.639$).

For AB022 and CA010, the differing effectiveness of mapping between samples (manifested as the sum of FPKM values) meant that samples had very different numbers of transcripts, N50 and N95 values (Table 2), although more than 90 transcripts were detected in each OMV sample. We therefore focused on the top 50 most abundant transcripts in each OMV transcriptome, as we had done for DK1622 OMVs, regardless of what proportion of the transcriptome those 50 transcripts represented.

For AB022 OMVs there were 26 transcripts in common among the top 50 most abundant transcripts of both replicates, and 14 such transcripts for CA010 (File S2, ESI†), compared to 13 for DK1622. Considering the six OMV transcriptomes of AB022, CA010 and DK1622 together, only two transcripts were found in the top 50 transcripts of all six samples (MXAN_1564; *ahpC* and MXAN_4815). Two transcripts were found in the top 50 transcripts of five transcriptomes (MXAN_1563; *ahpD* and MXAN_0934), and three more were in the top 50 of four OMV transcriptomes (MXAN_0457; *raiA*, MXAN_2072; *rpsO* and MXAN6754, an HSP60).

Surprisingly, the highly enriched DK1622 OMV transcript MXAN_4355 was not abundant in any of the AB022/CA010 OMV transcriptomes, and while the other highly-enriched DK1622 OMV transcript MXAN_0457; *raiA*, was found abundantly in both AB022 OMV transcriptomes, its enrichment in OMVs was only 1.8 fold compared to the AB022 cellular transcriptome. It seems that the majority of 'core' OMV transcripts observed in a strain are neither 'core' in other strains, nor consistently enriched relative to the cellular transcriptomes, in other strains

of the same species. However, transcripts encoding peroxidases, raiA family proteins, and ribosomal subunits do appear to be consistently found abundantly in OMVs.

3.5. ncRNAs in OMVs

To investigate whether any non-coding RNAs (ncRNAs) were packaged into OMVs, the abundance of 37 previously catalogued DK1622 ncRNAs in the OMV and cell transcriptomes was assessed by mapping transcripts against the DK1622 genome, as described previously.[25] Examples are shown in Fig. 2.

Twelve of the 20 ncRNAs found abundantly in the cell transcriptomes, were also found in the OMV transcriptomes of each strain, including the ncRNAs antisense to rRNA operons (Mxs005-Mxs007, Mxs024-Mxs026, Mxs031 and Mxs036), as well as the tmRNA, 6S rRNA, RNase P and signal recognition particle ncRNAs (SsrA, SsrS, RnpB and Ffs, respectively). It seems likely that these ncRNA were packaged passively into OMVs, and that the eight abundant DK1622 cellular ncRNAs which were not abundant/present in OMVs of any strains (Mxs001, Mxs002, Mxs016, Mxs023, Mxs033, Mxs037, MsDNA and Pxr), were somehow excluded from packaging.

Of the remaining 17 ncRNAs (Mxs004, Mxs008-Mxs010, Mxs012-Mxs015, Mxs017, Mxs019, Mxs021, Mxs022, Mxs028-30, Mxs034 and Mxs035), all were low abundance or entirely absent from all cell and OMV transcriptomes, except for Mxs028, which was abundant in both the cell and OMV transcriptomes, but only for AB022 (Fig. 2).

4. Discussion

In several cases, the OMVs secreted by Gram-negative bacteria are known to affect the biology of other organisms by delivering RNAs directly into target cells. As a first step to investigate whether myxobacterial OMVs promote killing of prey organisms or promote communication within swarms *via* the transport of RNAs, we undertook transcriptome sequencing of OMVs and cells isolated from multiple myxobacteria, including the model myxobacterium *M. xanthus* DK1622.

Comparing the profiles of the cellular and OMV transcriptomes gave only moderate to weak correlations, suggesting that the RNA cargo of OMVs is not simply a consequence of sampling the cellular transcriptome. Indeed, many abundant cellular transcripts were entirely absent from OMVs and *vice versa*. The cellular transcriptome profiles of different strains of *M. xanthus* correlated moderately with one another, however there was only weak correlation between the transcriptome profiles of replicate OMV preps, suggesting that OMV cargo packing is subject to significant stochasticity. The greater correlation between cellular transcriptomes than between OMV replicates may be a reflection of cells being subject to stronger selective pressures than the OMVs they produce.

Nevertheless, despite such variability, we were able to identify 13 'core' OMV transcripts in our datasets, which were abundant in both replicate OMV preps and whose abundance correlated strongly between replicates. The core OMV transcripts

Table 4 Correlations between the normalised FPKM values of cell and/or mean OMV transcriptomes for three strains of *M. xanthus*

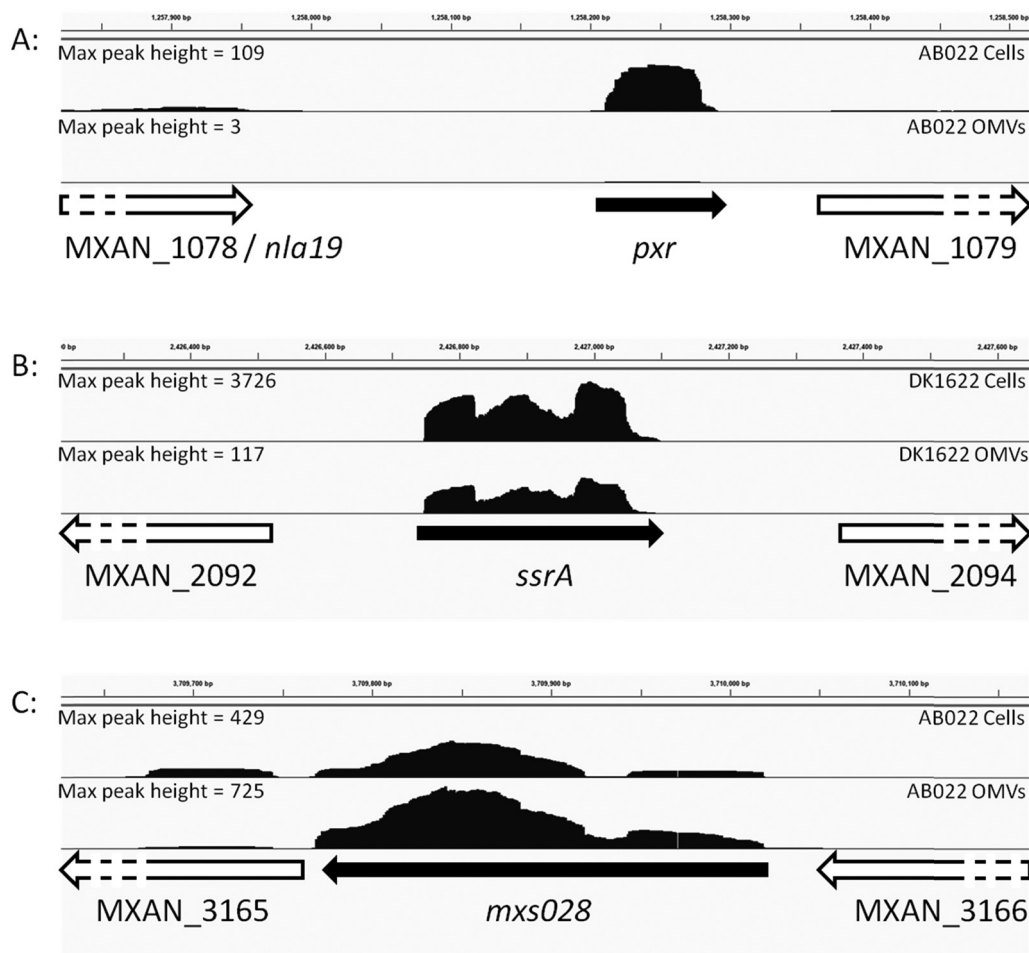|		OMVs		Cells		
Correlation coefficients (r)		CA010	DK1622	AB022	CA010	DK1622
OMVs	AB022	0.227	0.217	0.377	0.347	0.221
	CA010		0.202	0.441	0.639	0.468
	DK1622			0.333	0.262	0.331
Cells	AB022				0.56	0.418
	CA010					0.51

Fig. 2 Mapped transcripts for three example ncRNAs. ncRNAs are shown as black arrows and surrounding protein-coding genes as white arrows. In each panel the upper trace is from the cellular transcriptome and the lower from the OMV transcriptome. (A) *pxr* of AB022, (B) *ssrA* of DK1622, (C) *mxs028* of AB022.

of DK1622 had no obvious commonality of function, with the exception of several ribosomal RNAs, which was also observed by Chapagain *et al.* in the OMVs of *Flavobacterium psychrophilum*.[26] Core OMV transcripts were not consistently enriched in abundance relative to the cellular transcriptome, and only five of the core OMV transcripts were found among the core OMV transcripts of other *Myxococcus xanthus* strains: namely those encoding a protease (MXAN_0934), AhpC, AhpD and two family RaiA proteins.

This picture of variability, conservation and enrichment is similar to that obtained when studying the proteins of myxobacterial OMVs. The myxobacterial OMV proteome is enriched for particular proteins compared to the cellular proteome, and is highly variable when comparing between strains – a few core proteins are found in every strain's OMVs, but the majority of OMV proteins are strain-specific.[24,27] These shared features of myxobacterial OMV transcriptomes and OMV proteomes in turn mirror the myxobacterial pan-genome, which comprises a relatively small core genome, with a large and highly variable accessory genome.[28,29]

Enrichment of specific molecular species in OMVs compared to their relative abundance in the cell is indicative of cargo selection and usually assumed to be adaptive. A small number of core OMV transcripts were found to be enriched, but enrichment of those transcripts was not typically conserved, and many OMV transcripts were not enriched at all. We propose that in myxobacteria, RNA cargo selection is essentially passive, with the packaging of transcripts depending on their cellular abundance. However, it would seem that many cellular transcripts are actively impeded from being packaged, either as a consequence of specific exclusion mechanisms and/or because their physical properties inhibit their inclusion within OMVs.

The mechanism of selective cargo loading of OMVs is not at all clear, even for OMV proteins, which have been much more extensively studied than OMV RNAs.[3] While the mechanism of cargo selection is not understood, the proteins incorporated into OMVs are typically outer membrane or periplasm residents, so it is easy to imagine them being captured within an OMV as it blebs off a cell if they are in the right place at the right time. In contrast, RNAs are produced and reside in the cytoplasm, so in principle they must cross the inner membrane to enter budding OMVs. How this might occur is more difficult to imagine. The lack of widespread enrichment of OMV

transcripts suggests there is no active mechanism for RNA cargo selection/incorporation, but all sorts of factors could potentially impede their packaging into OMVs – *e.g.* how large they are, whether they are being actively translated, whether they form secondary structures, *etc.* We assessed whether there was differential packaging of different sized RNAs, but found no correlation between abundance or enrichment of OMV RNAs and their length.

Presumably, abundant OMV transcripts are particularly good at leaking through any exclusion mechanisms/barriers rather than being specifically packaged into OMVs. However, this does not preclude them providing a selective advantage during OMV-mediated predation or *via* intra-swarm communication. With passive OMV packaging, mutations increasing transcript synthesis (or induction of transcription in response to the presence of prey) would also increase the incorporation of the resulting transcript into OMVs, thereby conferring a selective advantage. Similarly, the lack of conservation of a transcript between myxobacterial OMVs doesn't preclude a role for the transcript in predation, as the presence/absence of genes within the accessory genome correlates with predatory activity.[30] It is also worth considering that myxobacterial OMVs may have a more important role in defence against nematode predation, rather than the killing of bacterial/fungal prey.[31]

Might there be functional roles for the core OMV transcripts in predation? If OMV transcripts can enter the target cell cytoplasm, they could potentially affect host processes. Pathogenic bacteria package small regulatory RNAs which impede the host response to infection, but they also package full length mRNAs which could potentially be translated by the host cell. The conserved core OMV transcriptome of DK1622 includes several proteins which if translated could potentially disrupt the prey's ability to defend itself from predation. MXAN_0934 is a protease, which if translated could potentially digest prey cell proteins. Similarly, RaiA proteins inhibit ribosome activity during starvation, preventing the initiation of translation, so RaiA and MXAN_4815 could potentially affect prey homeostasis by impeding the prey's ability to express predation resistance proteins.[32] Conversely, AhpC and AhpD are involved in the detoxification of hydroperoxides, and it seems unlikely that antioxidant expression might hinder the prey cell's ability to withstand predation.[33] It would be interesting to test whether the OMV transcripts mentioned above are detrimental to prey health (*e.g.* if expressed from an inducible plasmid), and whether their proteins can be detected in prey cells after co-incubation with *M. xanthus* OMVs. If such transcripts are involved in predation, we might also expect to see a reduced predatory activity of mutants carrying deletions of the genes encoding those transcripts and/or induction of transcript expression/packaging in response to the presence of prey.

In addition to identifying conserved OMV cargo transcripts for further study, this study has provided the first cellular transcriptomes for three myxobacterial strains (AB022, CA010 and DSM14675[T]), including the first RNA-seq dataset for a species of *Myxococcaceae* other than *M. xanthus* (*M. stipitatus*), which we hope will support broader research into myxobacterial biology.

Data availability

Sequence data has been added to the Short Read Archive under accession PRJNA1028607, accessible at **https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1028607**.

Author contributions

Conceptualization, DEW; data curation, MTS; formal analysis, DEW, MTS; funding acquisition, DEW, MTS; investigation, ASA, EJR, JHH; software, MTS; supervision, DEW; visualization, DEW, MTS; writing – original draft, DEW; writing – review & editing, ASA, EJR, JHH, MTS.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

References

1 M. G. Sartorio, E. J. Pardue, M. F. Feldman and M. F. Haurat, Bacterial Outer Membrane Vesicles: From Discovery to Applications, *Annu. Rev. Microbiol.*, 2021, **75**, 609–630.

2 M. F. Haurat, J. Aduse-Opoku, M. Rangarajan, L. Dorobantu, M. R. Gray, M. A. Curtis and M. F. Feldman, Selective sorting of cargo proteins into bacterial membrane vesicles, *J. Biol. Chem.*, 2011, **286**, 1269–1276.

3 N. Orench-Rivera and M. J. Kuehn, Differential Packaging Into Outer Membrane Vesicles Upon Oxidative Stress Reveals a General Mechanism for Cargo Selectivity, *Front. Microbiol.*, 2021, **12**, 561863.

4 P. Dauros-Singorenko, C. Blenkiron, A. Phillips and S. Swift, The functional RNA cargo of bacterial membrane vesicles, *FEMS Microbiol. Lett.*, 2018, **365**, fny023.

5 A. Ghosal, B. B. Upadhyaya, J. V. Fritz, A. Heintz-Buschart, M. S. Desai, D. Yusuf, D. Huang, A. Baumuratov, K. Wang, D. Galas and P. Wilmes, The extracellular RNA complement of Escherichia coli, *MicrobiologyOpen*, 2015, **4**, 252–266.

6 C. Blenkiron, D. Simonov, A. Muthukaruppan, P. Tsai, P. Dauros, S. Green, J. Hong, C. G. Print, S. Swift and A. R. Phillips, Uropathogenic Escherichia coli Releases Extracellular Vesicles That Are Associated with RNA, *PLoS One*, 2016, **11**, e0160440.

7 O. V. Alikina, O. A. Glazunova, A. A. Bykov, S. S. Kiselev, M. N. Tutukina, K. S. Shavkunov and O. N. Ozoline,

A cohabiting bacterium alters the spectrum of short RNAs secreted by Escherichia coli, *FEMS Microbiol. Lett.*, 2018, **365**, fny262.

8 A. Malabirade, J. Habier, A. Heintz-Buschart, P. May, J. Godet, R. Halder, A. Etheridge, D. Galas, P. Wilmes and J. V. Fritz, The RNA Complement of Outer Membrane Vesicles From *Salmonella enterica* Serovar Typhimurium Under Distinct Culture Conditions, *Front. Microbiol.*, 2018, **9**, 2015.

9 H. J. Lee, Microbe-Host Communication by Small RNAs in Extracellular Vesicles: Vehicles for Transkingdom RNA Transportation, *Int. J. Mol. Sci.*, 2019, **20**, 1487.

10 B. A. Stanton, Extracellular Vesicles and Host-Pathogen Interactions: A Review of Inter-Kingdom Signaling by Small Noncoding RNA, *Genes*, 2021, **12**, 1010.

11 K. Koeppen, T. H. Hampton, M. Jarek, M. Scharfe, S. A. Gerber, D. W. Mielcarz, E. G. Demers, E. L. Dolben, J. H. Hammond, D. A. Hogan and B. A. Stanton, A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles, *PLoS Pathog.*, 2016, **12**, e1005672.

12 H. Zhang, Y. Zhang, Z. Song, R. Li, H. Ruan, Q. Liu and X. Huang, sncRNAs packaged by Helicobacter pylori outer membrane vesicles attenuate IL-8 secretion in human cells, *Int. J. Med. Microbiol.*, 2020, **310**, 151356.

13 E. Furness, D. E. Whitworth and A. Zwarycz, Predatory Interactions between Myxobacteria and their Prey, in *The Ecology of Predation at the Microscale*, ed. E. Jurkevitch and R. Mitchell, Springer, 2020, DOI: 10.1007/978-3-030-45599-6_1.

14 S. Seef, J. Herrou, P. de Boissier, L. My, G. Brasseur, D. Robert, R. Jain, R. Mercier, E. Cascales, B. H. Habermann and T. Mignot, A Tad-like apparatus is required for contact-dependent prey killing in predatory social bacteria, *eLife*, 2021, **10**, e72409.

15 B. S. Arakal, D. E. Whitworth, P. E. James, R. Rowlands, N. P. T. Madhusoodanan, M. R. Baijoo and P. G. Livingstone, In Silico and In Vitro Analyses Reveal Promising Antimicrobial Peptides from Myxobacteria, *Probiotics Antimicrob. Proteins*, 2023, **15**, 202–214.

16 A. G. L. Evans, H. M. Davey, A. Cookson, H. Currinn, G. Cooke-Fox, P. J. Stanczyk and D. E. Whitworth, Predatory activity of Myxococcus xanthus outer-membrane vesicles and properties of their hydrolase cargo, *Microbiology*, 2012, **158**, 2742–2752.

17 P. G. Livingstone, R. M. Morphew, A. R. Cookson and D. E. Whitworth, Genome Analysis, Metabolic Potential, and Predatory Capabilities of Herpetosiphon llansteffanense sp. nov, *Appl. Environ. Microbiol.*, 2018, **84**, e01040-18.

18 A. S. Zwarycz, T. Page, G. Nikolova, E. J. Radford and D. E. Whitworth, Predatory Strategies of Myxococcus xanthus: Prey Susceptibility to OMVs and Moonlighting Enzymes, *Microorganisms*, 2023, **11**, 874.

19 P. G. Livingstone, A. D. Millard, M. T. Swain and D. E. Whitworth, Transcriptional changes when Myxococcus xanthus preys on Escherichia coli suggest myxobacterial predators are constitutively toxic but regulate their feeding, *Microb. Genomes*, 2018, **4**, e000152.

20 D. E. Whitworth, Interspecies conflict affects RNA expression, *FEMS Microbiol. Lett.*, 2018, **365**, fny096.

21 B. S. Goldman, W. C. Nierman, D. Kaiser, S. C. Slater, A. S. Durkin, J. A. Eisen, C. M. Ronning, W. B. Barbazuk, M. Blanchard, C. Field, C. Halling, G. Hinkle, O. Iartchuk, H. S. Kim, C. Mackenzie, R. Madupu, N. Miller, A. Shvartsbeyn, S. A. Sullivan, M. Vaudin, R. Wiegand and H. B. Kaplan, Evolution of sensory complexity recorded in a myxobacterial genome, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 15200–15205.

22 P. G. Livingstone, R. M. Morphew and D. E. Whitworth, Myxobacteria Are Able to Prey Broadly upon Clinically-Relevant Pathogens, Exhibiting a Prey Range Which Cannot Be Explained by Phylogeny, *Front. Microbiol.*, 2017, **8**, 1593.

23 A. S. Zwarycz and D. E. Whitworth, Myxobacterial Predation: A Standardised Lawn Predation Assay Highlights Strains with Unusually Efficient Predatory Activity, *Microorganisms*, 2023, **11**, 398.

24 A. S. Zwarycz, P. G. Livingstone and D. E. Whitworth, Within-species variation in OMV cargo proteins: the Myxococcus xanthus OMV pan-proteome, *Mol. Omics*, 2020, **16**, 387–397.

25 D. E. Whitworth and M. T. Swain, A survey of non-coding RNAs in the social and predatory myxobacterium Myxococcus xanthus DK1622, *Mol. Omics*, 2020, **16**, 492–502.

26 P. Chapagain, A. Ali and M. Salem, Dual RNA-Seq of *Flavobacterium psychrophilum* and Its Outer Membrane Vesicles Distinguishes Genes Associated with Susceptibility to Bacterial Cold-Water Disease in Rainbow Trout (*Oncorhynchus mykiss*), *Pathogens*, 2023, **12**, 436.

27 D. E. Whitworth, S. E. Slade and A. Mironas, Composition of distinct sub-proteomes in Myxococcus xanthus: metabolic cost and amino acid availability, *Amino Acids*, 2015, **47**, 2521–2531.

28 D. E. Whitworth, N. Sydney and E. J. Radford, Myxobacterial Genomics and Post-Genomics: A Review of Genome Biology, Genome Sequences and Related 'Omics Studies, *Microorganisms*, 2021, **9**, 2143.

29 J. Chambers, N. Sparks, N. Sydney, P. G. Livingstone, A. R. Cookson and D. E. Whitworth, Comparative Genomics and Pan-Genomics of the Myxococcaceae, including a Description of Five Novel Species: Myxococcus eversor sp. nov., Myxococcus llanfairpwllgwyngyllgogerychwyrndrobwllllantysiliogogogochensis sp. nov., Myxococcus vastator sp. nov., Pyxidicoccus caerfyrddinensis sp. nov., and Pyxidicoccus trucidator sp. nov, *Genome Biol. Evol.*, 2020, **12**, 2289–2302.

30 D. Sutton, P. G. Livingstone, E. Furness, M. T. Swain and D. E. Whitworth, Genome-Wide Identification of Myxobacterial Predation Genes and Demonstration of Formaldehyde Secretion as a Potentially Predation-Resistant Trait of *Pseudomonas aeruginosa*, *Front. Microbiol.*, 2019, **10**, 2650.

31 N. Mayrhofer, G. J. Velicer, K. A. Schaal and M. Vasse, Behavioral Interactions between Bacterivorous Nematodes and Predatory Bacteria in a Synthetic Community, *Microorganisms*, 2021, **9**, 1362.

32 D. E. Agafonov, V. A. Kolb, I. V. Nazimov and A. S. Spirin, A protein residing at the subunit interface of the bacterial ribosome, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 12345–12349.

33 T. Jaeger, Peroxiredoxin systems in mycobacteria, *Subcell. Biochem.*, 2007, **44**, 207–217.