Predatory activity of *Myxococcus xanthus* outer-membrane vesicles and properties of their hydrolase cargo

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The deltaproteobacterium *Myxococcus xanthus* predates upon members of the soil microbial community by secreting digestive factors and lysing prey cells. Like other Gram-negative bacteria, *M. xanthus* produces outer membrane vesicles (OMVs), and we show here that *M. xanthus* OMVs are able to kill *Escherichia coli* cells. The OMVs of *M. xanthus* were found to contain active proteases, phosphatases, other hydrolases and secondary metabolites. Alkaline phosphatase activity was found to be almost exclusively associated with OMVs, implying that there is active targeting of phosphatases into OMVs, while other OMV components appear to be packaged passively. The kinetic properties of OMV alkaline phosphatase suggest that there may have been evolutionary adaptation of OMV enzymes to a relatively indiscriminate mode of action, consistent with a role in predation. In addition, the observed regulation of production, and fragility of OMV activity, may protect OMV-producing cells from exploitation by *M. xanthus* cheating genotypes and/or other competitors. Killing of *E. coli* by *M. xanthus* OMVs was enhanced by the addition of a fusogenic enzyme (glyceraldehyde-3-phosphate dehydrogenase; GAPDH), which triggers fusion of vesicles with target membranes within eukaryotic cells. This suggests that the mechanism of prey killing involves OMV fusion with the *E. coli* outer membrane. *M. xanthus* secretes GAPDH, which could potentially modulate the fusion of co-secreted OMVs with prey organisms in nature, enhancing their predatory activity.

**INTRODUCTION**

The myxobacterium *Myxococcus xanthus* is a predatory member of the soil microbial community. It can lyse a wide variety of 'prey' micro-organisms, and can grow using prey lysis products as its sole nutrient source (Reichenbach & Dworkin, 1981; Velicer, 2008; Berleman & Kirby, 2009). Lysis of prey is thought to occur through extracellular digestion; lytic factors potentially involved include secondary metabolites, many of which possess antimicrobial activity, and digestive enzymes, which presumably play a role in both lysis and subsequent metabolism of lysis products prior to uptake by *M. xanthus*. Myxobacteria are known to produce a wide range of secondary metabolites, and their genomes encode large numbers of hydrolytic enzymes (Schneiker et al., 2007; Wenzel & Müller, 2009). Predation by *M. xanthus* also appears to be co-operative, at least under some experimental conditions. Rosenberg et al. (1977) showed that the growth rate of a shaken culture increases with cell density when nutrients are provided as protein (casein) rather than amino acids/peptides, and that growth rate correlates with secreted protease activity.

In addition to their biotechnological potential, the myxobacteria have received a great deal of research interest due to their 'multicellular' life cycle (Whitworth, 2008). As well as co-operative predation, they exhibit communal responses to starvation, manifested as macroscopic pattern formation, fruiting body formation, and collective sporulation (Kaiser et al., 2010). It has become apparent that interactions between strains with different 'social phenotypes' can have profound consequences for the survival of the population (Velicer & Vos, 2009). For instance, some strains of *M. xanthus* cheat on other strains, and the cost imposed on a population by cheaters can drive the population to extinction (Fiegna & Velicer, 2003). Cheating is a potential problem whenever an organism generates a publicly accessible resource, the 'public good', which cheats disproportionately consume at the expense of co-operative strains.

During predation, two forms of public good are generated by *M. xanthus*: lytic factors and the prey nutrients which...
they in turn liberate. Both of these resources will tend to migrate away from the producing cell, providing an opportunity for exploitation by others. Reducing the transport rate or half-life of lytic factors would restrict public goods to the immediate vicinity of the producing organism, lessening potential competition/exploitation (Whitworth, 2011). One mechanism to achieve this might be to package lytic factors into larger agglomerations such as vesicles, reducing transport rates.

Outer-membrane vesicles (OMVs) have been observed to be produced by all Gram-negative bacteria investigated (including M. xanthus), and similar vesicles are now known to be made by Gram-positive bacteria (Whitworth, 2011). OMVs are roughly spherical membrane-bounded structures, which are selectively laden with hydrodases and metabolites (Kulp & Kuehn, 2010; Whitworth, 2011). For instance, phosphatases (hydrodases that liberate inorganic phosphate from organophosphate molecules) are frequently found in OMVs (e.g. Bomberger et al., 2009; Horstman & Kuehn, 2000; Pierson et al., 2011). OMVs can lyse next to Gram-positive cells, releasing their contents next to a target cell. However, they can also fuse directly with the outer membrane of Gram-negative prokaryotes and eukaryotes (Kulp & Kuehn, 2010). The OMVs of Gram-negative bacteria are typically capable of killing other bacteria, leading to their initial description as ‘predatory MVs’ by Li et al. (1998).

Surprisingly little is known about the mechanisms of predation by M. xanthus. Although the predatory behaviour of M. xanthus and the predatory activity of bacterial OMVs are both well documented, the hypothesis that M. xanthus OMVs (mxOMVs) are involved in its predatory behaviour remains untested. In this work we investigated the predatory activity and composition of mxOMVs.

**METHODS**

**Bacterial strains and their cultivation.** Wild-type M. xanthus strain DK1622 (Kaiser, 1979) was used throughout, except when assaying phosphatase activity using p-nitrophenylphosphate (pNPP) as chromogenic substrate. Yellow pigments of wild-type M. xanthus (DKxanthenes) co-purified with OMVs and masked the production of p-nitrophenol. Therefore a strain unable to produce Dkxanthenes was used in phosphatase assays, kindly provided by Rolf Müller's laboratory. Strain PMAK88 carries an engineered point mutation in the eighth keto-reductase domain of dksN from the Dkxanthenes biosynthetic cluster, and is consequently unpigmented (Meiser et al., 2008). Double-Casitone Yeast Medium (DCY) was used as a rich medium for the growth and maintenance of M. xanthus at 31 °C [2 % casitone, 0.2 % yeast extract, 10 mM Tris (pH 8.0), 8 mM MgSO4, with 1.5 % agar for solid media]. *Escherichia coli* strain TOP10 carrying plasmid pCR2.1 (Invitrogen) was used as prey for *M. xanthus*. PhoA was purified from *E. coli* strain CW3747 (ATCC 27237), which constitutively expresses alkaline phosphatase, by the method of Nossal & Heppel (1966). *E. coli* strains were cultivated as described by Sambrook et al. (1989) on Luria–Bertani (LB) medium, which for TOP10 [pCR2.1] was supplemented with kanamycin (50 µg ml−1) and ampicillin (100 µg ml−1).

**Isolation of mxOMVs and supernatant.** Cells from late-exponential phase cultures of M. xanthus were subjected to centrifugation for 20 min at 10,400 g, before being washed and resuspended in TM buffer (50 mM Tris, pH 7.8, 10 mM MgSO4). After incubating for 18 h, cells were harvested as above. MxOMVs were then sedimented by centrifugation at 100,000 g for 1 h, washing with TM buffer and resedimenting. Pelleted mxOMVs were resuspended in a minimal volume of TM buffer and stored at 4 °C. All centrifugation was performed at 6 °C. MxOMV preparations were filtered using 0.2 µm pore-size syringe filters, while supernatant was filtered using an Amicon ultrafiltration system. These conditions typically yielded 3.0 g (wet weight) of mxOMVs per litre of culture (containing 91 mg protein, representing 1.50–1.85 % of the total culture biomass), more than twice the yield obtained without overnight incubation in TM.

**Characterization of mxOMV components.** SDS-PAGE was performed according to the method of Silhavy et al. (1984), except that samples were not boiled prior to loading onto the gel. Gels were activity-stained by immersing in freshly prepared staining solution (30 mM Tris/HCl, pH 9, 5 mg z-naphthyl pyrophosphate ml−1, 1 mg Fast Red TR ml−1) for 10 min, while Coomassie brilliant blue was used to stain all protein. Phosphatase assays used pNPP as substrate (typically between 4 mM and 4 µM) in 0.5 M Tris (a phosphate sink, preventing end product inhibition), measuring absorbance of p-nitrophenol at 420 nm (in a Uvicon spectrophotometer from Kontron Instruments). To determine *Km* values, substrate concentrations were varied, and kinetic parameters extracted from double-reciprocal plots (data were acquired in triplicate and the standard deviations were in all cases less than 10 % of the mean values). When assaying acid phosphatase activity, pH was maintained with an acetate buffer, and reactions were adjusted to pH 7.8 with NaOH immediately prior to measuring absorbance. Protein concentrations were determined according to the Lowry method.

Dkxanthenes absorb maximally at 400 nm (Meiser et al., 2006), and Dkxanthene concentrations were assayed spectrophotometrically at 400 nm as a function of the protein content of the samples. Values obtained for samples derived from the Dkxantheme mutant PMAK88 were subtracted from equivalent values for the wild-type, to gain a specific absorbance due to Dkxanthenes.

Zymograms were performed according to the method of Troebberg & Nagase (2004), with casein incorporated within the resolving gel. After electrophoresis under denaturing conditions, gels were incubated in ‘renaturing buffer’ for 20 min and then transferred to ‘developing buffer’ for 3 h. Staining of the casein and sample proteins was achieved using Coomassie brilliant blue.

Secretion pathways were predicted for proteins using a hierarchical approach. Proteins were sequentially tested using LipoP (type II lipoprotein signal peptides), SignalP (type I signal-peptides), TatP (twin-arginine translocase) and SecretoP (non-classical and leaderless secretion) at http://www.cbs.dtu.dk/services/, until a significant hit was obtained.

**Electron microscopy.** Samples were mixed 1:1 with fixative (2.5 % glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2; Agar Scientific), and incubated for 90 min (with fresh fixative every 30 min). Samples were then washed in 0.1 M sodium cacodylate, pH 7.2 (wash buffer), and resuspended in molten 2 % low temperature-gelling agarose (Sigma Aldrich). Drops of the sample/agarose mixture were placed on a polished microscope slide and cooled to 4 °C. Solidified drops were immersed in wash buffer, which was replaced after 30 min at 4 °C. Samples were then progressed through an alcohol series: 30, 50, 70, 80 % and 100 % overnight at 4 °C. They were then dehydrated by centrifugation into a 1:2 mixture of ethanol/LR White (hard grade) acrylic resin (London Resin Company), then into a 2:1 mixture, and finally into 100 % resin overnight at 60 °C. Sections of 60–80 nm were cut on a Reichert-Jung Ultracut E.
Ultramicrotome with a Diatome Ultra 45° diamond knife and collected on Gilder GS2X0.5 3.05 mm diameter nickel slot grids (Gilder Grids) float-coated with Butvar B98 polymer films (Agar Scientific). Sections were double-stained with uranyl acetate (Agar Scientific) and Reynold’s lead citrate (TAAB Laboratories Equipment) and observed using a JEM 1010 transmission electron microscope (JEOL) at 80 kV. The resulting images were photographed using Kodak 4489 electron microscope film and developed in a Kodak D-19 developer according to the manufacturer’s instructions.

**Killing plate assays.** Small amounts (10 µl, containing 2000 cells) of a diluted exponential phase culture of *E. coli* (washed and resuspended in TM buffer) were mixed with test substances (typically 30 µg of protein of culture fractions, in a total volume of 100 µl). Mixtures were incubated without shaking for 60 min at 30 °C prior to serial dilution and plating onto selective media (LB supplemented with kanamycin and ampicillin). After incubation overnight at 37 °C, the numbers of *E. coli* colonies on the plates were counted and the most probable number of *E. coli* in each original sample was calculated. A negative control was performed where the test substance was TM buffer. The number of *E. coli* survivors in each sample was normalized against those of the negative control and expressed as percentage survival relative to the negative control (100% survival). Assays were performed at least in triplicate, with at least three different mxOMV preparations. BSA and rabbit (Oryctolagus cuniculus) glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Sigma Aldrich) were dissolved in TM buffer and added to killing assays at a final concentration of 0.1 mg ml⁻¹.

**Flow cytometric analysis of predation.** Propidium iodide (PI; Sigma) was dissolved in Millipore MillQ (0.22 µm filtered) water to a concentration of 60 µg ml⁻¹ and stored at −20 °C. FITC (Sigma) was dissolved to a concentration of 1 mg ml⁻¹ in acetone and stored at 4 °C. These were added to samples at final concentrations of 6 µg PI ml⁻¹ and 3 µg FITC ml⁻¹. Samples were incubated at 25 °C in the dark for 15 min prior to analysis. Flow cytometric analyses were performed using a Partec PAS-III flow cytometer (Partec) under the control of software supplied by the manufacturer. Sheath fluid was prepared using MilliQ water. Cells were illuminated using a 488 nm argon ion laser and data collection was triggered by FITC fluorescence at 525 nm. PI fluorescence was collected via a 630 nm band pass filter. Offline analysis was performed using WinMDI (http://facs.scripps.edu/software.html). Control live and dead (70% ethanol) *E. coli* were prepared as described in the LIVE/DEAD BacLight Bacterial Viability kit manual from Invitrogen (http://tools.invitrogen.com/content/sfs/manuals/mp07007.pdf)

**MS.** Coomassie-stained gel fragments were processed and tryptically digested using the manufacturer’s recommended protocol, on a MassPrep robotic protein handling system. The extracted peptides from each sample were analysed by means of nano-liquid chromatography-electrospray ionization-MS/MS (nanoLC-ESI-MS/MS) using the NanoAcquity/Q-ToF Ultima Global instrumentation (Waters) with a 45 min LC gradient. All MS and MS/MS data were corrected for mass drift using reference data collected from human [Glu1]-Fibrinopeptide B (Sigma Aldrich) sampled each minute of data collection. The data were used to interrogate the *M. xanthus* database (October 2009) (http://www.ebi.ac.uk/integr8/EBI-Integr8-HomePage.do) using ProteinLynx Global Server v2.3. Protein identities for each sample were assigned if there were at least two peptide identifications per protein.

**RESULTS**

**Proteins secreted by planktonic *M. xanthus***

Two patterns of phosphatase activity have been reported in vegetatively grown *M. xanthus* and five genes in the *M. xanthus* genome appear to encode phosphatases (Weinberg & Zusman, 1990; Whitworth et al., 2008), however, which genes are responsible for observed phosphatase activities is unknown. While attempting to purify the vegetative alkaline phosphatase, our assays revealed that most (>99%) alkaline phosphatase activity in cultures of wild-type *M. xanthus* DK1622 was extracellular, suggesting that the enzyme was secreted.

To identify the secreted phosphatase, the cell-free supernatant from a culture of *M. xanthus* was concentrated by rotary evaporation and freeze-drying. Components of the concentrated supernatant were then separated by mildly denaturing SDS-PAGE, alongside purified PhoA (alkaline phosphatase) of *E. coli* (Fig. 1). PhoA can be stained in-gel; however, no in-gel staining was observed for *M. xanthus* samples. The Coomassie-stained supernatant protein profile showed several protein bands. Nine (Fig. 1, bands A–I) were excised from the gel and identified by MS.

Twelve proteins were identified (Table 1), including three hydrolases, two transport proteins and six putative lipoproteins (PLPs). Bioinformatics analyses suggested that 10 of the 12 proteins were targeted beyond the cytoplasm, while the remaining two proteins were predicted to be cytoplasmic. Every protein identified was either annotated as a hydrolase/transporter itself, or was found in a putative operon with a proposed transporter/hydrolase, implying a hydrolysis/transport-related function (Table 1). Five of the six PLPs were predicted to possess type II signal peptides, suggesting that the mature PLPs were lipoylated and membrane-associated. As autolysis does not happen significantly to *M. xanthus* cells less than 20 h after

![Fig. 1. Protein profile of concentrated *M. xanthus* culture supernatant.](image-url)
Table 1. Secreted proteins of *M. xanthus*

Gel bands are labelled as in Figs 1 and 6. Associated genes are potentially co-transcribed genes, with functions related to transport and/or hydrolysis. Role is defined as: H, hydrolysis; T, transport; HA, hydrolysis-associated (gene has an associated hydrolase); TA, transport-associated (gene has an associated transport gene). OMP, Outer-membrane protein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Band(s)</th>
<th>Protein size</th>
<th>Annotation</th>
<th>Secreted*</th>
<th>Domains†</th>
<th>Associated genes</th>
<th>Role(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Secretome gel, Fig. 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MXAN_6574</td>
<td>A, C, D</td>
<td>53 kDa</td>
<td>PLP</td>
<td>SecretomeP</td>
<td>None</td>
<td>Two PLPs, TonB-dependent receptor, two Fe transporters with permeases</td>
<td>TA</td>
</tr>
<tr>
<td>MXAN_7199</td>
<td>B, C, D</td>
<td>66 kDa</td>
<td>PLP</td>
<td>LipoP</td>
<td>None</td>
<td>Phosphatase</td>
<td>HA</td>
</tr>
<tr>
<td>MXAN_2290</td>
<td>C, D</td>
<td>62 kDa</td>
<td>PLP</td>
<td>LipoP</td>
<td>Cys-rich repeats × 6, fibro-slime domain§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MXAN_7040, FadL‡</td>
<td>C</td>
<td>42 kDa</td>
<td>Putative OMP P1</td>
<td>SignalP</td>
<td>Toluene_X transporter</td>
<td>Lipase</td>
<td>T</td>
</tr>
<tr>
<td>MXAN_5970‡</td>
<td>F</td>
<td>82 kDa</td>
<td>Peptidase (subtilisin-like)</td>
<td>SignalP</td>
<td>None</td>
<td></td>
<td></td>
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<tr>
<td>MXAN_6270</td>
<td>F</td>
<td>72 kDa</td>
<td>Uncharacterized protein</td>
<td>TatP</td>
<td>None</td>
<td>Polysaccharide deacetylase</td>
<td>HA</td>
</tr>
<tr>
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<td>F, G</td>
<td>113 kDa</td>
<td>PLP</td>
<td>LipoP</td>
<td>Cys-rich repeats × 12</td>
<td>OmpA homologue</td>
<td>TA</td>
</tr>
<tr>
<td>MXAN_6720</td>
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<td>11 kDa</td>
<td>PLP</td>
<td>LipoP</td>
<td>None</td>
<td>PLP, haloacid dehalogenase (HAD) hydrolase</td>
<td>HA</td>
</tr>
<tr>
<td><strong>MxOMV zymogram, Fig. 6</strong></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>MXAN_1056</td>
<td>G</td>
<td>48 kDa</td>
<td>N-Succinylarginine dihydrolase</td>
<td>NS</td>
<td>AstB (SuccArg hydrolase)</td>
<td></td>
<td>H</td>
</tr>
<tr>
<td>MXAN_1450, Oar‡</td>
<td>G</td>
<td>122 kDa</td>
<td>TonB-dependent receptor</td>
<td>TatP</td>
<td>TonB</td>
<td>PLP</td>
<td>T</td>
</tr>
<tr>
<td>MXAN_3971</td>
<td>G</td>
<td>57 kDa</td>
<td>PLP</td>
<td>LipoP</td>
<td>None</td>
<td>Phospholipase, RNase</td>
<td>HA</td>
</tr>
<tr>
<td>MXAN_2815</td>
<td>H</td>
<td>36 kDa</td>
<td>GAPDH</td>
<td>NS</td>
<td>GAPDH (Rossmann)</td>
<td>SecG</td>
<td>H, TA</td>
</tr>
<tr>
<td>MXAN_1450, Oar‡</td>
<td>J, K, L</td>
<td>122 kDa</td>
<td>TonB-dependent receptor</td>
<td>TatP</td>
<td>TonB</td>
<td>PLP</td>
<td>T</td>
</tr>
<tr>
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<td>J</td>
<td>51 kDa</td>
<td>PLP</td>
<td>LipoP</td>
<td>Delta-60 repeat domain × 5</td>
<td>TonB-dependent receptor</td>
<td>TA</td>
</tr>
<tr>
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<td>K</td>
<td>24 kDa</td>
<td>PLP</td>
<td>LipoP</td>
<td>None</td>
<td>TonB-dependent receptor</td>
<td>TA</td>
</tr>
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<td>MXAN_5855</td>
<td>K, L</td>
<td>42 kDa</td>
<td>Hypothetical protein</td>
<td>SignalP</td>
<td>None</td>
<td>Acetate-CoA ligase, acetate-cation symporter</td>
<td>TA</td>
</tr>
</tbody>
</table>

*Prediction of secretion pathways was undertaken as described in Methods [LipoP, type II signal peptidase (lipoprotein) pathway; SignalP, type I signal peptidase pathway; SecretomeP, non-classical secretion; TatP, twin-arginine translocase pathway; NS, not secreted].
†Domains were identified using CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).
‡Identified in mxOMVs by Kahnt et al. (2010).
§Fibro-slime domains are sugar-binding domains found in many hydrolases, particularly β-glycosidases.
starvation (Berleman et al., 2006), the most likely explanation for the large number of membrane proteins identified in the culture supernatant was that they were components of mxOMVs.

Microscopy of mxOMV production

Images of mxOMVs have been published previously (Kahnt et al., 2010; Palsdottir et al., 2009; Whitworth, 2011); however, no images have yet captured mxOMV production. To visualize mxOMV production, a culture of M. xanthus was embedded in agar, and cross-sections were imaged by electron microscopy. During imaging many instances were observed where M. xanthus cells were apparently in the process of forming a vesicle. Fig. 2(a, b) shows M. xanthus cells surrounded by myxovesicles of 50–100 nm diameter, some of which are apparently broken, while others remain intact. Occasionally mxOMVs appeared to be linked to the cell surface by a tether, as reported elsewhere (Palsdottir et al., 2009), and vesicles could also appear densely stained, implying that mxOMVs are right-side out and filled with cargo (Fig. 2a, b). When cultures were starved of nutrients for 16 h, a subpopulation of imaged cells (around 1%) provided evidence of a novel mechanism of mxOMV production, more complex than merely budding from the outer membrane, as generally proposed for Gram-negative bacteria (Kulp & Kuehn, 2010). Circular structures (protovesicles) with a diameter of 100–200 nm were observed at the surface of several cells (Fig. 2c, d), apparently held pinched by an invagination of the double membrane. In many images the ‘pinch’ was elongated and in extreme cases formed a septum within the cell (Fig. 3). From static images it was difficult to deduce whether the observed vesicles were being formed or absorbed by cells. The images shown in Fig. 3 could almost represent a pseudo time-series of vesicle formation, coincident with septation. If the images in Fig. 3 were snapshots of vesicle absorption rather than formation, absorption would be coincident with cell–cell fusion, a much less likely scenario.

On its extracellular side, the protovesicle was apparently bounded by a single membrane. In some images, the interior of the protovesicle appeared contiguous with the periplasm, whereas in others (Fig. 2c) protovesicle contents appeared to be separated from the periplasm. In images where the cell wall peptidoglycan could be seen clearly between the outer and inner membranes (Fig. 2c), there usually appeared to be no obvious peptidoglycan layer within the pinched region.

Killing of E. coli by mxOMVs

The predatory life-style of M. xanthus and the earlier description of OMVs as predatory (Li et al., 1998; Mashburn-Warren & Whiteley, 2006) led us to test mxOMVs for the ability to kill prey (E. coli). We developed an assay wherein known numbers of E. coli were mixed

Fig. 2. Electron micrographs of cross-sections through M. xanthus and mxOMVs. (a) mxOMVs surrounding an M. xanthus cell. Black arrows indicate dark-staining vesicles, suggestive of material packed within the mxOMV. (b) In some cases, mxOMVs appear to remain attached to cells by tethers (black arrows). (c) An M. xanthus cell with a protovesicle (black arrow), held by a pinched-in region of the cell surface (large white arrow). The peptidoglycan layer (white arrow with black edge) is clearly visible between the outer and inner membrane, except in the vicinity of the pinch. (d) Another example of a protovesicle (black arrow), apparently held pinched by the cell surface (white arrow). Bars: (a, b) 200 nm; (c, d) 100 nm. Samples imaged in (a) and (b) were cells growing in rich medium, whilst those in (c) and (d) had been starved.

Fig. 3. Series of electron micrographs of sections through M. xanthus. ‘Pinches’ associated with vesicle formation can be elongated, ultimately forming septa within the cell, supporting the notion that these images represent snapshots of vesicle formation rather than vesicle absorption. Bars, 200 nm.
with test substances prior to serial dilution and plating onto selective media. The number of resulting *E. coli* colonies was counted and the most probable number of *E. coli* in each sample calculated. A negative control was also performed where the test substance was buffer (TM), and the number of *E. coli* survivors in each sample was normalized against the negative control and expressed as percentage survival. Student’s *t* test was used to interpret results.

In the killing assays, addition of purified mxOMVs significantly (*P*<0.001) reduced the number of *E. coli* colonies by nearly 75% (Fig. 4). Supernatant (lacking mxOMVs) also exhibited significant killing activity (*P*<0.05), although less than mxOMVs, and there was an additive effect on killing when mxOMVs and supernatant were added together. Using this assay we also observed killing of a *Pseudomonas* strain by mxOMVs (data not shown). Light microscopy confirmed a reduction in the numbers of intact *E. coli* cells after the addition of mxOMVs, while electron microscopy showed cells resembling the ‘lysed cells’ of Li *et al.* (1998) in the presence of mxOMVs.

Potentially, cells, cell fragments, mxOMVs and/or supernatant proteins could have contaminated each of our various sample preparations. We took special efforts to eliminate or control for such possibilities. Cells and mxOMVs were washed extensively to remove contaminating proteins from the supernatant, and to remove mxOMVs from cells. In addition, filtration was used to remove mxOMVs from supernatant, and to remove cells from mxOMV preparations. Preparations of mxOMVs and concentrated supernatant appeared to be devoid of cells (lysed or intact) and cellular debris when visualized by light and electron microscopy. As a control, French-pressing was employed to break open any vesicles and cells. Killing assays demonstrated that intact cells had no detectable predatory activity (neither did French-pressed cells), while mxOMVs lost their killing activity upon French-pressing (Fig. 4). The predatory activity of supernatant was unaffected by French-pressing. In addition, preliminary quantitative proteomic analyses have found that the top 10 most abundant cellular proteins (representing one-third of all cellular proteins) are undetectable in mxOMV and supernatant proteomes (Susan Slade, University of Warwick, personal communication). Therefore we can be confident that the predatory activities of our mxOMV and supernatant samples were not due to contaminating cells, mxOMVs, or cellular and/or vesicular proteins.

The predatory activity of mxOMVs was also assessed using flow cytometric analysis of *E. coli* treated with viability-dependent fluorophores (López-Amorós *et al.*, 1997; Assunção *et al.*, 2006; Davey, 2011). Live and dead *E. coli* were prepared and stained with FITC and PI. Live cells were mixed with mxOMVs, and after incubating for 10 min at room temperature, samples were subjected to flow cytometric analysis. A plot of fluorescence at 630 nm against fluorescence at 520 nm shows that live and dead *E. coli* exhibited distinct patterns of red and green fluorescence (Fig. 5). After incubation with mxOMVs, the population of live *E. coli* cells gave a fluorescence profile typical of dead cells, confirming that mxOMVs are able to kill *E. coli*, and in a relatively short amount of time.

In eukaryotes, the glycolytic enzyme GAPDH has an additional fusogenic enzymic activity, stimulating the fusion of membranes, including the fusion of vesicles with cell membranes (Glaser & Gross, 1995). Rabbit GAPDH and BSA alone had no effect on *E. coli* survival or death; however, GAPDH, but not BSA, enhanced the predatory activity of mxOMVs (Fig. 4). This suggests that the killing of *E. coli* by mxOMVs is mediated through the fusion of vesicles with the *E. coli* outer membrane.

**Cargo of mxOMVs**

mxOMVs were highly pigmented (yellow), due to the incorporation of DKxanthenes secondary metabolites (Meiser *et al.*, 2008). The relative amount of DKxanthenes (per milligram protein) in mxOMVs and cells was determined, and DKxanthene levels were found to be only 1.5 times higher in OMVs than in cells (hundreds of times higher than the relative concentration in cell- and mxOMV-free supernatant). This suggests that DKxanthenes incorporation into mxOMVs is passive, with no evidence for either active incorporation into or exclusion from mxOMVs.

Preliminary experiments described above showed that >99% of alkaline phosphatase activity in an *M. xanthus* vegetative culture was extracellular. We subsequently separated cells and mxOMVs from supernatant by differential centrifugation, French-pressed the samples, and assayed phosphatase activity as a function of sample volume. It was found that 95% of the culture activity was co-localized with mxOMVs, 4% was found in the supernatant, while only around 1% was associated with cells. Taking into account the relative amounts of cells and mxOMVs in a culture (1 g mxOMVs per ~66 g of cells), this represents an enrichment in (activity/volume) of approximately 104-fold in mxOMVs relative to intracellular levels, suggesting active packaging of alkaline phosphatase(s) into mxOMVs.

The kinetic properties of mxOMV phosphatases were then determined, at pH 5.2 (acid), pH 7.2 (neutral) and pH 8.5 (alkaline); pH optima previously defined for *M. xanthus* phosphatases (Weinberg & Zusman, 1990). Under acid, neutral and alkaline conditions, specific activities of 1.8, 0.7 and 3.5 nmol min⁻¹ mg⁻¹ were observed, respectively, when saturated with substrate (calculated from *V*max values). The apparent *Km* for pNPP was 7 μM under acidic conditions, 4 μM at neutral pH and 2 mM at pH 8.5. No stimulation of activity was observed upon the addition of magnesium to the assays (5 or 10 mM), suggesting that the enzyme(s) were magnesium-independent, and prolonged incubation of mxOMVs at 30 °C and...
reassaying gave an apparent half-life of phosphatase activity in mxOMVs of around 20 h (around 24 h at room temperature).

*M. xanthus* is known to secrete proteases (Rosenberg *et al.*, 1977), so we tested mxOMV proteins for protease activity using zymography. MxOMV proteins were resolved by SDS-PAGE using gels incorporating casein, and then proteins within the gels were renatured and stained with Coomassie blue, with regions containing proteases showing up as areas of reduced casein staining (Fig. 6). Three bands were visible within a zone of casein hydrolysis (which was sensitive to the presence of the protease inhibitor PMSF), corresponding to mxOMV proteins of between 50 and 250 kDa (Fig. 6). MS identified four proteins within those three bands (Table 1). While none of the four proteins was annotated as a protease, two of them were lipoproteins encoded immediately adjacent to TonB-dependent receptors (MXAN_1451 and MXAN_5024), while a third protein was one of the adjacent TonB-dependent receptors (MXAN_1450).

**DISCUSSION**

Bacterial OMVs are potential biomolecule delivery vehicles, vaccine components and antibiotics (Khandelwal & Banerjee-Bhatnagar, 2003; Kim *et al.*, 2008; Sanders & Feavers, 2011). OMVs generally exhibit lytic activity against a wide range of bacteria, leading to their description as ‘predatory’, even though studied OMVs are produced by non-predatory bacteria (Li *et al.*, 1998; Mashburn-Warren & Whiteley, 2006). Myxobacteria have a predatory lifestyle, and we have observed that mxOMVs can kill *E. coli* and *Pseudomonas* strains. To our knowledge, this is the first report of OMV-mediated killing by a predatory organism. Killing is likely to be due to membrane damage, which is sufficient to enable the entry of the viability-dependent stains PI and FITC (López-Amorós *et al.*, 1997; Davey 2011).
Our assay results also demonstrated that the mxOMV-free supernatant of an *M. xanthus* culture possesses predatory activity, additive to that of mxOMVs. In the environment, prey killing by *M. xanthus* may involve both mxOMVs and secreted factors, potentially with different prey organisms exhibiting different susceptibilities to mxOMVs and soluble factors. Individual myxobacterial strains have different prey ranges (Morgan *et al.*, 2010; Pham *et al.*, 2005), and it would be interesting to determine whether prey specificity is a consequence of selective targeting by mxOMVs, or differential resistance/susceptibility to mxOMV cargo.

Electron microscope images revealed large quantities of mxOMVs adjacent to *M. xanthus* cells, with many touching the cell membrane, presumably being blebbed from the outer membrane. However, a subset of cells appeared to produce mxOMVs by an alternative mechanism, at specialized cellular structures (‘pinches’). Potentially the two processes might result in distinct subpopulations of mxOMVs, as suggested for OMVs of other species (Kulp & Kuehn, 2010). In the majority of cases the peptidoglycan layer appeared perturbed at pinches, and it may be that pinch-mediated mxOMV production occurs at areas of peptidoglycan weakness, a feature of OMV production suggested by other studies (reviewed by Kulp & Kuehn, 2010).

As shown before (Kahnt *et al.*, 2010), the protein profiles of mxOMVs and broken intact cells are very different (data not shown), implying selective incorporation/exclusion of proteins from mxOMVs (Kulp & Kuehn, 2010). Various secreted enzymic activities have been observed for *M. xanthus*, including protease/peptidase, amidase, glucosaminidase and lysozyme activities (reviewed by Berleman & Kirby, 2009). The annotation of the secreted proteins that we identified by MS (Table 1) included examples of all of those activities, except for glucosaminidase activity, and we confirmed protease activity of mxOMV proteins through zymography. Alkaline phosphatase activity was found to be almost exclusively associated with mxOMVs (further suggesting a lack of contamination during sample preparation), and is likely to be due to the presence of AIP2/MXAN_1389, which is proposed to be a PhoD-family alkaline phosphatase (Whitworth *et al.*, 2008). MXAN_1389 has been identified before in mxOMVs (Kahnt *et al.*, 2010), although we did not identify it here. Phosphate nutrition is of vital importance to *M. xanthus*; the absence of phosphate from rich media is sufficient to trigger multicellular development, and during fruiting body formation phosphatases are expressed which appear to cannibalise phosphate from autolysed cells (Moraleda-Muñoz *et al.*, 2003; Pham *et al.*, 2006). The enrichment of alkaline phosphatase activity in mxOMVs implies that most prey phosphatase liberation during predation is mediated by mxOMVs.

\[ K_m \text{ values of 7 \mu M, 4 \mu M and 2 mM were observed for } \]
\[ \text{phosphatase activity against pNPP under acidic, neutral and alkaline conditions, respectively. A comparison of our } \]
\[ \text{results with those of Weinberg & Zusman (1990) is not meaningful, as we were assaying phosphatases in mxOMVs } \]
\[ \text{rather than cells, our mxOMV-producing cells had been } \]
\[ \text{resuspended in buffer for a day prior to the assays, and our } \]
\[ \text{assays included Tris in all buffers to prevent end-product } \]
\[ \text{inhibition. In the enzyme database BRENDA (Scheer } \]
\[ \text{et al., 2011), wild-type alkaline phosphatase of } E. \ coli \]
\[ \text{has been ascribed } K_m \text{ values for pNPP between 8 and 20 \mu M under alkaline conditions. This suggests that the } M. \ xanthus \]
\[ \text{enzyme has a significantly lower affinity for substrate than } \]
\[ \text{PhoA of } E. \ coli, \text{ which may relate to its role in predation. A } \]
\[ \text{higher } K_m \text{ value for an enzyme would typically suggest a } \]
\[ \text{relative physiological abundance of the substrate, and a } \]
\[ \text{decreased } k_{cat}/K_m \text{ (specificity constant), which in turn } \]
\[ \text{implies a broader substrate range, arguably more appropriate } \]
\[ \text{for an enzyme whose function is to indiscriminately hydrolyse phosphoryl esters during predation. } \]

Extracellular hydrolases such as phosphatases are a form of public goods, which can be exploited by cheaters or competitors (Travisano & Velicer, 2004). Many strategies are employed by organisms to reduce the impact of such competition, ensuring that they get the choice share of any public resource. Cells can ensure that public goods that they generate stay nearby by reducing the transport/diffusion of these goods (for instance by packaging hydrolases into OMVs) and/or reducing their longevity (Whitworth, 2011). Another strategy is to regulate production, so that public resources are only made when there is competition for resources, helping reduce the cost of public good production (Kümmerli & Brown, 2010).

Purified *E. coli* alkaline phosphatase PhoA is stable at room temperature for several months (Malamy & Horecker, 1966), whereas mxOMV alkaline phosphatase activity is lost relatively quickly (half-life of 20 h). Whether the *M.
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Myxobacterial outer-membrane vesicles


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