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Preparation of Outer Membrane Vesicles from *Myxococcus xanthus*

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[Abstract] Outer membrane vesicles (OMVs) represent a unique sub-cellular compartment of bacteria that may act as a scaffold for various extracellular activities, including intercellular signaling. *Myxococcus xanthus* (*M. xanthus*) is a predatory bacterium that engages in cell-cell behaviors such as fruiting body formation and contact dependent lysis of other microbes. The OMVs of *M. xanthus* have been shown to have an elaborate architecture of chains and tubes that can connect cells within a biofilm. These higher order OMV structures have been shown to contain proteins exchanged for community behaviors and small molecules that have antibiotic activities, and may help facilitate directed exchange. *M. xanthus* OMVs allow material transfer between neighboring cells for motility and predation.

Materials and Reagents

1. 50 ml plastic tubes for handling harvested cultures (VWR International, catalog number: 62406-200)
2. 0.45 µm syringe filters (VWR International, catalog number: 28145-477)
3. 0.22 µm syringe filters (VWR International, catalog number: 28144-050)
4. 30 ml syringes (VWR International, catalog number: 66064-760)
5. 1.5 ml Eppendorf tubes (VWR International, catalog number: 89213-152)
6. 200 mesh formvar coated TEM grids (Electron Microscopy Sciences, catalog number: EMS200-Cu)
7. Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Coverglass Base (Thermo Fisher Scientific)
8. *Myxococcus xanthus* wild type strain DZ2 (UC Regents, Berkeley)
9. Phosphate buffered saline (PBS) (VWR International, catalog number: 97064-158)
10. Fluorescent lipid dye FM 4-64 (Life Technologies, Molecular Probes®, catalog number: T-13320)
    Note: Currently, it is "Thermo Fisher Scientific, Molecular Probes™, catalog number: T-13320".
11. Uranyl acetate (Electron Microscopy Sciences)
12. 10 mM MOPS (pH 7.6)
13. 2 mM $MgSO_4$

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14. 10% (w/w) Bacto casitone
15. 5% (w/w) Bacto yeast extract
16. CYE Media (see Recipes)

Equipment

1. Sterile, side arm 250 ml Erlenmeyer Flasks
2. Shaking incubator (*e.g.,* Thermo Fisher Scientific, model: MaxQ4000)
3. Vortexer
4. Centrifuge with capacity for 25 ml cultures, 5,000 *x g* (Refrigeration not required)
5. Ultra-centrifuge with capacity for 1 ml samples, 140, 000 *x g* required and refrigeration required. (*e.g.,* GMI, Beckman Coulter, model: L8-70M**)**
6. Ultra-centrifuge tubes (fit to machine specifications)
7. -80 °C freezer
8. Fluorescence plate reader (*e.g.,* Tecan Trading AG, model: Vission-100)
9. Transmission Electron Microscope (Philips/FEI, model: 5350 NE Dawson Creek Drive), capable of imaging negatively-stained samples at voltages ranging between 80 kv and 200 kV equipped with a 2 k x 2 k CCD camera
10. Autoclave for sterilizing media and flasks (or access to sterile media and growth vessels).

Software

1. Digital Micrograph software and 2 k x 2 k CCD camera (Gatan Inc.)

Procedure

1. In order to obtain a cell free preparation of outer membrane vesicles and vesicle chains, *M. xanthus* strain DZ2 (wild type) cells were harvested from restreaks on CYE Petri plates, 3-21 days old and inoculated into 250 ml Erlenmeyer flasks with 25 ml of liquid CYE media (10:1 volume of flask:volume of culture ratio should be maintained for aerobic cultures of *M. xanthus*). A single 25 ml culture is sufficient for isolating ~200 µg of OMVs for further analysis.
2. Cultures were grown with shaking at 200 rpm, 32 °C for 2 days to an OD_{600} = 2.0 (~2 x 10^9 cells/ml) in a Thermo Scientific MaxQ4000 shaking incubator.
3. Cultures were transferred to 50 ml centrifuge tubes and vortexed for 30 sec to disperse aggregates and release vesicles. Note that for *M. xanthus*, large cell aggregates are common, and this duration of vortexing may not be needed when

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adapting to other bacterial species. Cultures should be handled at room temperature for steps 3-6, as cold temperature disrupts cell viability and impinges on fractionation.

4. The vortexed culture was centrifuged for 10 min at 5,000 *x g* to pellet whole cells.
5. The supernatant was filtered through a 0.45 µm syringe filter to remove any remaining cells.
6. Filtrate was further centrifuged as in step 4, then passed through 0.22 µm syringe filters to remove debris.
7. Cell free filtrate was then subjected to 140,000 *x g* centrifugation at 4 °C using an ultracentrifuge for 1 h to harvest outer membrane vesicles, vesicle chains and membrane tubes, that will be in the resulting pellet and resuspended in 1 ml of PBS.
8. Samples were then saved at -80 °C or subjected immediately to analysis. Samples were analyzed by two methods described below to (a) monitor the purity of vesicle fractions by fluorescent lipid dye binding and (b) monitor the purity and quality of vesicle fractions by electron microscopy. Additional analyses can also be performed, for instance, proteomic analyses have indicated that the OMV protein fraction is consistent with little to no contamination from any other large protein complexes.
    a. Sample aliquots were mixed with fluorescent lipid dye FM 4-64 (purchased as a 100 µg dry stock, stored at -20 °C until use) to a final volume of 100 µl and final concentration of 16 µM. The relative concentration of vesicle samples was determined using black 96 well plates and a fluorescence plate reader by exciting at 515 nm, measuring emission at 640 nm. Five independent samples were analyzed for each biological preparation, as mixtures are heterogeneous. 16 µM FM 4-64 in PBS should be used as a negative control, while whole cell fractions can be utilized as positive controls. Fluorescence units may vary, but successful preparations should be 10-40 fold or higher than negative control readings. 1:1 Serial dilutions of samples may be required if signal saturation occurs. Yield increases with increasing cell density and with stable surface grown cultures (biofilms) relative to aerobic liquid cultures but ~200 µg or more is obtainable with this protocol.
    b. Negative-stained TEM samples were prepared by applying 3 µl of the OMV samples in 3 replicates to 200 mesh formvar coated TEM grids, allowing 3 min to settle and rinsing briefly in distilled water. A 2.0 % solution of Uranyl acetate was applied to cover the grid before immediately blotting off and air drying. Samples were imaged on a FEI CM200 microscope with a 2k x 2k Gatan CCD camera or on a JEOL 1200 microscope, operated at 200 kV or 120 kV, respectively, with quality judged by the presence of vesicles and vesicle chains and the absence of cells or cellular debris. For more detail on EM procedures see Remis *et al.* (2014a), for a video of EM procedures please see Rames *et al.* (2014b).

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Notes

Other EM analysis methods [SEM, resin-embedded sections TEM, cryo-EM of frozen-hydrated vesicle samples (Remis *et al.*, 2014)] are also feasible for this assessment, but negative stain TEM is the simplest technique that provides this information. Light microscopy alone, due to its limited resolution, typically fails to provide accurate assessments of vesicle purifications.

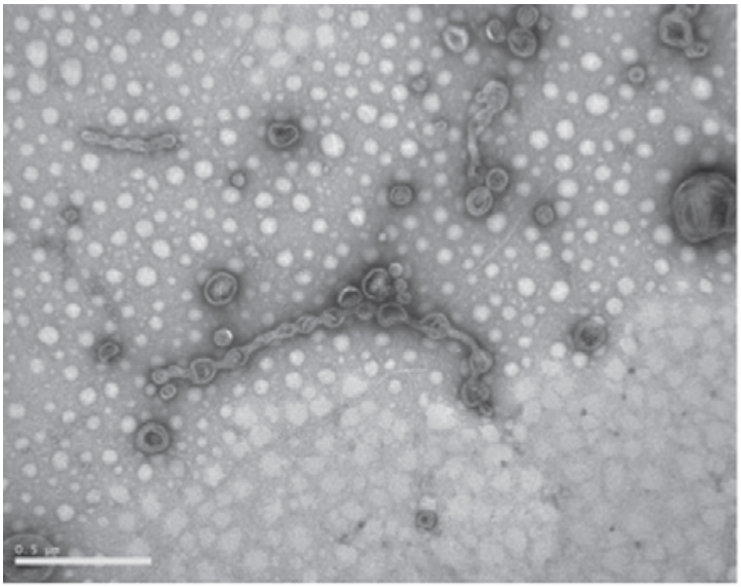

Figure 1. TEM image of *M. xanthus* OMVs (from Remis *et al.*, 2014) showing isolated OMVs as well as OMV chains

Recipes

1. CYE Media
   10 mM MOPS (pH 7.6)
   2 mM $MgSO_4$
   10% (w/w) Bacto casitone
   5% (w/w) Bacto yeast extract
   Sterilized by autoclaving

Acknowledgments

This protocol was adapted from previous work (Berleman *et al.*, 2014; Palsdottir *et al.*, 2009; Remis *et al.*, 2014). This work was Supported by Lab directed research development funds from the Office of Biological and Environmental Research of the US Department of Energy under contract number DE-AC02-05CH11231 (to Manfred Auer) and the US Department of Energy VFP program (to James E. Berleman).

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