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Bacterial Social Networks: Structure and composition of *Myxococcus xanthus* outer membrane vesicle chains

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Summary

The social soil bacterium, *Myxococcus xanthus*, displays a variety of complex and highly coordinated behaviors, including social motility, predatory rippling and fruiting body formation. Here we show that *M. xanthus* cells produce a network of outer membrane extensions in the form of vesicles and vesicle chains that interconnect cells. We observed peritrichous display of vesicles and vesicle chains and increased abundance in biofilms compared to planktonic cultures. By applying a range of imaging techniques, including 3D Focused Ion Beam Scanning Electron Microscopy (FIB/SEM), we determined these structures to range between 30–60 nm in width and up to 5 µm in length. Purified vesicle chains consist of typical *M. xanthus* lipids, fucose, mannose, N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) carbohydrates and a small set of cargo protein. The protein content includes CglB and Tgl membrane proteins transferred in a contact-dependent manner. Most significantly, the 3D organization of cells within biofilms indicates that cells are connected via an extensive network of membrane extensions that may connect cells at the level of the periplasmic space. Such a network would allow the transfer of membrane proteins and other molecules between cells, and likely provides a mechanism for the coordination of social activities.

Introduction

Recent decades have seen a profound change in our concepts of the growth of bacteria, in that the notion of groups of individual planktonic cells has given way to the concept of

integrated microbial communities (Hibbing *et al.*, 2010). This structural and functional coordination of cells can be mediated by diffusible chemical signals (Costerton, 2007; Davies *et al.*, 1998). Additionally, Mashburn & Whitely (2005) have shown that the limitations of radial diffusion can be overcome by packaging these signals in vesicles for direct, targeted communication between cells of the same species.

Myxococcus xanthus is a model system for coordinated social behavior, as individual cells must communicate in a targeted manner that ensures that signals are transferred to their intended recipients without interference from other organisms in their complex soil habitat (Berleman & Kirby, 2009; Whitworth, 2011). Freely diffusing signals for aggregation, such as cAMP used by social amoeba (O'Day and Keszai, 2011), or autoinducing homoserine lactones have not been identified in *M. xanthus*. The question remains as to how signals are communicated in *M. xanthus* social behaviors. Transfer of signals via individual vesicles is one possibility as this would result in undiluted material transfer and could constitute a “message in a bottle” that could be read at any time. An even more satisfying mechanism would be a direct targeting of a receiving cell via a connection that allows both the cell-to-cell transfer of membrane proteins, and possibly cell-to-cell diffusion of small chemical signals, secluded from the rest of the microbial community.

Previous studies have shown that cells of *M. xanthus* produce large numbers of vesicles, to which we and others have ascribed the potential to transfer membrane proteins that “stimulate” social activity (Palsdottir *et al.*, 2009; Kahnt *et al.*, 2010). Previous results implicated that outer membrane fusion between *M. xanthus* cells could explain this protein transfer, but direct observation of fused cells has still not been observed (Nudleman *et al.*, 2005). Recent work by Wei *et al.* (2011) confirms that intercellular transfer of lipoprotein from outer but not inner membrane occurs, but the molecular mechanism of transfer is unclear. Dubey and Ben-Yehuda (2011) have shown that cytoplasmic contents, including non-conjugative plasmids, may in certain cases be transferred via bridges between cells of *Bacillus subtilis*, between *B. subtilis* and *Staphylococcus aureus*, and even between *B. subtilis* and the distantly related cells of *E. coli*. Shetty *et al.* (2011) have shown by negative stain and thin section TEM as well as by cryo-electron tomography evidence for a hollow protein tube that can deliver vesicles over relatively large distances, but due to the limitations of conventional sectioning, cell-cell connections were difficult to observe. In addition, intercellular “nanowire” structures bridge some cells but are thought to function as a conduit for redox reactions between cells and extracellular metabolites (Gorby *et al.*, 2010; Reguera *et al.*, 2005). Observations of nanowires and nanotubes by scanning electron microscopy (SEM) are complicated by eutectic drying artifacts such that controversies have arisen concerning their structure and function (Miller *et al.*, 1983; Gorby *et al.*, 2008). A study by Dohnalkoya *et al.* (2011) attributed intercellular linkages, often found in SEM images of bacteria, to be critical point drying artifacts of EPS. Observations of intercellular structures by transmission electron microscopy (TEM) are complicated by the small volume of ultrathin sections typical for TEM imaging, making it difficult to follow long extracellular extensions from cell to cell. Thicker sections suffer from the superposition of structures in projection images precluding an unambiguous identification of cell-to-cell connections.

Here, we apply ultra-rapid freezing preparative methods, and advanced 3D electron microscopy imaging techniques that provide a new perspective on cell to cell structures. We observed the presence of novel types of outer membrane-derived extracellular membrane extensions in the form of vesicle chains. Cryo-EM analysis suggests the presence of carbohydrate-based surface structure, possibly lipopolysaccharides (LPS). Proteomics analysis reveals the presence of two outer membrane proteins, Tgl and CglB, known to be transferred in a cell-to-cell contact dependent manner. Vesicle chain appendages, while present in planktonic cells, were found to be up-regulated in biofilms, where they form an extensive membrane-enclosed network, that may link cells at the level of the periplasmic space. Given the faithful sample preservation and the detailed high-resolution 3D analysis used for this study, the detection of these lipid-based cell-to-cell connections provides a new perspective on bacteria in biofilms. These vesicular connections constitute intercellular bridges that have the potential of transferring materials between cells, enabling biofilms of this bacterial species to function as a dynamic multicellular organism.

Results

Observation of extracellular bridges in *M. xanthus* biofilms

Previous results indicated that *M. xanthus* produces extracellular vesicle structures that may help mediate multicellular behaviors such as aggregating into fruiting bodies (Palsdottir, *et al.*, 2009). We analyzed the early stages of biofilm formation in *M. xanthus* with SEM microscopy to determine if extracellular vesicles are observed (Fig. 1). At time 0, small clusters of cells are observed, with cells covered in peritrichously organized tubes that are 0.5 – 3 microns long and ~50 nm wide. These dimensions and organization indicate that they are not Type IV pili, but they appear quite different from spherical vesicles observed previously. Over the course of 48 hours, cell aggregates increase in size and the tubes are observed bridging spaces between cells (Fig. 1d-i). These images indicate that there may be higher order structures connecting cells than previously thought, but connectivity between bacteria have been challenged in the past and attributed as a sample preparation artifact.

To unambiguously detect and describe these tubular connections, we utilized a novel method for analysis of biological samples, known as Focused Ion Beam Scanning Electron Microscopy (FIB/SEM) (Heymann, *et al.*, 2009, Wei *et al.*, 2012). For FIB/SEM, samples are embedded in resin similar to samples prepared for TEM, and thus circumvent the possible complication of SEM critical point drying artifacts such as formation of eutectic “bridges” (Miller *et al.*, 1983, Dohnalkova *et al.*, 2011). While images are not as sharp as TEM images, FIB/SEM analysis provides ~300 cubic μm total volume (Fig 2)., FIB/SEM imaging of *M. xanthus* biofilms reveals a 3D network of intercellular connections, some of which appear as tubes (Fig 2a), others appear more like vesicle chains (Fig 2b). Several examples of cells connected by extracellular appendages were observed in the FIB/SEM 3D data set. However, the large number of intercellular connections can only be appreciated by rendering the 3D volume (Figure 2c and d). (For a movie of the image stack through this 3D volume as well as a 3D rendering, please see supplemental movie S1 and S2, respectively). For clarity, *M. xanthus* cells in the biofilm are colored yellow and the extracellular appendages colored in red. These appendages range from small vesicles budding/blebbing

off the outer membrane, to spherical extracellular vesicles, to the long, filamentous appendages extending out from the cell surface and often connecting two neighboring cells. The observed size of the appendages indicates a diameter of ~ 40-60 nm and a length from 0.5 μm (next neighbor connections) to 5 μm or longer. The 3D FIB/SEM reconstruction confirms the SEM observation of intercellular bridges, but neither technique can reveal the fine ultrastructure and composition of these bridges.

Structural analysis of extracellular membrane appendages

To further examine these extracellular membrane appendages, we examined planktonic cultures and biofilm samples with a variety of technical approaches, including *in vivo* live-cell fluorescent imaging of lipophilic dye (FM4-64)-labeled bacteria (Figure 3a), ultra-rapid plunge freezing (cryofixation) followed by cryo-TEM projection imaging (Figure 3b,c) of vitrified, frozen-hydrated whole-mount bacteria, cryo-tomography of biochemically isolated vesicle chain samples (Figure 3d), room-temperature electron tomography of high-pressure frozen, freeze-substituted and resin-embedded biofilm sections (Figure 3e), as well as room temperature whole-mount TEM projection imaging of negatively stained cells (Figure 3f). Throughout all these approaches, we consistently found the extracellular appendages to be ~30-60 nm in diameter, and either appeared as chains of closely spaced and physically interacting vesicles (Fig 1b,d), periodically constricted vesicle chains (Fig 1e, f) or tubes (Fig 1c). Cryo-tomograms of purified vesicle chains reveal that each vesicle chain displays a remarkably uniform vesicle diameter size while there were significant differences in vesicle diameter between different chains (Fig 1d). For slice-by-slice inspection of a 3D TEM tomographic volume of 150 nm ultrathin high pressure frozen, freeze substituted biofilm sections and vitrified isolated vesicle chains, see Supplemental Movie S3 and Supplemental Movie S4, respectively.

Using epifluorescence light microscopy of planktonic cells labeled with the lipophilic dye FM4-64 we verified the *in vivo* presence of lipid-based extracellular appendages up to 10 μm in length emanating from *M. xanthus* cells (Figure 3a) but such structures were rare in planktonic culture cells. Cryo-TEM projection imaging shows higher resolution of structures with vesicle chains (Figure 3b). We also observed the type-IV pili extending from the cell pole (3b insert), indicating faithful preservation and high image quality. The dual membrane structure of the Gram-negative *M. xanthus* cell envelope is also clear, whereas the vesicle chains consist of only a single membrane. In the cryo-preserved samples, the outer surfaces of both cells and vesicles display short, hair-like protrusions that possibly represent the lipopolysaccharide (LPS) O-antigen side chains that are only present on the outer leaflet of outer membrane. These protrusions are not observed on the inner leaflet or the phospholipid bilayer inner membrane, and like all carbohydrates (in contrast to lipids and proteins) do not stain with osmium tetroxide, which was used as a stain for the resin embedded samples.

In addition to the vesicle chains observed (Fig 3b), there are other similar structures observed by the same method such as tubes with a uniform diameter (Fig 3c). The 3D structure of purified vesicle fraction confirms the observation of chains (Fig 3d) as well as periodically constricted tubes (Fig 3e and 3f) that may represent intermediate structures. Interestingly, we could not detect any large protein complexes at these sites of constriction,

suggesting that these sites of constrictions might be regulated by proteins within the membranes itself although spontaneous vesiculation cannot be completely ruled out..

Compositional analysis of purified vesicles and membrane appendages

To determine the chemical composition of vesicles, we purified the vesicle fraction and examined it for its carbohydrate and lipid content (Figure 4). Vesicles were purified by vortexing cultures of *M. xanthus* to sever vesicles and membrane appendages off the cells. Cells were removed by pelleting at low speed centrifugation and culture supernatants were passed through a 0.22 μm filter to remove any remaining cells. Vesicles were then harvested by ultracentrifugation and resuspended in phosphate buffer. Using negative stain electron microscopy, we monitored fractions throughout the biochemical separation of vesicles and lipid appendages from the bacterial cells (Figure 4a). We found no significant bacterial inner or outer membrane contamination as would be indicated by the presence of ghost cells or large lipid sheets, but instead almost exclusively found vesicles and vesicle chains.

To assess carbohydrate content, we performed acid hydrolysis of the purified vesicle fraction followed by Fluorophore Assisted Carbohydrate Electrophoresis (FACE) analysis (Starr *et al.*, 1996). FACE analysis revealed the presence of several sugar moieties (Fig 4b). Among these carbohydrate moieties we identified neutral sugars such as fucose and mannose, as well as N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc). Fucose, while common in eukaryotic surface sugars, is rare in bacterial surface oligosaccharides. GlcNAc has previously been characterized as an abundant component of the extracellular material of *M. xanthus*, and is associated with pilus retraction (Li *et al.*, 2003). However, addition of vesicle fraction to liquid cultures of *M. xanthus* did not lead to Type IV pilus-dependent cell agglutination (data not shown), suggesting the mere presence of GlcNAc is not sufficient to trigger pili retraction.

Lipid analysis of vesicle fractions was performed by Fatty acid methyl esterification (FAME) analysis (Figure 4c). FAME analysis indicates the vesicle fraction of *M. xanthus* to be complex in composition with the most abundant fatty acid to be the branched chain 15:0 iso at 41.7 % of the total fatty acid content. In total, nearly 80 % of the content is 15 and 17 carbon chain fatty acids, for comparison, *E. coli* typically uses 14 and 12 carbon chain fatty acids in LPS. It is important to note that this analysis does not distinguish inner leaflet phospholipids from outer leaflet LPS (Figure 4d).

Up-Regulation of Vesicle Structures in Biofilms

We hypothesized that physical connections between bacterial cells might be profoundly influenced by their local environment and possibly by the culturing conditions. A small portion of cells under planktonic growth conditions produce vesicle chains but such structures are unlikely to result in stable connections due to the agitation of the medium, whereas cells living under the stationary conditions of a biofilm can potentially form more permanent and likely more effective structures. When we compared the amount of vesicles from equal amounts of cells derived from liquid culture versus biofilm growth conditions (Figure 5a), we found a significantly higher abundance in biofilms compared to planktonic cells by fluorescence quantification of lipid content (Figure 5a), although morphology of

appendages appeared similar (Figure 5b-e). Moreover, isolated cells from planktonic samples rarely show vesicle chains (Figure 5d) when examined by electron microscopy of negatively stained or cryo-samples, whereas vesicle chains are abundant when small groups of cells are aggregated (Figure 5e). We further noticed that vesicle chains are frequently found in biofilm samples by TEM of thin sections (Figure 5f).

Functional analysis of vesicle fractions

To determine what physiological function(s) *M. xanthus* vesicle, vesicle chains and tubes may have, we assayed for motility (Figure 6a-d) and predation (Figure 6f-i) as well as for protein content (Figure 6e). In order to test for the role of extracellular membrane appendages in adventurous gliding motility, we examined gliding cells on agar both by phase contrast bright fields optical microscopy as well as fluorescence imaging of FM4-64 labeled cells (Figure 6a-d). By tracking the movement of motile cells by phase contrast light microscopy and comparing with the detection of vesicle chains upon fluorescence imaging, we found that there was no correlation. While some moving cells have detectable vesicle chains at their tail end, other moving cells do not. SDS-PAGE analysis of vesicle fractions revealed 50 or more total proteins in the vesicle fraction, in agreement with previous studies. Employing protein mass spectrometry of discrete SDS gel bands from purified vesicles and vesicle chains, we detected Tgl and CglB (Figure 6e). Both proteins, i.e. Tgl and CglB, have been previously shown to be transferred between cells in order to stimulate Social (S) and Adventurous (A) motility, respectively (Nudleman *et al.* 2005; Rodriguez & Spormann, 1999). Most of the proteins detected are lipoproteins of unknown function, but we consistently did not detect OmpA (MXAN_5152) or the F0F1 ATP synthase (MXAN_6921), which are abundant in outer and inner membranes, respectively, indicating that the vesicle fraction has a distinct protein population, with little contamination.

It has recently been proposed that vesicles could mediate the killing of *E. coli* and other prey organisms through the delivery of toxic proteins or antibiotics (Whitworth, 2011; Evans *et al.*, 2012). To determine if our vesicle preparations contain lytic activity, we utilized fluorescently tagged *E. coli* strain β 2155 to quantify prey cell lysis. *E. coli* cultures were harvested and washed and added to black 96 well plates with CFL agar media. Vesicle fractions ("open circles") and whole *M. xanthus* cells ("X") were added in triplicate to the *E. coli* and output fluorescence monitored for 4 days (Figure 6f, open circle). Changes in fluorescence observed indicate that the vesicle fractions contain a portion of the lytic activity of wildtype cells, causing ~50 % of loss of *E. coli* cell signal. In addition, *E. coli* colonies (Fig 6g) are rapidly killed when mixed with *M. xanthus* cells, leading to complete clearing and the formation of fruiting aggregates (Figure 6h), whereas partial clearing is observed when *E. coli* is mixed with *M. xanthus* vesicle fraction (Fig 6i). These data suggest that one function of vesicles is to help mediate prey cell lysis, but there may be other functions as well.

Discussion

The observation of a discrete network of intercellular bridges in *M. xanthus* has several potential impacts. One possibility is that these bridges mediate the exchange of nutrients or

signals, another is that bridges help to physically bind cells together during social behaviors. Connections between cells are common in filamentous bacteria, but it is intriguing to consider that organisms such as *M. xanthus* may be able to regulate when and where these intimate connections between cells are formed. These bridges could be responsible for the observation of raft formation in gliding *M. xanthus* cells, the establishment of aggregates during fruiting body formation and in the detection and lysis of prey bacteria. If signals and nutrients are exchanged, this may also help to coordinate local responses such as the simultaneous transition from vegetative cells to spores within fruiting bodies. More work is required to determine how much of a chemical and physical role these structures play, but these two aspects are not mutually exclusive.

The use of FIB/SEM 3D imaging was particularly revealing as it allowed to visualize the previously unknown extent of cell-to-cell connections within a biofilm of *M. xanthus*. We conclude that these structures constitute a network that can connect virtually all of the cells in a local area of a biofilm, often by multiple connections. While the image of the lone planktonic bacterial cell has long since been replaced by the idea of cells embedded together in a cohesive matrix, the observation of vesicle chains connecting bacterial cells within a biofilm, again provides a new perspective on cells in a bacterial community connecting to one another (Figure 7). In light of the recent demonstration (Dubey & Ben-Yehuda, 2011) that cells of *B. subtilis* may under certain conditions exchange cytoplasmic proteins with each other and with cells of other species, via connections of undefined structure, we cannot help but to see the similarities of such cell-to-cell transfer to the network we have observed in *M. xanthus* biofilms. Indeed, recent work by Sanchez (2011) has pointed out that this newly discovered cytoplasmic continuity between cells in a microbial biofilm community links bacteria to higher plants that use plasmodesmata to produce elaborate multicellular syncytia.

But the credibility of descriptions of extracellular filamentous or tubular structures like nanotubes, particularly when shown by traditional SEM approaches, always hinges on the avoidance of sample preparation artifacts. Because such small membrane appendages are notoriously difficult to preserve faithfully with conventional approaches, and require high resolution imaging as their fine details lie beyond the limits of optical microscopy, the combination of cryo-EM, cryo-tomography and FIB/SEM techniques that we deployed here is very powerful as it can unambiguously demonstrate the presence and fine 3D structure of these vesicle chains. Our results indicate that while present at low levels in planktonic bacteria in liquid culture, vesicle chains are substantially up-regulated in groups of cells and biofilms, where they can be found in high abundance.

The membrane-based network we have discovered in *M. xanthus* has physical properties that differ from the much more rigid pili and the somewhat controversial nanowire structures, which mediate processes such as conjugation and electron sharing respectively in biofilm communities (Helmuth *et al.*, 1978; Gorby *et al.*, 2006). In contrast to the self-assembled protein structure of pili and nanowires, membranes would provide a fluid bridge between cells, within which individual proteins and hydrophobic molecules can move laterally in a sheet of possibly LPS or phospholipids, and thus form a dynamic continuum throughout the community. The presence of eukaryotic-like sugar moieties such as fucose on vesicles also

leads to the possibility of inter-kingdom communication/antagonism. Elongated membranes also define a fluid-filled space, with a barrier of variable permeability so that an array of membrane bound vesicles may provide increased surface area for metabolite exchange, or even connecting the periplasmic spaces of cells. To our knowledge, this is the first study that clearly demonstrates lipid appendages-based prokaryotic cell-to-cell bridges, whereas all previous claims of cell-cell bridges were suggested to be protein-based.

Our finding of CglB and Tgl as part of the vesicle fraction suggests a role for these structures in the exchange of proteins essential for motility. The contact-mediated transfer of outer membrane proteins in *M. xanthus* has previously been proposed through membrane fusion during pole-pole cell collisions (Nudleman *et al.*, 2005). While we find no evidence that vesicle chains in themselves are essential for motion, exchange of vesicles between cells would readily explain the phenomenon of motile *M. xanthus* cells being able to stimulate motility by transferring key proteins to certain strains of non-motile cells. CglB was also found in vesicles by Kahnt and colleagues (2010), and their work supports our proposition that vesicle secretion and fusion may be one of the underlying mechanisms of protein transfer. It is interesting to note that adjacent cells have been shown to exchange signals via vesicles for *Pseudomonas* (cytoplasmic contents), as well as *Bacillus*, and *M. xanthus* (Mashburn & Whiteley, 2005; Wei, *et al.*, 2010). Because we used virtually artifact-free cryo-preservation and 3D cryo-tomography as well as novel advanced 3D FIB/SEM imaging approaches, we were able to preserve and visualize structures that are difficult to discern by traditional approaches. Structure and function of other bacterial biofilms will hopefully be reexamined with this in mind, to determine how often bacterial cells should be seen as members of integrated multi-cellular communities.

Experimental Procedures

Cell growth conditions

M. xanthus strain DZ2 (wild type) cells were grown in liquid culture shaking at 200 rpm, 32 degrees Celsius in CYE media consisting of 5 mM MOPS pH 7.6, 2 mM MgSO₄, 0.5% (w/w) Bacto Casitone, 0.25% (w/w) Bacto Yeast Extract or on 1.5% agar plates with the same components. The biofilms grown on agar were allowed to mature for 2-4 days before imaging.

Transmission electron microscopy (TEM) and Cryo-TEM

Negative stained TEM samples were prepared using 2% uranyl acetate, followed by blotting off excessive stain and air drying of the sample. Alternatively, cells were placed onto glow-discharged lacey carbon grids (Ted Pella 01881). The grids were blotted and plunged into liquid ethane using a Vitrobot (FEI, Hillsboro, OR) before being transferred and stored in liquid nitrogen until imaged by cryo-EM. Images and tomographic tilt series were acquired by a 2k × 2k camera (Gatan Inc., Pleasanton, CA) at 20,000 magnification on a Philips CM200FEG or JEOL-3100 electron microscope with an underfocus value of 1 or 10µm, respectively.

High pressure frozen TEM (HPF-TEM)

Biofilms grown on 1.5% agar CYE plates were cryofixed in a Bal-Tec HPM010 high-pressure freezer (2,100 bars, 5 to 7 milliseconds). Using the Leica automated freeze-substitution system AFS (Leica Microsystems, Vienna, Austria), cryofixed specimens were freeze-substituted in anhydrous acetone containing 1% osmium tetroxide and 0.1% uranyl acetate and infiltrated with Epon-Araldite following established protocols (Palsdottir et al. 2009). Specimens were flat-embedded between two microscopy slides and polymerized at 60°C over 1 to 2 days. Resin-embedded biofilm samples were remounted under a dissecting microscope for precise orientation.

Focused ion beam scanning electron microscopy (FIB/SEM)

For Focused-Ion-Beam / Scanning Electron Microscopy (FIB/SEM) analysis, biofilm samples were prepared similar to HPF-resin-section TEM samples, but were fixed at room temperature in a solution of 4% paraformaldehyde, 0.1% ruthenium red and 1% osmium in PBS for 30 minutes before being rinsed three times with ddH₂O prior to high pressure freezing. This approach allows for sufficient sample contrast needed for FIB/SEM analysis, while avoiding dehydration artifacts typically encountered in conventionally prepared samples (McDonald & Auer, 2006). Conventional benchtop and microwave-assisted EM sample processing yielded similar results for the presence of vesicle chains, yet resulted in overall lower preservation due to extraction artifacts (data not shown). For FIB/SEM imaging we employed a Zeiss Auriga FIB/SEM workstation equipped with a Cobra Gallium column (probe size <2nm) and a Gemini electron column (probe size < 0.8nm). An energy-selective in-lens high-sensitivity backscattered-electron detector (EsB) was used for image acquisition. Slicing was achieved by using 30kV focused Ga ion beam with a probe current of 240 pA. Images were recorded after each round of ion beam milling using the SEM beam at 1.5 keV with an energy filter bias set at 750eV. Data acquisition occurred in an automated way using the ZEISS SmartSEM software, with an XY pixel size of 3 nm and Z step size of 5 nm, resulting in typical volumes of 10 µm by 8 µm by 15 µm

TEM-Tomography

For tomography 200nm thin biofilm sections were cut with a microtome and imaged in an FEI Tecnai F30 microscope operating at 300 kV (Boulder Laboratory for 3D Electron Microscopy of Cells, University of Colorado). Binned 2k by 2k tilt series were collected on 4k by 4k charge-coupled-device (CCD) Gatan camera (Gatan, Inc., Pleasanton, CA) every 1° from +70° to -70°, using the UCSF tomography or SerialEM software package. The nominal setting of defocus was 2.0 µm and the pixel size of the data corresponded to 1 nm. Series were aligned with the help of either 10-nm or 15-nm gold fiducial markers (BBi Research, Inc., WI), and 3D reconstructed using the IMOD software package. IMOD was also used for data inspection and analysis. Where appropriate, 3D volume data were inspected interactively using the volume and surface rendering tools VOLUME ROVER (Bajaj et al., 2003) and CHIMERA (Pettersen et al., 2004; <http://www.cgl.ucsf.edu/chimera>).

Time-lapse fluorescence microscopy

For time-lapse microscopy, cells from 32°C culture were spotted on a thin fresh 0.5 % agar CYE pad atop of a slide and stained by 20 µg/ml FM4-64 solution (Molecular probes, Inc.), before immediately putting on a cover slip and imaging by epifluorescence microscopy after allowing cells 10 minutes to begin moving again. Time-lapse phase contrast images of moving cells were taken every 90 sec with a fluorescent image taken at the end of series.

Quantification of *E. coli* prey cell lysis

For qualitative determination of prey cell lysis, *M. xanthus* and *E. coli* were cultured as described previously (Berleman *et al.*, 2007). Vesicles were purified from *M. xanthus* cultures that were grown to mid log phase in CYE media, washed and resuspended in liquid CFL media and incubated 24 h at 32 C in 6 well plates. Whole cell and vesicle preps were added to CFL plates in 10 µl aliquots, allowed to dry, and washed. *E. coli* cell cultures were added directly on top at the same volume. For quantitative assays, *E. coli* strain β 2155 was transformed with pGEMT plasmid (Promega) carrying a constitutively expressed mCherry fluorescent protein. 200 µl aliquots of molten CFL agar were pipetted in each well of a Black Corning 96 well Polystyrol plate with a flat, clear bottom and allowed to solidify. 10 µl aliquots of *E. coli* cells were pipetted in each well and 10 µl aliquots of either *M. xanthus* or purified vesicles were pipetted on top of them. Fluorescent measurements were taken 0, 1, 2, 3 and 4 days after plating. Fluorescence levels were measured using a Tecan Infinite M1000 microplate reader. The fluorescence reader followed a filled-circle pattern when measuring each well for a total of 12 measurements per well. An average of the readings across multiple samples was used to determine lytic rate.

Vesicle chain purification

To obtain a cell free sample of vesicle structures, 25 mL batches of 2.0 OD₆₀₀ liquid *M. xanthus* cultures were vortexed for 30 seconds. The vortexed culture was centrifuged for 10 minutes at 5000g to pellet out the whole cells. The supernatant was filtered through 0.45 µm and 0.22 µm syringe filters to remove cell debris. Cell free supernatant was subjected to 140,000g for 1 h, and the resulting pellet resuspended in 1 mL of PBS. Sample aliquots were subjected to electron microscopy analysis to monitor the presence and purity of vesicle fractions.

Vesicle & Vesicle Chain Quantification

Samples were grown in liquid CYE media to an OD₆₀₀ of 0.6 and 1.2 OD or were grown on CYE agar plates for 1 and 3 days. Samples were normalized by wet weight and a total of five independent samples were analyzed. Samples were resuspended in PBS and mixed with FM 4-64 (Molecular Probes, www.invitrogen.com) and using a plate-reader (Tecan, Vission-100, AFAB Lab, LLC, Frederick, MD) relative concentration of vesicle samples was determined.

In-gel Tryptic Digestion and Protein Identification

The purified membrane vesicles were solubilized with SDS lysis buffer and separated on SDS-PAGE gel. Visible bands were cut from the Coomassie stained gel and subjected to

tryptic digestion using a ProGest robot (DigiLab, Inc, www.digilabglobal.com). The resulting tryptic peptides were extracted from the gel with 10% formic acid and peptide mixtures were analyzed by nanoLC-ESI-MSMS utilizing a 2DLC NanoLC system (Eksigent) interfaced with the LTQ XL linear ion trap mass spectrometer (ThermoFisher) equipped with the Advance Captivespray source (Michrom Bioresources). Data was searched with an in-house Mascot v2.2 search engine (Matrix Science) against a custom *M. xanthus* protein sequence database (downloaded from UniProt) with common contaminants appended; a reversed version was concatenated to the database to follow false discovery rate (14935 sequences; 5626671 residues). Peaklists were generated with the Mascot Daemon (Matrix Sciences) using the "ThermoFinnigan LCQ/DECA RAW file" (Thermo Fisher) data import filter; search was limited to doubly- and triply-charged precursors. The following search parameters were utilized: precursor mass tolerance 0.8 Da; fragment mass tolerance 0.8 Da; tryptic digestion; 1 missed cleavage; fixed modification: Cys-carbamidomethyl; variable modifications: deamidation (Asn and Gln), Met-sulfoxide and Pyro-glu (N-term Gln).

Fluorophore-assisted carbohydrate electrophoresis (FACE) for monosaccharide composition analysis

The monosaccharide composition for the vesicle fraction was analyzed using the protocol provided by Glyko, Inc. (Hayward, CA) Briefly, neutral sugar hydrolysis was performed by incubating 50 µl of purified vesicle fraction with 50 µl trifluoroacetic acid (4N) at 100 °C for 5 h. Amine sugars were analyzed using Hydrochloric acid (8N) at 100 °C for 1 h. On hydrolysis, the samples were dried in a centrifugal vacuum evaporator with no heat. The hydrolyzed samples were labeled overnight at 37 °C with Mono labeling dye, dried and resuspended in labeling solvent for subsequent electrophoresis on a polyacrylamide gel. A Mono ladder standard for monosaccharides was run on the gel along with the hydrolyzed samples. The gel was photographed in a GelDoc (Bio-Rad, Hercules, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Bajaj, Chandrajit; Yu, Zeyun; Auer, Manfred. Volumetric Feature Extraction and Visualization of Tomographic Molecular Imaging. *J Struct Biol.* Nov; 2003 144(1-2):132–143. [PubMed: 14643216]
- Berleman, James E.; Kirby, John R. Multicellular Development in *Myxococcus xanthus* Is Stimulated by Predator-prey Interactions. *J Bacteriol.* Aug; 2007 189(15):5675–5682. doi:10.1128/JB.00544-07. [PubMed: 17513469]

- Berleman, James E.; Kirby, John R. Deciphering the Hunting Strategy of a Bacterial Wolfpack. *FEMS Microbiol Rev.* Sep; 2009 33(5):942–957. doi:10.1111/j.1574-6976.2009.00185.x. [PubMed: 19519767]
- Costerton JW, Montanaro L, Arciola CR. Bacterial Communications in Implant Infections: a Target for an Intelligence War. *Int J Artificial Organs.* Sep; 2007 30(9):757–763.
- Davies, DG.; Parsek, MR.; Pearson, JP.; Iglewski, BH.; Costerton, JW.; Greenberg, EP. *Science.* Vol. 280. New York, N.Y.: Apr 10. 1998 The Involvement of Cell-to-cell Signals in the Development of a Bacterial Biofilm.; p. 295-298.
- Dohnalkova, Alice C.; Marshall, Matthew J.; Arey, Bruce W.; Williams, Kenneth H.; Buck, Edgar C.; Fredrickson, James K. Imaging Hydrated Microbial Extracellular Polymers: Comparative Analysis by Electron Microscopy. *Appl Environ Microbiol.* Feb; 2011 77(4):1254–1262. doi:10.1128/AEM.02001-10. [PubMed: 21169451]
- Dubey, Gyanendra P.; Ben-Yehuda, Sigal. Intercellular Nanotubes Mediate Bacterial Communication. *Cell.* Feb 18; 2011 144(4):590–600. doi:10.1016/j.cell.2011.01.015. [PubMed: 21335240]
- El-Naggar, Mohamed Y.; Wanger, Greg; Man Leung, Kar; Yuzvinsky, Thomas D.; Southam, Gordon; Yang, Jun; Ming Lau, Woon; Nealson, Kenneth H.; Gorby, Yuri A. Electrical Transport Along Bacterial Nanowires from *Shewanella Oneidensis* MR-1. *PNAS.* Oct 19; 2010 107(42):18127–18131. doi:10.1073/pnas.1004880107. [PubMed: 20937892]
- Evans, Alun G L.; Marie Davey, Hazel; Cookson, Alan; Currinn, Heather; Cooke-Fox, Gillian; Stanczyk, Paulina J.; Edward Whitworth, David. Predatory Activity of *Myxococcus xanthus* Outer Membrane Vesicles and Properties of Their Hydrolase Cargo. *Microbiology (Reading, England).* Sep 13.2012 doi:10.1099/mic.0.060343-0.
- Gorby Y, McLean J, Korenevsky A, Rosso K, El-Naggar MY, Beveridge TJ. Redox-reactive Membrane Vesicles Produced by *Shewanella*. *Geobiology.* Jun; 2008 6(3):232–241. doi:10.1111/j.1472-4669.2008.00158.x. [PubMed: 18498526]
- Gorby, Yuri A.; Yanina, Svetlana; McLean, Jeffrey S.; Rosso, Kevin M.; Moyles, Dianne; Dohnalkova, Alice; Beveridge, Terry J., et al. Electrically Conductive Bacterial Nanowires Produced by *Shewanella Oneidensis* Strain MR-1 and Other Microorganisms. *PNAS.* Jul 25; 2006 103(30):11358–11363. doi:10.1073/pnas.0604517103. [PubMed: 16849424]
- Helmuth R, Achtman M. Cell-cell Interactions in Conjugating *Escherichia Coli*: Purification of F Pili with Biological Activity. *PNAS.* Mar; 1978 75(3):1237–1241. [PubMed: 349561]
- Heymann, Jurgen A W.; Shi, Dan; Kim, Sang; Bliss, Donald; Milne, Jacqueline L S.; Subramaniam, Sriram. 3D Imaging of Mammalian Cells with Ion-abrasion Scanning Electron Microscopy. *J Struct Biol.* Apr; 2009 166(1):1–7. doi:10.1016/j.jsb.2008.11.005. [PubMed: 19116171]
- Hibbing, Michael E.; Fuqua, Clay; Parsek, Matthew R.; Brook Peterson, S. Bacterial Competition: Surviving and Thriving in the Microbial Jungle. *Nature Reviews. Microbiology.* Jan; 2010 8(1): 15–25. doi:10.1038/nrmicro2259.
- Kahnt, Jörg; Aguiluz, Kryssia; Koch, Jürgen; Treuner-Lange, Anke; Konovalova, Anna; Huntley, Stuart; Hoppert, Michael; Søgaard-Andersen, Lotte; Hedderich, Reiner. Profiling the Outer Membrane Proteome During Growth and Development of the Social Bacterium *Myxococcus xanthus* by Selective Biotinylation and Analyses of Outer Membrane Vesicles. *J Proteome Res.* Oct 1; 2010 9(10):5197–5208. doi:10.1021/pr1004983. [PubMed: 20687614]
- Li, Yinuo; Sun, Hong; Ma, Xiaoyuan; Lu, Ann; Lux, Renate; Zusman, David; Shi, Wenyan. Extracellular Polysaccharides Mediate Pilus Retraction During Social Motility of *Myxococcus xanthus*. *PNAS.* Apr 29; 2003 100(9):5443–5448. doi:10.1073/pnas.0836639100. [PubMed: 12704238]
- Mashburn, Lauren M.; Whiteley, Marvin. Membrane Vesicles Traffic Signals and Facilitate Group Activities in a Prokaryote. *Nature.* Sep 15; 2005 437(7057):422–425. doi:10.1038/nature03925. [PubMed: 16163359]
- Miller KR, Prescott CS, Jacobs TL, Lassignal NL. Artifacts Associated with Quick-freezing and Freeze-drying. *J Ultrastruct Res.* Feb; 1983 82(2):123–133. [PubMed: 6338243]
- Nudleman, Eric; Wall, Daniel; Kaiser, Dale. *Science.* Vol. 309. New York, N.Y.: Jul 1. 2005 Cell-to-cell Transfer of Bacterial Outer Membrane Lipoproteins.; p. 125-127.doi:10.1126/science.1112440

- Palsdottir, Hildur; Remis, Jonathan P.; Schaudinn, Christoph; O'Toole, Eileen; Lux, Renate; Shi, Wenyuan; McDonald, Kent L.; William Costerton, J.; Auer, Manfred. Three-dimensional Macromolecular Organization of Cryofixed *Myxococcus xanthus* Biofilms as Revealed by Electron Microscopic Tomography. *J Bacteriol.* Apr; 2009 191(7):2077–2082. doi:10.1128/JB.01333-08. [PubMed: 19168614]
- Pathak, Darshankumar T.; Wei, Xueming; Bucuvalas, Alex; Haft, Daniel H.; Gerloff, Dietlind L.; Wall, Daniel. Cell Contact-dependent Outer Membrane Exchange in *Myxobacteria*: Genetic Determinants and Mechanism. *PLoS Genetics.* 2012; 8(4):e1002626. doi:10.1371/journal.pgen.1002626. [PubMed: 22511878]
- Pettersen, Eric F.; Goddard, Thomas D.; Huang, Conrad C.; Couch, Gregory S.; Greenblatt, Daniel M.; Meng, Elaine C.; Ferrin, Thomas E. UCSF Chimera--a Visualization System for Exploratory Research and Analysis. *J Comp Chem.* Oct; 2004 25(13):1605–1612. doi:10.1002/jcc.20084. [PubMed: 15264254]
- Reguera, Gemma; McCarthy, Kevin D.; Mehta, Teena; Nicoll, Julie S.; Tuominen, Mark T.; Lovley, Derek R. Extracellular Electron Transfer via Microbial Nanowires. *Nature.* Jun 23; 2005 435(7045):1098–1101. doi:10.1038/nature03661. [PubMed: 15973408]
- Rodriguez AM, Spormann AM. Genetic and Molecular Analysis of *cglB*, a Gene Essential for Single-cell Gliding in *Myxococcus xanthus*. *J Bacteriol.* Jul; 1999 181(14):4381–4390. [PubMed: 10400597]
- Sanchez, Cesar. Cellular Microbiology: Bacterial Networking. *Nature Reviews. Microbiology.* Apr. 2011 9(4):229.
- Shetty, Ameesha; Chen, Shicheng; Tocheva, Elitza I.; Jensen, Grant J.; Hickey, William J. Nanopods: a New Bacterial Structure and Mechanism for Deployment of Outer Membrane Vesicles. *PloS One.* 2011; 6(6):e20725. doi:10.1371/journal.pone.0020725. [PubMed: 21687732]
- Starr CM, Masada RI, Hague C, Skop E, Klock JC. Fluorophore-assisted Carbohydrate Electrophoresis in the Separation, Analysis, and Sequencing of Carbohydrates. *J Chroma. A.* Jan 12; 1996 720(1-2):295–321.
- Wei, Xueming; Pathak, Darshankumar T.; Wall, Daniel. Heterologous Protein Transfer Within Structured *Myxobacteria* Biofilms. *Mol Microbiol.* Jul; 2011 81(2):315–326. doi:10.1111/j.1365-2958.2011.07710.x. [PubMed: 21635581]
- Whitworth, David E. *Myxobacterial Vesicles Death at a Distance?* *Adv Appl Microbiol.* 2011; 75:1–31. doi:10.1016/B978-0-12-387046-9.00001-3. [PubMed: 21807244]

Figure 1. SEM imaging time course of biofilm and fruiting body formation

M. xanthus cells were grown in submerged culture on silicon wafers. Samples were removed and examined with SEM at 0 h (a-c), 24 h (d-f) and 48 h (g-i). Over this time frame, cells are observed to go from small dispersed clumps to large aggregates. Peritrichous membrane appendages are observed on cells at all time points. Scale bars represent 10 μm (d,g), 2 μm (a), 1 μm (b,e,h), 0.5 μm (c,i), and 0.2 μm (f).

Figure 2. 3D FIB/SEM imaging of the biofilm structure of *M. xanthus* reveals the prevalence of intercellular appendages

M. xanthus biofilms were grown at a surface-air interface for 4 days at 32 C, embedded in resin and imaged with the macromolecular resolution of 3D FIB/SEM imaging of an ~300 cubic μm total volume. The FIB/SEM image reveals the abundance of vesicle chains and tubes (a-d). An ~500 nm slice through the reconstructed 3D volume reveals that cells are often linked to neighboring cells through membrane connections (a-b). Such connections can be vesicle chain-like (a) or tubular (b) and these membrane-based structures mediate cell-to-cell connectivity. Surface rendering of the 3D volume (c-d) shows the high frequency of such connections (shown in red) between neighboring cells (shown in yellow) in an ~8 μm thick volume of *M. xanthus* biofilm.

Figure 3. Ultrastructure analysis of vesicle chains

The vesicle chains are seen as long extracellular appendages (a) when living cells are stained with the lipid bilayer dye FM4-64 and visualized by epifluorescence light microscopy. These lipid structures are resolved as vesicle chains/tubes (b and c) when cryofixed and visualized by TEM. Vesicle chains appear to be single membrane structures with regular size and shape (b, white line inset) are distinct from pili (b, dotted line inset) and lack the double membrane structure of the cell (b, solid black line inset). The lumen of the vesicle chains can be seen to be continuous in some cases (c, white line inset). Fast-frozen, artifact-free, preparations (d) show each chain made up of vesicles of similar size, whereas vesicle size can differ between distinct chains. A 1 nm thin slice through a 3D tomogram of a HPF/FS, resin-embedded sample (e) and a negatively stained whole-mount projection image (f) both reveal a continuous lumen inside the constricted vesicle chains. Scale bars represent 5 μm (a), 50 nm (b, c) 100 nm (d, e and f).

Figure 4. Compositional analysis of vesicle fractions

Cell-free vesicle chain preparations were obtained and confirmed by negative staining (a). The purified vesicle fraction contains both vesicle chains and free vesicles. The vesicle fraction was subjected to analysis for total carbohydrate and lipid content. Carbohydrate analysis by Fluorophore Assisted Carbohydrate Electrophoresis (FACE) (b) indicates Mannose and Fucose are abundant neutral sugars in vesicle preparations (Lane 2, controls lane 1) while amine sugars, N-acetylgalactosamine and N-acetylglucosamine (Lane 3) were detected in addition to several unknown amine sugars when vesicle fractions were analyzed for acetylated sugars. Lipid composition was determined by Fatty Acid Methyl Esterification (FAME) analysis (c) indicating that 15- and 17-length carbon chains predominate, and characteristic myxobacteria lipids such as 16:1 w5c were also detected. (d) A vesicle chain with (arrows) hair-like densities covering it are observed when planktonic cells are imaged in thin vitreous ice using whole cell cryo-TEM, these structures likely represent LPS.

Figure 5. Vesicle chains are enriched under biofilm growth conditions

Vesicle chains were purified from planktonic cell cultures or biofilm communities grown on nutrient rich agar plates. After cell mass normalization we quantified FM4-64 intensity to determine membrane structures concentration (a). Aliquots of purified vesicle chains from both planktonic cell cultures (b) and biofilms (c) were examined by whole mount TEM to exclude cell membrane contamination. Negative staining of individual *M. xanthus* cells fresh from culture solution reveals that such cells rarely show vesicle chains (d), whereas cells resuspended from biofilms frequently show many vesicle chains (e). Vesicle chains are frequently observed in *M. xanthus* biofilms as revealed by TEM imaging of ultrathin biofilm sections (f).

Figure 6. Functional analysis of vesicle chains

M. xanthus cells labeled with FM4-64 were added to agar coated slides and time-lapse phase contrast microscopy (a-c) was used to track cell movement. After a total of 180 seconds, cells were imaged by fluorescence microscopy (d) to visualize vesicle chains. Arrows point to two examples of motile cells, showing that the presence or absence of a detectable vesicle chain has no bearing on cell movement. SDS-PAGE separation followed by Mass Spectrometry was used to ID protein content with known cell-cell transferred Tgl and CglB proteins specifically identified at their expected sizes (29 and 45 kD) in the vesicle fraction (e). *M. xanthus* cells and vesicle fractions were mixed with mCherry-labeled *E. coli* in a predation assay to determine if the vesicle fraction mediates lysis of *E. coli* cells (f-i). Quantification of viable *E. coli* by fluorescence, indicates the vesicle fraction has partial lytic activity (“open circles”) relative to *E. coli* only (“closed circles”) and *E. coli* incubated with *M. xanthus* (“X”) (f). *E. coli* lysis was also observed by light microscopy after 24 h incubation of *E. coli* cells on agar plates alone (g) with *M. xanthus* cells (h) and with vesicle fraction (i).

Figure 7. Model for the role of vesicles and vesicle chains in *M. xanthus* biofilms

We propose that cells maintain connections and possibly exchange signals through vesicles and other extracellular appendages. These structures are covered by a carbohydrate, possibly LPS, which may play a role in cell-cell recognition and intercellular material transfer. Free vesicles provide a diffusion free mechanism of signal transmission, while vesicle chains mediate direct intercellular contacts, creating a tightly-knit multicellular community.