Shewanella oneidensis MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components

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Bacterial nanowires offer an extracellular electron transport (EET) pathway for linking the respiratory chain of bacteria to external surfaces, including oxidized metals in the environment and engineered electrodes in renewable energy devices. Despite the global, environmental, and technological consequences of this biotic–abiotic interaction, the composition, physiological relevance, and electron transport mechanisms of bacterial nanowires remain unclear. We report, to our knowledge, the first in vivo observations of the formation and respiratory impact of nanowires in the model metal-reducing microbe Shewanella oneidensis MR-1. Live fluorescence measurements, immunolabeling, and quantitative gene expression analysis point to S. oneidensis MR-1 nanowires as extensions of the outer membrane and periplasm that include the multiheme cytochromes responsible for EET, rather than pilin-based structures as previously thought. These membrane extensions are associated with outer membrane vesicles, structures ubiquitous in Gram-negative bacteria, and are consistent with bacterial nanowires that mediate long-range EET by the previously proposed multistep redox hopping mechanism. Redox-functionalized membrane and vesicular extensions may represent a general microbial strategy for electron transport and energy distribution.

Significance

Bacterial nanowires from Shewanella oneidensis MR-1 were previously shown to be conductive under nonphysiological conditions. Intense debate still surrounds the molecular makeup, identity of the charge carriers, and cellular respiratory impact of bacterial nanowires. In this work, using in vivo fluorescence measurements, immunolabeling, and quantitative gene expression analysis, we demonstrate that S. oneidensis MR-1 nanowires are extensions of the outer membrane and periplasm, rather than pilin-based structures, as previously thought. We also demonstrate that the outer membrane multiheme cytochromes MtrC and OmcA localize to these membrane extensions, directly supporting one of the two models of electron transport through the nanowires; consistent with this, production of bacterial nanowires correlates with an increase in cellular resducate activity.
display them. Intense debate still surrounds the molecular makeup, identity of the charge carriers, and interfacial electron transport mechanisms responsible for the high electron mobility of bacterial nanowires. *Geo bacter* nanowires are thought to be type IV pili, and their conductance is proposed to stem from a metallic-like band transport mechanism resulting from the stacking of aromatic amino acids along the subunit PilA (15). The latter mechanism, however, remains controversial (13, 16). In contrast, the molecular composition of bacterial nanowires from *Shewanella*, the best-characterized facultatively anaerobic metal reducer, has never been reported. *Shewanella* nanowire conductance correlates with the ability to produce outer membrane redox proteins (10), suggesting a multistep redox hopping mechanism for EET (17, 18).

The present study addresses these outstanding fundamental questions by analyzing the composition and respiratory impact of bacterial nanowires in vivo. We report an experimental system allowing real-time monitoring of individual bacterial nanowires from living *Shewanella oneidensis* MR-1 cells and, using fluorescent redox sensors, we demonstrate that the production of these structures correlates with cellular reductase activity. Using a combination of gene expression analysis, live fluorescence measurements, and immunofluorescence imaging, we also find that the *Shewanella* nanowires are membrane- rather than pilin-based, contain multitheme cytochromes, and are associated with outer membrane vesicles. Our data point to a general strategy wherein bacteria extend their outer membrane and periplasmic electron transport components, including multitheme cytochromes, micrometers away from the inner membrane.

**Results and Discussion**

**In Vivo Imaging of Nanowire Formation.** Previous reports demonstrated increased production of bacterial nanowires and associated redox-active membrane vesicles in electron acceptor (O2)-limited *Shewanella* cultures (7, 10, 19). To directly observe this response in vivo, we subjected *Shewanella oneidensis* MR-1 to O2-limited conditions in a microliter-volume laminar perfusion flow imaging platform (Materials and Methods) and monitored the production and growth of extracellular filaments from individual cells with fluorescent microscopy. The cells and attached filamentous appendages were clearly resolved (Fig. 1 and Movies S1–S4) at the surface–solution interface using NanoOrange, a merocyanine dye that undergoes large fluorescence enhancement upon binding to proteins (20, 21). This dye was previously used to label bacterial nanowires recovered from chemostat cultures (7, 22). In all our experiments, the production of filaments coincided with the formation of separate or attached spherical membrane vesicles, another observation consistent with previous electron and atomic force microscopy measurements of *Shewanella* nanowires (19). In Fig. 1A, a leading membrane vesicle can be clearly seen emerging from one cell 20 min after switching to anaerobic flow conditions, followed by a trailing filament. These proteinaceous vesicle-associated filaments were widespread in all of the *S. oneidensis* MR-1 cultures tested; the response was displayed by 65 ± 8% of all cells (statistics obtained by monitoring 6,466 cells from multiple random fields of view in six separate biological replicates). As a representative example, Movie S5 shows an average length of 2.5 μm and reaching up to 9 μm (100 randomly selected filaments from six biological replicates).

The filaments described here were the only extracellular structures observed in our experiments, and possess several features of the conductive bacterial nanowires previously reported in O2-limited chemostat cultures. Specifically, the dimensions of these filaments (10, 23, 24), their association with membrane vesicles (19), and their production during O2 limitation (7) led us to conclude that these structures are the bacterial nanowires whose conductance was previously measured ex situ under dry conditions (10). Additionally, when we labeled cells grown in O2-limited chemostat cultures with the same fluorescent dyes, we observed identical structures with the same composition as the perfusion cultures reported here (see below).

**The Production of *S. oneidensis* MR-1 Bacterial Nanowires Is Correlated with an Increase in Cellular Reductase Activity.** To directly measure the physiological impact bacterial nanowire production has on *S. oneidensis*, we labeled cells with RedoxSensor Green (RSG) in the perfusion imaging platform described above. RSG is a fluorogenic dye that yields green fluorescence upon interaction with bacterial reductases in the cellular electron transport chain, and has been previously demonstrated to be an indicator of active respiration in pure cultures and environmental samples (25–27). Because the redox-sensing ability of RSG was not previously characterized in *Shewanella*, we first confirmed that electron donor (lactate)-activated respiration increases RSG fluorescence in aerobic cultures relative to starved controls (SI Appendix, Fig. S2), and that the addition of specific electron transport inhibitors abolishes RSG fluorescence (SI Appendix, Fig. S3). *S. oneidensis* MR-1 cells displayed...
a significant increase in RSG fluorescence concomitant with nanowire production (Fig. 1 B and C and Movie S6), indicating increased respiratory activity compared with nearby control cells that did not produce nanowires in the same field of view under identical perfusion conditions. DMSO, which is required extracellularly by Shewanella (28), was available as a terminal electron acceptor in all RSG-labeled experiments. However, no fluorescence was detected along nanowires from Shewanella cells (30, 31). FM 4-64 has also been widely used in bacterial cells to study the endocytic pathway, as extensively characterized in eukaryotic cells (32, 33), except in a few bacterial species where flagella are coated in membrane sheetsheds (34). To our surprise, the entire length of the Shewanella nanowires was clearly stained with this reliable lipid bilayer dye (Fig. 1B, red channel, and Fig. 2), indicating that membranes are a substantial component of Shewanella nanowires, contrary to previous suggestions that these structures are pilin based. We stained cells producing both nanowires and membrane vesicles with NanoOrange and FM 4-64FX, demonstrating that proteins and lipid colocalized on these extracellular structures, consistent with being derived from the cell envelope (Fig. 2A). Most known bacterial vesicles are composed primarily of outer membrane and periplasm. To determine whether Shewanella nanowires contain periplasm, we expressed either GFP fused to the charged head that is anchored at the membrane surface (29, 30).

HydA (Pirbadian et al. PNAS Early Edition 2023). We then used qPCR to measure changes in the expression of key genes necessary for type IV pilus assembly: pilA, encoding the type IV major pilin subunit; mshA, encoding the mnnose sensitive hemagglutinin (ms) pilin major subunit; and pilT, encoding type IV minor pilin proteins. Expression of all these pilin genes either remained constant or decreased after electron acceptor limitation, when nanowire production was observed (Fig. 3A). Furthermore, mutants lacking the type IV pilin major subunit (ΔpilA) or both the type IV and ms pilus biogenesis systems (ΔpilM–QΔmmsH–Q) (37) produced bacterial nanowires and displayed an increase in reductase activity in the perfusion imaging platform (Fig. 3B and SI Appendix, Fig. S6)—a response identical to wild-type S. oneidensis MR-1. The chemostat qPCR and perfusion pilus-deletion observations both support the conclusion that Shewanella nanowires are distinct from pili.

Most known bacterial vesicles have previously been observed to be associated with Shewanella nanowires (19) (Fig. 1A). To test the extent of membrane involvement in nanowire formation, we labeled S. oneidensis MR-1 cells with the membrane stain FM 4-64FX. This styryl dye is membrane-selective as a result of a lipophilic tail that inserts into the lipid bilayer and a positively charged head that is anchored at the membrane surface (29, 30). The amphiphihic nature of this molecule hinders it from freely crossing the membrane into the cellular interior except through the endocytic pathway, as extensively characterized in eukaryotic cells (30, 31). FM 4-64 has also been widely used in bacterial cells and shown to specifically label membranes but not extracellular protein filaments such as flagella (32, 33), except in a few bacterial species where flagella are coated in membrane sheetsheds (34). To our surprise, the entire length of the Shewanella nanowires was clearly stained with this reliable lipid bilayer dye (Fig. 1B, red channel, and Fig. 2), indicating that membranes are a substantial component of Shewanella nanowires, contrary to previous suggestions that these structures are pilin based. We stained cells producing both nanowires and membrane vesicles with NanoOrange and FM 4-64FX, demonstrating that proteins and lipid colocalized on these extracellular structures, consistent with being derived from the cell envelope (Fig. 2A). Most known bacterial vesicles are composed primarily of outer membrane and periplasm. To determine whether Shewanella nanowires contain periplasm, we expressed either GFP fused to the charged head that is anchored at the membrane surface (29, 30).

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Why were the membrane and periplasmic components of these structures overlooked in previous studies? One important factor is the difficulty of isolating the appendages and separating them from cells before downstream compositional analysis (e.g., liquid chromatography–tandem mass spectrometry). In fact, membrane and periplasmic proteins were previously identified in a study focused on developing optimal methods for removing the Shewanella filaments, although it was not possible to rule these proteins out as an artifact of the shearing procedure (38). The present in vivo microscopy work circumvents some of the previous artifact problems by fluorescently labeling specific cellular components (protein, lipid, and periplasm) on intact nanowires attached to whole cells. In light of these new results, we revisited the chemostat samples from our previous conductance study, and noted that in those samples, SEM images also revealed vesicular morphologies, non-uniform cross-sections, and diameters >5 nm (larger than pili; SI Appendix, Fig. S7A and the source-drain devices in ref. 10). These suggestive links did not become clear until the present in vivo results. We fixed samples from O2-limited chemostat cultures and labeled them with FM 4-64FX. The nanowires from chemostat cultures contained both protein and membrane (SI Appendix, Fig. S7B), further demonstrating that these structures are the same as those observed in the perfusion cultures in vivo. Though the net patterns of gene expression measured in the planktonic chemostat cultures may differ from the surface-attached perfusion cultures, it is important to stress that identical membrane extension phenotypes were observed in both these experiments where O2 served as the sole electron acceptor in limiting concentrations.

Localization of the Decaheme Cytochromes MtrC and OmcA Along Nanowires. As a metal reducer, Shewanella has evolved an intricate EET pathway to traffic electrons from the inner membrane, through the periplasm, and across the outer membrane to external electron acceptors, including minerals and electrode. This pathway includes the periplasmic decaheme cytochrome MtrA, as well as the outer membrane decaheme cytochromes MtrC and OmcA, which may interface to soluble redox shuttles or, via solvent-exposed hemes, directly to the insoluble terminal acceptors (39). Previous work has shown that S. oneidensis cells lacking mtrC and omcA produce nanowires that are not capable of electron transport (10). In light of the structural finding that Shewanella nanowires are outer membrane and periplasmic...
extensions, we examined whether expression of these periplasmic and outer membrane cytochromes increases during nanowire production. We measured the expression levels of *mtrA*, *mtrC*, *omcA*, and *dmsE*, a periplasmic decaheme cytochrome required for maximal extracellular respiration of DMSO (28), during and after the transition to O2 limitation in chemostat cultures. These EET components had significantly increased expression in response to electron acceptor limitation (Fig. 3 C and SI Appendix, Fig. S8). To determine whether these cytochromes are indeed components of the membrane extensions, we performed immunofluorescence with MtrC- and OmcA-specific antibodies (40) following in vivo observation of the target nanowires in the perfusion imaging platform (Movies S7 and S8). We observed MtrC and OmcA localization at the periphery of the cell, as expected. We also observed clear localization of these cytochromes along the membrane-stained bacterial nanowires (25 of 35 nanowires labeled with anti-MtrC and 19 of 22 nanowires labeled with anti-OmcA), whereas no fluorescence was detected from ΔmtrC/omcA negative control cells or their membrane extensions (none of 22 nanowires labeled with anti-MtrC and none of 20 nanowires labeled with anti-OmcA; Fig. 3 D).

Though the conductance of *Shewanella* nanowires was previously only demonstrated under nonphysiological conditions (10), the data reported here are consistent with membrane extensions that could function as nanowires to mediate EET from live cells. Localization of MtrC and OmcA to these membrane extensions provides the most compelling evidence to date, and directly supports the proposed multistep redox hopping mechanism (13, 17, 18), allowing long-range electron transport along a membrane network of heme cofactors that line *Shewanella* nanowires (Fig. 4). We have shown that *S. oneidensis* nanowires contain periplasm (Fig. 2B); therefore, it is also possible that periplasmic proteins and soluble redox cofactors may contribute to electron transport through these extensions.

**Intermediate Steps in Nanowire Formation.** The extension of outer membrane filaments, and their functionalization with electron transport proteins, may represent a widespread strategy for EET. Virtually all Gram-negative bacteria produce outer membrane vesicles, and can alter the rate of production and composition of those vesicles in response to various stress conditions (19, 41). More recent electron microscopy reports describe membrane vesicle chains and related membrane tubes (also referred to as periplasmic tubules) that form cell–cell connections in the social soil bacterium *Myxococcus xanthus* (42) as well as the...
phototrophic consortium Chlorochromatium aggregatum (43). Consistent with these reports, we observed both a transition from vesicle chains to smoother filaments (Movie S9), as well as nanowires connecting separate Shewanella cells (Movie S4).

To gain a clearer picture of the role of membrane vesicles in Shewanella nanowire formation, we performed atomic force microscopy (AFM) on the same bacterial nanowires after observing their growth with fluorescent imaging under perfusion flow conditions. Such observations were not possible in steady-state chemostat cultures where the nanowire growth is not confined to the surface–solution interface. Perfusion was stopped and the samples were fixed quickly after observing the early signs of nanowire production, allowing us to examine the initial stages of nanowire formation with nanoscale resolution using AFM (Fig. 5). We measured the nanowires to be 10 nm in diameter under dry conditions, consistent with previous observations (10, 23, 24) and roughly corresponding to two lipid bilayers. In addition, we were able to resolve different morphologies corresponding to different stages of nanowire formation (Fig. 5), consistent with the chain-to-filament transition in Movie S9. The morphologies observed ranged from vesicle chains (Fig. S4) to partially smooth filaments incorporating vesicles (Fig. 5B), consistent with SEM imaging in SI Appendix, Fig. S7A), and finally continuous filaments (Fig. 5C). In addition to possibly mediating EET up to micrometers away from the inner membrane, the vesiculation and extension of outer membranes into the quasi one-dimensional morphologies observed here increase the surface area-to-volume ratio of cells. This shape change can present a significant advantage, increasing the likelihood cells will encounter the solid-phase minerals that serve as electron acceptors for respiration.

Given the ubiquity of membrane vesicles and related extensions in Gram-negative bacteria, the localization of electron transport proteins along membrane extensions in a manner consistent with bacterial nanowires that could mediate extracellular electron transport, and the finding of nanowire-based cell–cell connectivity, our results raise the intriguing possibility of redox-functionalized membrane extensions as a general microbial strategy for EET and cell–cell signaling. Our study motivates further experimental and theoretical work to build a detailed understanding of the full biomolecular makeup, electron transport physics, and physiological impact of bacterial nanowires.

Materials and Methods

Cell Growth Conditions. All Shewanella strains were grown aerobically in LB broth from a frozen (~80 °C) stock, at 30 °C, up to an OD600 of 2.4–2.8. The cells from these precultures were pelleted by centrifugation at 4226 × g for 5 min, washed twice, and finally resuspended in a defined medium consisting of 30 mM Pipes, 60 mM sodium DL-lactate as an electron donor, 28 mM NH4Cl, 1.34 mM KCl, 4.35 mM NaH2PO4, 7.5 mM NaOH, 30 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 0.05 mM ferric nitrilotriacetic acid. In addition, vitamins, amino acids, and trace mineral stock solutions were used to supplement the medium as described previously (7). The medium was adjusted to an initial pH of 7.2.

Perfusion Chamber Conditions. Washed cells were slowly injected into the perfusion chamber (d = 250 µm, w = 9,580 µm, l = 7,110 µm, described in detail in SI Appendix) and the flow was stopped to allow the cells to settle on the coverslip with a surface density of ~100 cells in an 83 × 66-µm field of view while being imaged by the inverted microscope. After inoculation, sterile defined medium (100 mL in an inverted 135-mL sealed glass serum bottle) was connected to the perfusion chamber inlet and passed through at a flow rate of 5 ± 1 µL/s that remained constant for 3 h. As previously detailed (7, 19), transition to electron-acceptor limitation was required to promote the production of bacterial nanowires and membrane vesicles; this was accomplished using one of two methods. In the first method, the experiment is started with fully aerobic medium flow before switching to supply bottles containing medium made anaerobic by boiling and purging with 100% N2. This method has the advantage of providing a precise time point for entering acceptor (O2) limitation (Fig. 1A and Movies S1 and S4). In the second method, O2 becomes limited close to the coverslip surface at high cell density, even with aerobic medium, as a result of the laminar flow (no mixing between adjacent layers) and no-slip condition (zero velocity at the surface); this is confirmed with the simple calculation outlined in SI Appendix. Using method 2, nanowires and membrane vesicles were consistently observed after a lag period of 90–120 min from the start of perfusion flow (Movies S2 and S3). We observed higher nanowire production rates and better consistency using this method, possibly because the slower transition exposed the surface-bound cells to a wide range of acceptor concentrations, compared with the abrupt transition in the first method. Both methods resulted in identical membrane extensions as observed using membrane fluorescence (FM 4-64FX), protein fluorescence (NanoOrange), and the accompanying increase in reductase activity (RedoxSensor Green).

Immunofluorescence with MtrC or OmcA Antibody. S. oneidensis MR-1 and OmcA immunofluorescence (SI Appendix, Table S2) were used in the perfusion chamber experiment as described above. As soon as the nanowires were produced and observed through staining by FM 4-64FX, the media flow was stopped and the chamber was opened under sterile medium such that the coverslip remained hydrated. The sample (coverslip with attached cells) was fixed with 4% (vol/vol) formaldehyde solution in PBS for 1 h at room temperature (RT). After rinsing in PBS, the sample was incubated in 0.15% glycine at RT for 5 min to quench free aldehyde groups and reduce background fluorescence. The sample was then transferred to a blocking solution of 1% BSA in PBS for 5 min, and reacted with the diluted polyclonal rabbit-raised MtrC or OmcA-specific primary antibody (40) at RT for 30 min (MtrC Ab: 2.6 µg/mL, OmcA

Fig. 5. Correlated atomic force microscopy (AFM) and live-cell membrane fluorescence of bacterial nanowires. (A–C) Tapping AFM phase images of S. oneidensis MR-1 cells after producing bacterial nanowires in the perfusion flow system. The sample is fixed and air-dried before AFM imaging. (Scale bars: 2 µm.) (Inset) In vivo fluorescence images of the same cells/nanowires at the surface/solution interface in the perfusion platform. The cells and the nanowires are stained by the membrane stain FM 4-64FX. (Scale bars: 1 µm.) The morphologies observed range from vesicle chains (A) to partially smooth filaments incorporating vesicles (B), which is consistent with SEM imaging of chemostat samples (SI Appendix, Fig. S7A), and, finally, continuous filaments (C). See also Movie S9, which captures the transition from a vesicle chain to a continuous bacterial nanowire.

Chemostat Growth and qPCR Analysis of the Transition from Electron-Donor to Electron-Acceptor Limitation. 5. oneidensis MR-1 was grown in chemostat medium (SI Appendix, Table S3) at 30 °C and 20% dissolved oxygen tension using continuous flow bioreactors (BioFlo 110; New Brunswick Scientific) with a dilution rate of 0.05 h−1 and an operating liquid volume of 1 L, as previously described (7). After 48 h of this aerobic growth, a reference sample was taken. The dissolved oxygen tension was then manually dropped to 0% by adjusting the N2/air mixture entering the reactor, and the first sample after electron acceptor limitation (t = 0) was taken at this time. O2 served as the sole terminal electron acceptor throughout the experiment. Samples were subsequently taken at 15-min intervals for 1 h. At each time point, 10 mL of cells was harvested in ice-cold 5% citrate-saturated ethanol in ethanol to prevent further transcription and protect the RNA. Samples were taken from each independent biological replicate (different chemostats). Total RNA was prepared using a hot phenol extraction, as previously described (36). Five micrograms of total RNA from each of the time points described above was used as input for reverse-transcriptase reactions with the SuperScript III First-Strand Synthesis System, as per the manufacturer's protocol (Life Technologies). Subsequent cDNA was then diluted and used as template in qPCR experiments with SYBR Select Master Mix (Life Technologies). Fold change in gene expression relative to the reference sample was calculated by 2−ΔΔCT from at least four reactions of three independent biological replicate samples, using rpoB for normalization. Similar results were obtained using rpoB for normalization. The sequences used for each gene are shown in SI Appendix, Table S4.

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Ab: 1.9 μg/mL, both in 1% BSA/PBS. After rinsing four times in PBS, the sample was incubated with anti-rabbit FITC-conjugated secondary antibody (Thermo Scientific Pierce antibodies, catalog no. 31635; 7.5 μg/mL in 1% BSA/ PBS) at RT for 30 min. Finally, the coverslip was rinsed twice in PBS before immunofluorescence imaging in the green channel. To perform immunofluorescence on the same cells and nanowires observed during live imaging (Movies S7 and S8 and Fig. 3D), we modified the coverslips with surface scratches that acted as fiducial markers.

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Supporting Information

Pirbadian et al. 10.1073/pnas.1410551111

Movie S1. Time-lapse movie of nanowire growth and outer membrane vesicle blebbing by Shewanella oneidensis MR-1 in the perfusion platform. Green fluorescence is the result of protein staining by NanoOrange. In this movie, the cell produced a nanowire as well as an attached outer membrane vesicle. The nanowires grew in the direction of perfusion flow (5 μL/s). The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. (Scale bar: 5 μm.)

Movie S1

Movie S2. Time-lapse movie of nanowire growth by S. oneidensis MR-1 in the perfusion platform. Green fluorescence is the result of protein staining by NanoOrange. In this movie, the cell produced a nanowire that is 6 μm long. The nanowires grew in the direction of perfusion flow (5 μL/s). The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. (Scale bar: 5 μm.)

Movie S2
Movie S3. Time-lapse movie of nanowire growth by *S. oneidensis* MR-1 in the perfusion platform. Green fluorescence is the result of protein staining by NanoOrange. The nanowires grew in the direction of perfusion flow (5 μL/s). In this movie, a cell produced two nanowires, one is 6 μm and the other is 13 μm long. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. (Scale bar: 5 μm.)

Movie S4. Time-lapse movie capturing the connection of two *S. oneidensis* MR-1 cells via a nanowire in the perfusion platform. Green fluorescence is the result of protein staining by NanoOrange. The nanowires grew in the direction of perfusion flow (5 μL/s). The cell on the left produced two nanowires that approached the cell on the right as they grew longer. After some time, the cell–cell connection is broken. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. (Scale bar: 5 μm.)
Movie S5. Time-lapse movie of nanowire growth in a representative population of *S. oneidensis* MR-1 cells in the perfusion platform. Red fluorescence is the result of membrane staining by FM 4-64FX. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. (Scale bar: 10 μm.)

Movie S6. Time-lapse movie of nanowire growth and its impact on the redox activity of a *S. oneidensis* MR-1 cell. The movie shows the combined fluorescence from the membrane stain FM 4-64FX (red) and RedoxSensor Green (RSG; green). The production of a nanowire (membrane extension) can be observed in the red channel, along with a significant increase in the cell's reductase activity observed in the green channel. The interval between two consecutive frames is 5 min, but is reduced to 500 ms in the movie. (Scale bar: 5 μm.)
Movie S7. Time-lapse movie of the growth of the nanowire later observed by MtrC-specific immunofluorescence in Fig. 3D. Red fluorescence is the result of membrane staining by FM 4-64FX. The specific nanowire seen here was immunofluorescently imaged again (Fig. 3D) after reacting with the primary MtrC-specific antibody and FITC-conjugated secondary antibody. This movie shows that the target structure in Fig. 3D was in fact a nanowire that was produced under electron–acceptor-limiting conditions inside the perfusion chamber. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. (Scale bar: 2 μm.)

Movie S7

Movie S8. Time-lapse movie of the growth of the nanowire later observed by OmcA-specific immunofluorescence in Fig. 3D. Red fluorescence is the result of membrane staining by FM 4-64FX. The specific nanowire seen here was immunofluorescently imaged again (Fig. 3D) after reacting with the primary OmcA-specific antibody and FITC-conjugated secondary antibody. This movie shows that the target structure in Fig. 3D was in fact a nanowire that was produced under electron–acceptor-limiting conditions inside the perfusion chamber. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. (Scale bar: 5 μm.)

Movie S8
Movie S9. Time-lapse movie capturing the transition from a vesicle chain to a continuous bacterial nanowire from a *S. oneidensis* MR-1 cell. Red fluorescence is the result of membrane staining by FM 4-64FX. The interval between two consecutive frames is 5 min, but is reduced to 500 ms in the movie. (Scale bar: 5 μm.)

Movie S9

Other Supporting Information Files

SI Appendix (PDF)
Supporting Information for

*Shewanella oneidensis* MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components

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This file includes:

- SI Materials and Methods
- Tables S1 to S4
- Figs. S1 to S8
- Captions for Videos S1 to S9

Other Supporting Information for this manuscript includes the following:

- Videos S1 to S9
SI Materials and Methods

Perfusion chamber. A laminar flow Vacu-Cell incubation chamber (C&L Instruments, model VC-LFR-25) was used in all perfusion and live microscopy experiments. The chamber was constructed from black polyether ether ketone (PEEK, custom order) and modified with an additional port (0.0805 in), besides the inlet and outlet ports, for direct injection of reagents (e.g. stains) into the chamber. An inverted serum bottle was used to supply medium to the perfusion chamber, and a bubble trap (Omnifit, model 006BT) placed upstream of the chamber. The flow of media from the serum bottle was established either by peristaltic pumping or by pressurizing the supply bottle’s headspace with air or N₂, depending on whether aerobic or anaerobic conditions were desired. Using a vacuum line, the perfusion chamber was sealed against a 43 mm × 50 mm No.1 coverslip, forming a small (250 μm deep, 17.4 μL volume) laminar flow rectangular chamber. The entire perfusion set-up was placed on a Nikon Eclipse Ti-E inverted microscope, equipped with a drift correction unit (Nikon Perfect Focus System) for maintaining focus at the coverslip-medium interface during time-lapse imaging.

O₂ limitation in the perfusion chamber. For plane Poiseuille flow between two parallel plates (w = 9,580 μm) separated by distance d (250 μm), the volumetric flow rate (5 μL/s in our experiment) is given by \( Gwd^3/12\mu \), where \( \mu \) is the viscosity, allowing us to estimate \( G \), the pressure gradient in the flow direction \( x \). In addition, the fluid velocity profile between the plates is given by \( Gy(d-y)/(2\mu) \), where \( 0 \leq y \leq d \). Since the cells are located at the coverslip \( (y = 0) \), we assume that the cells interact with the 1 μm-thick layer of media adjacent to the coverslip (no mixing from upper layers) where the average flow velocity \( v \) is 25 μm/s. The O₂ concentration will consequently diminish in the \( x \) direction according to \( C(x) = C_0 - \alpha \times x/v \), where \( C_0 \) is the inlet concentration (~8 mg/L at 100% of air saturation) and \( \alpha \) is the cellular consumption rate which we estimate from previous studies of per cell respiration rates (1) and our surface cell density to be 0.63 mg/L/s. From this calculation, we find that the O₂ concentration drops below 10% of air saturation at \( x = 290 \) μm and reaches 0% at \( x = 320 \) μm. A similar analysis for lactate reveals that the donor concentration only drops to 96.9% of its inlet value (60 mM) at the outlet \( (x = 7,110 \) μm). In other words, the majority of the surface-bound cells are acceptor, rather than donor, limited.

Fluorescence microscopy. To visualize the nanowires/vesicles by fluorescence microscopy, either the protein stain NanoOrange® (Life Technologies) (Fig. 2A) or the membrane stain FM® 4-64FX (Life Technologies) (Fig. 2A) was used. For each experimental run, we added 100 µL of the NanoOrange reagent or 25 µg of FM 4-64FX (dissolved in 200 µL of deionized water) to the autoclaved serum bottle containing the 100 mL of perfusion medium. Both of these fluorescent dyes allowed staining of cells, membrane vesicles, and bacterial nanowires.

Fluorescence imaging of NanoOrange and FM 4-64FX was done in the FITC (Nikon filter set B-2E/C) and TRITC (Nikon filter set G-2E/C) excitation/emission channels, respectively. The exposure time in both cases was 500 ms. The excitation and emission windows for both channels are described in Table S1. NanoOrange fluorescence was visible in both FITC and TRITC, whereas FM 4-64FX was only bright in TRITC.

Since NanoOrange has significant emission in the TRITC channel, we were not able to simultaneously stain structures with FM 4-64FX and NanoOrange. We began with FM 4-64FX in the
medium, and imaged the nanowires produced by either wild-type MR-1 or the pilus-deletion mutant strains (Table S2) in TRITC. We then stopped the medium flow, manually injected 10 mL of NanoOrange solution (20 µL NanoOrange reagent diluted in cell medium), and imaged the same structures again in the FITC channel.

**RedoxSensor Green assay.** RedoxSensor Green (from BacLight™ RedoxSensor™ Green Vi- tality Kit, Life Technologies, catalog # B34954) was used to assess cellular respiration activity. Because NanoOrange and RedoxSensor Green (RSG) reagents both fluoresced in the same channel (FITC), we could not use NanoOrange and RSG simultaneously in the same experiment. Instead, to test the impact of nanowire production on cellular reductase activity, we used FM 4-64FX and RSG to simultaneously visualize the nanowires and track the reductase activity, respectively. In these experiments, 0.3 µM RSG (30 µL of 1 mM RSG reagent in 100% DMSO) was added to the 100 mL of flow medium in the supply bottle, along with FM 4-64FX.

To measure the intensity of RSG in cells, brightness and contrast of images were linearly adjusted, equally for all cells, to capture the entire range of RSG intensity in all cells. The images were then exported to MATLAB where the average pixel intensity (API, arbitrary units) of every cell was extracted by manual selection of the cell periphery and calculating the API of the pixels inside the selected periphery.

**Verification of RedoxSensor Green as a probe for active respiration in Shewanella.** The redox sensing functionality of RedoxSensor Green (RSG) was verified in Shewanella by observing fluorescence change in response to substrate activation (Fig. S2), as previously demonstrated for environmental samples (2). *S. oneidensis* MR-1 cells were taken from LB culture in mid-log phase (OD$_{600}$ = 1.5), washed twice and starved by incubation in defined medium without lactate or any other electron donor, for 19 hours at 30°C and 150 rpm. Following starvation, the cells were split into two subcultures. The first subculture was activated with 20 mM lactate, while the second culture remained starved. Both subcultures were sampled at multiple time points (1, 4, 5.5, and 7 hours after starvation) and imaged with the same microscope settings (fluorescence channel, exposure time and intensity) following incubation with 1 µM RSG reagent at 30°C for 40 min. Six randomly selected fields of view were imaged and analyzed at each time point.

Fluorescence intensity of cells was analyzed by image processing functions in MATLAB. The positions and exact periphery of cells were located using reflection mode (without fluorescence) and used to create a mask. The mask from the reflection image was then fit on top of the RSG fluorescence image to remove pixels lying outside the cells. The average pixel intensity (API) of the masked fluorescence image was calculated and averaged over the six fields of view, for both starved and activated samples at each time point.

The effect of three electron transport chain (ETC) inhibitors on RSG fluorescence was monitored to further confirm and localize the redox sensing functionality of RSG in *Shewanella*, as previously reported for other bacteria (3). Rotenone, antimycin A, and sodium azide were selected because they affect respiration by inhibiting different ETC components. Mid-log *S. oneidensis* MR-1 cells (OD$_{600}$ = 1.5) from LB cultures were washed twice and incubated in the defined medium with 1 mM rotenone, 20 µM antimycin A, 10 mM sodium azide, or no inhibitor (control) for 15 min at 30 °C. The RSG reagent (1µM) was added to each sample and incubated for 30 min at 30 °C. Cells from all samples were placed on a coverslip and imaged by fluorescence microscopy (Fig. S3) using the same settings (fluorescence channel, exposure time and intensity).

**Cytoplasmic and periplasmic green fluorescent protein (GFP) imaging.** To express GFP in the cytoplasm, *S. oneidensis* MR-1 was transformed with plasmid p519ngfp (4, 5). The resulting
strain was grown in LB augmented with 50 µg/mL kanamycin ahead of use in the perfusion flow imaging experiment as described above for the *S. oneidensis* MR-1. No kanamycin was added to the washing or perfusion medium.

To localize GFP to the periplasm, *S. oneidensis* MR-1 was transformed with plasmid pHGE-\(Ptac\)TorAGFP (Table S2) as described previously (6). pHGE-\(Ptac\)TorAGFP is an IPTG-inducible plasmid expressing GFP fused to the twin-arginine translocation (Tat) signal peptide from *E. coli* TorA. Fusion to the Tat signal peptide enables GFP to be exported to the periplasm (6). This *S. oneidensis* periGFP strain was grown in LB with 50 µg/mL kanamycin, and 0.1 mM IPTG was added to induce the expression of TorA\(sp\)-GFP at OD\(_{600}\) = 0.4 ahead of use in the perfusion imaging experiment as described above. Since the Tat system exports fully folded proteins from the cytoplasm, the cellular fluorescence pattern of this *S. oneidensis* periGFP strain is not necessarily limited to the periplasm only, and can change over time depending on the induction, export rate, or post-export cleavage of the signal peptide (6). Therefore, to assess the successful periplasmic localization of GFP prior to use in the perfusion experiments (Fig. 2B), we imaged this strain on 3% agar on a slide (no nutrients or further IPTG induction) which resulted in the characteristic peripheral fluorescence pattern consistent with periplasmic localization (Fig. S4).

**Atomic force microscopy following live perfusion flow imaging.** Following in vivo imaging of vesiculation and nanowire growth under perfusion flow conditions, samples were imaged by atomic force microscopy (AFM) to correlate fluorescence and nanoscale topography data (Fig. 5). Each sample (coverslip and attached cells) was fixed in 2.5% glutaraldehyde overnight at 4 °C. The sample was rinsed four times in deionized water and left to air dry. The coverslip was then cut and prepared for AFM imaging. The AFM imaging was performed using a tapping mode tip (Asylum Research, silicon probe model AC240TS with 2 N/m nominal spring constant) on a Veeco Innova instrument. Scratch marks previously placed on the coverslips acted as fiducial markers, allowing us to perform AFM on the same cells and nanowires observed during live cell growth.

**Strains and plasmids generated for this study.** \(\Delta pilA\): To generate a chromosomal deletion of *pilA*, 1 kb DNA fragments that flank this gene were amplified using the primers pilAF (5'-GCATTGGCATGTGATGAT), pilAR (5'-ATGTAAGCCTGTGGTGGGCATTTTCTCGCTCCAATACAG), pilBF (5'-CCCACCACAGGCTTACAT), and pilBR (5'-TTCGCCCACCATTACCCAG). The 1 kb fragments were used as template to amplify a 2 kb fragment using cross-over PCR. The resulting 2 kb DNA that lacks *pilA* was cloned into pER21 and used to generate chromosomal deletion mutant as described previously (7).

pHydA-YFP: pProbeNT-YFP was constructed by replacing the EcoRI-NsiI fragment encoding GFP in pProbeNT (8), with the EcoRI-NsiI fragment encoding YFP from pEYFP-C1 (Clonotech laboratories). A PCR product encoding hydA gene (SO_3920) and its upstream promoter was generated by amplification of *Shewanella oneidensis* MR-1 genomic DNA with primers 5'-gggcTCTAGATCGACCCGATAATCT-3' and 5'-gggcGAGCTCGAGCCGATGGAAGGC-3'. The PCR product was digested with SstI and \(XbaI\) (sites underlined in primer sequence) and then cloned into same sites of pProbeNT-YFP, yielding pHydA-YFP. *S. oneidensis* HydA-YFP, was grown in LB augmented with 50 µg/mL kanamycin ahead of use in the perfusion flow imaging experiment as described above for the wild-type. No kanamycin was added to the washing or perfusion medium.
Table S1. Wavelength windows of the fluorescence channels used in the perfusion chamber experiments.

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<th>Filter set name</th>
<th>Excitation Wavelengths</th>
<th>Filter Wavelengths</th>
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<td>505 nm (longpass, LP)</td>
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Table S2. Strains and plasmids used in this study.

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<th>Description</th>
<th>Source</th>
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<td>MR-1</td>
<td><em>Shewanella oneidensis</em>, wild-type</td>
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<tr>
<td>ΔmtrC/omcA</td>
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<td>MR-1 ΔpilA</td>
<td>This work</td>
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<tr>
<td>ΔpilM-Q/mshH-Q</td>
<td>MR-1 ΔpilMNOPQ, ΔmshHIJKLMNOPQ</td>
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<table>
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<th>Plasmid</th>
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<td>pHGE-PtacTorAGFP</td>
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<td>(6)</td>
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<tr>
<td>p519ngfp</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, Plac/Pnpt-2, mob&lt;sup&gt;+&lt;/sup&gt;, GFP (cytoplasmic GFP)</td>
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<tr>
<td>pPROBE-NT</td>
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<td>(8)</td>
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<tr>
<td>pHydA-YFP</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, gfp replaced with hydA-yfp in pPROBE-NT</td>
<td>This work</td>
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Table S3. Chemostat medium.

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<td>CaCl₂ (after autoclaving)</td>
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<td>Ferric nitritoltriacetic acid (after autoclaving)</td>
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* (10)
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References:


Fig. S1 Distribution of *Shewanella oneidensis* MR-1 nanowire length. Histogram of nanowire length (100 randomly selected nanowires from 6 separate biological replicates). The average is 2.5 μm.
Fig. S2 Verification of RedoxSensor Green functionality by comparing starved and electron-donor-activated *Shewanella oneidensis* MR-1 cells. A starved culture was divided into two subcultures, one of which remained starved while the other was activated with lactate as an electron donor. (A) Quantitative bar plot of fluorescence intensity of RSG in cells from subculture with electron donor (gray) and without electron donor (red) at various time points. Control refers to the initial sample that was starved for 19 hours. For each time point, images were collected from six random fields of view. The bar plot shows the average fluorescence ±SEM. (B) Representative reflection (left) and RSG fluorescence (right) images of cells from the control sample that was starved for 19 hours (top panel) and the subculture with added lactate after 7 hours of activation with lactate (bottom panel). The lactate-activated cells are all visible with fluorescence, in contrast to the starved cells not visible with fluorescence.
**Fig. S3 The effect of electron transport chain inhibitors on RedoxSensor Green fluorescence.** *Shewanella oneidensis* MR-1 cells were incubated with no ETC inhibitor (control), 1 mM rotenone, 20 µM antimycin A, or 10 mM sodium azide. Following staining with RedoxSensor Green (RSG), both reflection and RSG fluorescence images were collected. The top panel shows the control sample without any ETC inhibitor. The second panel from the top shows cells inhibited by rotenone, with cells significantly darker than the control sample. The third panel, from the sample inhibited by antimycin A, also shows cells darker than the control. These images indicate that RedoxSensor Green is capable of detecting the drop in respiration activity caused by rotenone and antimycin A inhibiting the ETC. However, cells inhibited by sodium azide are as bright as the control cells, indicating that RSG interacts with the ETC at a point downstream of antimycin A’s site of inhibition (cyt bc1 complex), but upstream of azide’s site of inhibition (cyt c oxidase).
Fig. S4 Cells containing periplasmic and cytoplasmic Green Fluorescent Protein (GFP). (A) Fluorescence image of *Shewanella oneidensis* MR-1 constitutively expressing GFP with no signal sequence for export, resulting in a uniform cellular fluorescence pattern throughout the cytoplasm. (B) *Shewanella oneidensis* MR-1 expressing GFP fused with the twin-arginine translocation (Tat) signal peptide from the *E. coli* TorA. Cells were imaged on an agar pad following LB growth and IPTG induction. Due to the presence of the signal peptide, the fusion protein is exported to the periplasm, resulting in fluorescence limited primarily to the periphery of the cell. Scale bars are 5 µm.
Fig. S5 Localization of the periplasmic [Fe-Fe] hydrogenase large subunit HydA along bacterial nanowires. Images are of *Shewanella oneidensis* MR-1 cells expressing HydA fused to yellow fluorescent protein (HydA-YFP) under the control of the native *hydA* promoter. The perfusion flow imaging platform was used to monitor bacterial nanowire production (arrows). The red (left) and green (right) channels show fluorescence from the membrane stain FM 4-64FX and YFP, respectively. Brightness/contrast adjustment and bilinear interpolation was applied equally to the entire image on the right to highlight the HydA-YFP fluorescence. Scale bars are 5µm.
Fig. S6 Nanowire production by ΔpilM-Q/mshH-Q and its impact on cell respiration indicated by RedoxSensor Green fluorescence. Images show combined fluorescence from membrane stain FM 4-64FX (red) and RedoxSensor Green (green) before (top) and after (bottom) nanowire production. The production of extracellular structures was correlated with a sudden increase in redox sensing fluorescence, similar to the wild-type strain. Scale bars are 5µm.
Fig. S7 O$_2$ limited chemostat cultures contain membranous and vesicle-associated filaments. (A) Scanning electron microscopy of *Shewanella oneidensis* MR-1 from an O$_2$-limited chemostat which was previously used for nanowire conductance measurements (1). Scale bar is 500 nm. (B) Fluorescence image of an O$_2$-limited chemostat sample stained by the membrane stain FM 4-64FX. A representative membranous filament is observed connecting two MR-1 cells. Brightness/contrast adjustment and bilinear interpolation was applied equally to the entire inset image to highlight the filament. Scale bar is 2 μm.
Fig. S8 Profiling of dmsE expression during transition from electron donor to electron acceptor limitation. Shewanella oneidensis MR-1 cells were grown under aerobic conditions (dissolved oxygen tension of 20%) at 30°C in a chemostat for 48 hours before the dissolved oxygen tension was reduced to 0% (t = 0). Samples were taken from the chemostat right before reducing the DO tension (t < 0, reference sample), at t = 0 and in 15-minute intervals for an hour. Following RNA purification and qPCR, the fold change in gene expression relative to the reference sample was calculated by $2^{-\Delta\Delta CT}$ from at least four reactions of three independent cultures, using recA for normalization.
Video S1. Time-lapse movie of nanowire growth and outer membrane vesicle blebbing by *Shewanella oneidensis* MR-1 in the perfusion platform. Green fluorescence is the result of protein staining by NanoOrange. In this movie, the cell produced a nanowire as well as an attached outer membrane vesicle. The nanowires grew in the direction of perfusion flow (5 μL/s). The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. Scale bar is 5 μm.

Video S2. Time-lapse movie of nanowire growth by *Shewanella oneidensis* MR-1 in the perfusion platform. Green fluorescence is the result of protein staining by NanoOrange. In this movie, the cell produced a nanowire that is 6 μm long. The nanowires grew in the direction of perfusion flow (5 μL/s). The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. Scale bar is 5 μm.

Video S3. Time-lapse movie of nanowire growth by *Shewanella oneidensis* MR-1 in the perfusion platform. Green fluorescence is the result of protein staining by NanoOrange. The nanowires grew in the direction of perfusion flow (5 μL/s). In this movie, a cell produced two nanowires, one is 6 μm and the other is 13 μm long. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. Scale bar is 5 μm.

Video S4. Time-lapse movie capturing the connection of two *Shewanella oneidensis* MR-1 cells via a nanowire in the perfusion platform. Green fluorescence is the result of protein staining by NanoOrange. The cell on the left produced two nanowires that approached the cell on the right as they grew longer. After some time, the cell-cell connection is broken. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. Scale bar is 5 μm.

Video S5. Time-lapse movie of nanowire growth in a representative population of *Shewanella oneidensis* MR-1 cells in the perfusion platform. Red fluorescence is the result of membrane staining by FM 4-64FX. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. Scale bar is 10 μm.

Video S6. Time-lapse movie of nanowire growth and its impact on the redox activity of a *Shewanella oneidensis* MR-1 cell. The movie shows the combined fluorescence from the membrane stain FM 4-64FX (red) and RedoxSensor Green (RSG, green). The production of a nanowire (membrane extension) can be observed in the red channel, along with a significant increase in the cell’s reductase activity observed in the green channel. The interval between two consecutive frames is 5 min, but is reduced to 500 ms in the movie. Scale bar is 5 μm.

Video S7. Time-lapse movie of the growth of the nanowire later observed by MtrC-specific immunofluorescence in Fig. 3D. Red fluorescence is the result of membrane staining by FM 4-64FX. The specific nanowire seen here was immunofluorescently imaged again (Fig. 3D) after reacting with the primary MtrC-specific antibody and FITC-conjugated secondary antibody. This movie shows that the target structure in Fig. 3D was in fact a nanowire that was produced under electron-acceptor-limiting conditions inside the perfusion chamber. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. Scale bar is 2μm.
Video S8. Time-lapse movie of the growth of the nanowire later observed by OmcA-specific immunofluorescence in Fig. 3D. Red fluorescence is the result of membrane staining by FM 4-64FX. The specific nanowire seen here was immunofluorescently imaged again (Fig. 3D) after reacting with the primary OmcA-specific antibody and FITC-conjugated secondary antibody. This movie shows that the target structure in Fig. 3D was in fact a nanowire that was produced under electron-acceptor-limiting conditions inside the perfusion chamber. The interval between two consecutive frames is 5 min, but is reduced to 200 ms in the movie. Scale bar is 5 µm.

Video S9. Time-lapse movie capturing the transition from a vesicle chain to a continuous bacterial nanowire from a *Shewanella oneidensis* MR-1 cell. Red fluorescence is the result of membrane staining by FM 4-64FX. The interval between two consecutive frames is 5 min, but is reduced to 500 ms in the movie. Scale bar is 5 µm.