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Targeted Protein Degradation of Outer Membrane Decaheme Cytochrome MtrC Metal Reductase in *Shewanella oneidensis* MR-1 Measured Using Biarsenical Probe CrAsH-EDT$_2$

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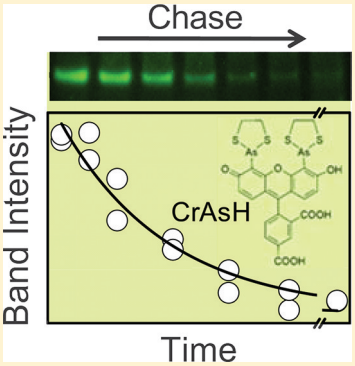
Supporting Information

ABSTRACT: Development of efficient microbial biofuel cells requires an ability to exploit interfacial electron transfer reactions to external electron acceptors, such as metal oxides; such reactions occur in the facultative anaerobic Gram-negative bacterium *Shewanella oneidensis* MR-1 through the catalytic activity of the outer membrane decaheme c-type cytochrome MtrC. Central to the utility of this pathway to synthetic biology is an understanding of cellular mechanisms that maintain optimal MtrC function, cellular localization, and renewal by degradation and resynthesis. In order to monitor trafficking to the outer membrane, and the environmental sensitivity of MtrC, we have engineered a tetracysteine tag (i.e., CCPGCC) at its C-terminus that permits labeling by the cell impermeable biarsenical fluorophore carboxy-FlAsH (CrAsH) of MtrC at the surface of living *Shewanella oneidensis* MR-1 cells. In comparison, the cell permeable reagent FlAsH permits labeling of the entire population of MtrC, including proteolytic fragments resulting from incorrect maturation. We demonstrate specific labeling by CrAsH of engineered MtrC (MtrC*) which is dependent on the presence of a functional type 2 secretion system (T2S), as evidenced by T2S system *gspD* or *gspG* deletion mutants which are incapable of CrAsH labeling. Under these latter conditions, MtrC* undergoes proteolytic degradation to form a large 35−38 kDa fragment; this degradation product is also resolved during normal turnover of the CrAsH-labeled MtrC protein. No MtrC protein is released into the medium during turnover, suggesting the presence of cellular turnover systems involving MtrC reuptake and degradation. The mature MtrC localized on the outer membrane is a long-lived protein, with a turnover rate of 0.043 h^{-1} that is insensitive to O_2 concentration. Maturation of MtrC is relatively inefficient, with substantial rates of turnover of the immature protein prior to export to the outer membrane (i.e., 0.028 h^{-1}) that are consistent with the inherent complexity associated with correct heme insertion and acylation of MtrC that occurs in the periplasm prior to its targeting to the outer membrane. These latter results suggest that MtrC protein trafficking to the outer membrane and its subsequent degradation are tightly regulated, which is consistent with cellular processing pathways that target MtrC to extracellular structures and their possible role in promoting electron transfer from *Shewanella* to extracellular acceptors.



As a facultative anaerobe, *Shewanella oneidensis* is noted for its remarkably versatile respiratory metabolism that enables it to use O_2, or in its absence, an extensive array of terminal electron acceptors that include nitrate, fumarate, trimethylamine *N*-oxide, dimethyl sulfoxide, thiosulfate, and both soluble and insoluble forms of Fe(III) and Mn(III/IV).[1] This metabolic flexibility of *Shewanella* appears to rely on a highly branched electron transport network that provides different terminal electron acceptors under different growth conditions.[2] In particular, the ability to reduce extracellular metals enables *Shewanella* its key role in biogeochemical cycling of Fe and Mn.[3] Metal reduction in *Shewanella* involves efficient movement of electrons from their source of generation at the cytoplasmic membrane through several electron transfer proteins (i.e., CymA and MtrA) to the extracellular surface of the outer membrane where the *c*-type decaheme cytochromes, OmcA (SO1779) and MtrC (SO1778), mediate the reduction of Fe^{3+} or Mn^{4+}.[4−8] An X-ray structure of a homologue of these decaheme cytochromes has recently been solved,[9] which demonstrates an interdomain construction that suggests electron transfer can occur between domain elements. Such a mechanism is consistent with dynamic structural measurements that detect enhanced domain movements linked to the binding of MtrC with solid metal oxides linked to electron transfer function.[10]

Decaheme outer membrane cytochromes are relatively unique features in *Shewanella* as compared with other Gram-negative bacteria in which terminal reductases are localized exclusively to the cytoplasmic inner membrane or periplasm. As lipoproteins, OmcA and MtrC are anchored by single acyl chains to the outer membrane to form a high-affinity and stable (2:1) heterotrimeric protein complex, in which MtrC acts as the centerpiece, assisting in the correct localization of OmcA to the outer membrane, where this complex can directly bind and

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reduce insoluble metals, e.g., hematite.[1,8,10,11] While the individual OmcA and MtrC proteins can be readily separated following detergent solubilization and anion exchange chromatography,[7,8,12] they retain high affinity following co-reconstitution irrespective of the tagging sequence[8] which is consistent with the facile cross-linking of these proteins in native membranes.[13,14] OmcA and MtrC exhibit functional redundancies by retaining independent metal reducing activities in deletion mutants where only one of these cytochromes is present; however, together, OmcA and MtrC exhibit synergism in their activities.

The cellular pathways that target these cytochromes from their site of synthesis in the cytoplasm through the periplasm for maturation to their ultimate destination at the cell surface involve the Type II secretory (T2S) system, as evidenced by experiments with T2S deletion mutants.[15,16] Correct maturation and assembly of OmcA and MtrC on the outer membrane surface are required for their ability to catalyze interfacial electron transfer to solid metal oxides[10,17,18] as well as the electrical conductivity properties of extracellular polymeric substances (EPS) and/or nanowires.[19,20] Extracellular electron transfer, including to electrode surfaces in microbial fuel cells, requires the presence of functional outer membrane cytochromes.[21] Our goal is to define the role of the decaheme cytochromes in the metabolic switching between different terminal electron acceptors under high and low O_2 tensions that alter the cellular content of MtrC, with the long-term goal of understanding the stability and targeting mechanisms important to synthetic biology applications involving, for example, chemical sensing through engineered electrical conductivity between living cells and electronic detectors as well as the development of biofuel cells.[10,21,22]

Measurements of the environmental stability of MtrC require an ability to differentiate immature protein (estimated to represent as much as 40% of total protein)[15,23] from mature protein following secretion and assembly on the outer membrane of *Shewanella*. To accomplish this, we have synthesized and deployed complementary fluorescent probes that differ in membrane permeability to selectively bind a tetracysteine binding sequence engineered at the C-termini of MtrC (Figure 1). Using the highly charged carboxy-FlAsH (CrAsH), we are able to selectively label mature MtrC localized exclusively on the outer membrane, while the cell permeable FlAsH targets the entire population of MtrC for labeling. Antibodies against MtrC protein or a V5 tag located near the C-terminus provide complementary measurements of changes in MtrC abundance, which include immature forms of MtrC localized in the periplasm where heme insertion and acylation occur prior to translocation of the mature protein across the outer membrane for assembly in the metal reductase supramolecular complex.[24] We find that upon induction of MtrC expression that: i) there is a modest (35% to 85%) increase in abundance and ii) that turnover of MtrC is very slow irrespective of dissolved O_2 tension. A substantial fraction of newly synthesized MtrC is degraded prior to maturation (0.028 h^{-1}). Following assembly with the Mtr electron transfer complex in the outer membrane, turnover involves targeted reuptake and proteolysis (0.043 h^{-1}) irrespective of O_2 concentrations. The inherent stability of MtrC to environmental change coupled with the targeted degradation by as yet unidentified cellular machinery suggests that the localization of MtrC with extracellular polymeric substances involves an active targeting and cellular regulation that is consistent with the utility of the Mtr complex in bioenergy and biosensor applications.[19−22]

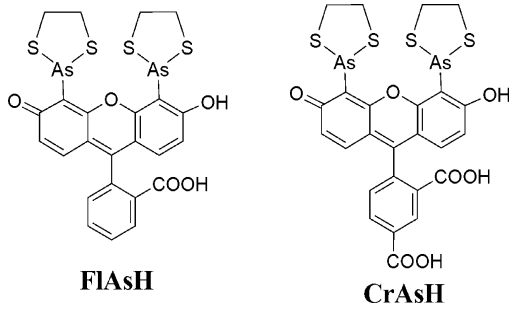

Figure 1. Structures of biarsenic fluorophores, CrAsH-EDT_2 and FlAsH-EDT_2. FlAsH-EDT_2 represents a fluorescein scaffold derivatized with two As(III) groups (4′,5′-bis(1,3,2-dithioarsolan-2-yl)-fluorescein); each arsenic is capped with an ethanedithiol (EDT). Similarly, CrAsH-EDT_2 represents 6-carboxy-FlAsH-EDT_2.

MATERIALS AND METHODS

Reagents and *Shewanella* Mutant Strains. Carboxy-FlAsH (i.e., CrAsH) was synthesized as previously described.[25,26] *Shewanella oneidensis* MR-1 mutants were developed that express the gene encoding MtrC (SO1778) or, alternatively, CFP (cyanofluorescent protein) with an appended C-terminal 52-amino acid sequence consisting of (in the following order) a tetracysteine tag (AREACCPGCCK) adjacent to a V5 epitope (KGGRADPAFLYKVVINSKLEGK-PIPNPLLGL) and a His_6 sequence as previously described.[8] This genetic construct was cloned into the pBAD202/D-TOPO (Invitrogen) expression system which contains a promoter of the *araBAD* (arabinose) operon, which allows control of protein expression through its responsiveness to arabinose (up-regulation) and glucose (down-regulation). Plasmids were transformed into either wild-type *S. oneidensis* MR-1 or knockout mutants lacking genes encoding wild-type MtrC ($MtrC_{wt}$), GspD, or GspG, as previously described.[15,18,27] In addition, wild-type *S. oneidensis* was transformed with the pBAD plasmid that lacks insertion of any exogenous gene (empty vector). The pBAD202/D TOPO vectors were selected using kanamycin (30 $\mu g\ mL^{-1}$). Expressed recombinant MtrC protein is referred to as MtrC*.

FlAsH Labeling of Tetracysteine-Tagged Calmodulin. A calmodulin mutant containing the tetracysteine motif in helix A, where E6, E7, A10, and Ell were all mutated to cysteines as described previously, was labeled with 1.0 μM FlAsH-EDT_2, 50 mM HEPES (pH 7.5), 140 NaCl, 1 mM β-mercaptoethanol, and 1 mM TCEP for up to 2 h.[28] FlAsH-EDT_2 binding was monitored from the resulting fluorescence enhancement, with excitation at 500 nm and emission at 530 nm.

FLAsH Labeling of Intracellular CFP-C4. *S. oneidensis* MR-1 expressing CFP with an engineered tetracysteine tag (i.e., CFP-C4) was cultured in LB medium at 30 °C until the optical density reached 0.8 before addition of 1 mM arabinose with FlAsH-EDT_2 or CrAsH-EDT_2, 0.2 mg/mL protamine, 20 μM Disperse Blue 3 for 2 h. The cells were centrifuged, washed with 20 mM HEPES (pH 7.5), and 150 mM NaCl buffer and were either resuspended in 20 mM HEPES (pH 7.5) for fluorescence spectral measurements or lysed for electrophoresis. Cell lysis involved incubation at 37 °C for 2 h with 5 mg/mL (chicken egg white) lysozyme in 50 mM HEPES (pH

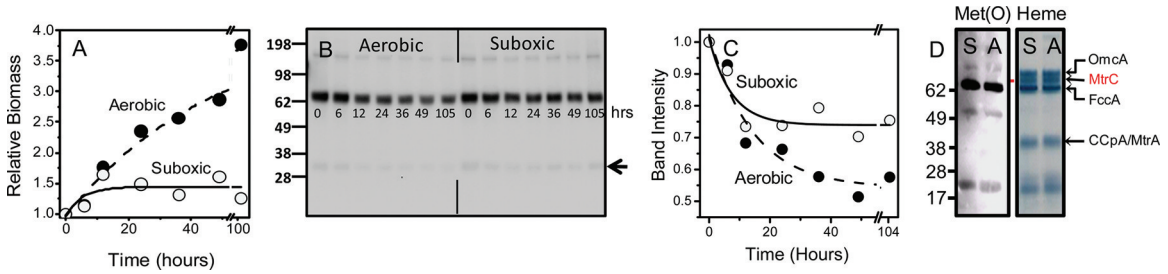

Figure 2. Turnover rates of MtrC are slow compared to growth rate. Relative biomass (total mg of protein) of *Shewanella oneidensis* MR1 (panel A), following 2 h induction of MtrC* expression using arabinose (1 mM) prior to growth under aerobic (●) or suboxic (○) conditions, as defined in Materials and Methods. Immunoblots using antibodies directed against wild-type MtrC (panel B) and associated densitometric changes in MtrC abundance (panel C) following cell lysis and electrophoretic protein separation at indicated times. Arrow in panel B denotes 30−35 kDa immunoreactive fragment of MtrC detected by antibodies. Lines represent nonlinear least-squares fits to an exponential decay, where rates are 14 ± 5 h (aerobic) and 7 ± 4 h (suboxic), with respective abundances of MtrC after 4 days corresponding to 54 ± 4% and 74 ± 4% of maximal abundance observed following induction. Immunoblot against methionine sulfoxide [Met(O)] oxidative damage or measured heme content (panel D) following 12 h of growth under aerobic (A) or suboxic (S) conditions. Abundant heme proteins include previously identified OmcA (78.6 kDa), MtrC (71.2 kDa), and FccA (62.4 kDa) and CCpA/MtrA;[23] red line indicates electrophoretic migration of MtrC identified using authentic standards. FccA comigrates with strong band of methionine sulfoxide staining. In all cases 10 μg of lysate proteins were applied to each lane of the 12% Bis/Tris (Invitrogen) electrophoresis gel; when appropriate, after electrophoresis the gel was cut in half for either immunoblotting or heme staining. Positions of molecular mass markers are indicated.

7.5), 150 mM NaCl, 5% Triton X-100, removing unbroken cells and cell debris by centrifugation at 16000*g* at 4 °C for 5 min. Protein concentrations were assayed with the Bradford assay using bovine serum albumin as the standard.

CrAsH Labeling of Cell Surface MtrC*. *S. oneidensis* was grown aerobically in LB medium at 30 °C until the optical density reached 1.0 before adding 1 mM arabinose to induce protein expression. After another 2 h incubation, 10 μM CrAsH-EDT_2, 2 mM tris (2-carboxyethyl)phosphine (TCEP), and 5 mM β-mercaptoethanol (β-ME) were added. Cells were further incubated under these conditions for up to 2 h. Subsequently, cells were collected by low-speed centrifugation (6000*g* for 10 min) prior to washing in 50 mM HEPES (pH 7.5), 150 mM NaCl.

Electrophoresis and Immunoblotting. Electrophoretic separation of proteins utilized 4−12% Bis/Tris gels (Invitrogen). Fluorescence images of the gels were obtained with a Kodak Imager with the excitation filter set at 465 nm and emission filter at 530 nm. The resulting band intensities were analyzed with the Java-based image processing program, ImageJ. For immunoblotting, proteins were transferred from gels to 0.45 μm pore size nitrocellulose membranes (Invitrogen) at 30 V for 2 h using transfer buffer obtained from Invitrogen with 10% methanol. Blots were probed with primary antibodies, either anti-V5 antibody conjugated with horseradish peroxidase (Invitrogen) diluted 1:5000 or anti-MtrC polyclonal antibody diluted 1:10000 followed with an anti-rabbit IgG secondary antibody diluted 1:2000.[13,15,29] Immunoblots against methionine sulfoxide used 1:2000 dilution of antimethionine sulfoxide rabbit polyclonal antibody (Oxford Biomedical Research, Oxford, MI). Antibody binding was visualized from densitometry of bands detected by chemiluminescence (ECL Western Blotting Reagents by GE Healthcare).

Heme Staining. Heme containing proteins were detected from the blue color formed by the peroxidase activity of heme proteins acting on 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma T5525) or TMB dihydrochloride (Sigma T8768). TMB was made fresh in methanol at a final concentration is 6.3 mM, mixing 3 parts TMB/methanol with 7 parts 0.25 M sodium acetate (pH 5.0). After a brief rinse in distilled water, the electrophoresis gel was stained in heme stain in the dark for up to 2 h with gentle mixing. For final color development, the gel was incubated in 30 mM H_2O_2 for 30 min, before a final wash with a buffer of 3 parts isopropanol and 7 parts 0.25 M acetate (pH 5.0) for at least 1 h, changing the buffer 1−2 times.

Determination of *in Vivo* MtrC Turnover Rates. Outer membrane MtrC* was induced with arabinose prior to its pulse labeling with CrAsH-EDT_2 (see above). Labeled cells were washed twice, each time centrifuging at 6000*g* for 10 min prior to resuspension in fresh LB medium. Subsequently, cultures were grown in 50 mL Falcon tubes with shaking (250 rpm), either tightly sealed with air in the headspace (suboxic conditions) or open to the air (aerobic conditions). Cells were grown in this manner, removing aliquots at various times, which were quick frozen and stored at −20 °C. Cells were thawed and lysed, removing unbroken cells and debris with a low speed centrifugation at 6000*g* for 10 min. The resulting supernatant (lysate) was applied for electrophoresis, loading identical volumes in each lane in order to normalize for fluorescence dilution due to cell growth. After electrophoretic separation of lysate proteins by SDS-PAGE, fluorescence on the gel was visualized with a Kodak Imager with the excitation filter set at 465 nm and emission filter at 530 nm, prior to staining the gel with Coomassie blue. In order to monitor CrAsH-labeled MtrC* in the cell media, the conditioned medium from cultured cells was concentrated with a Microcon YM50 centrifugal filter (Millipore) to the same protein concentration as that of the cell lysate and then applied for electrophoresis. In the case of monitoring total levels of MtrC protein over time (with antibodies), identical protein loads were applied to each electrophoresis gel lane.

Fluorescence Measurements. Fluorescence measurements made in cuvettes used a FluoroMax-2 fluorometer (SPEX, Edison, NJ) with an excitation and emission wavelengths as indicated in each figure legend with excitation and emission slits set at 5 nm. In all cases the sample temperature was maintained at 25 °C. Images of cells were collected using a Nikon Eclipse Ti inverted microscope equipped with a Nikon CFI Plan Apochromat 60× oil immersion objective. The camera was a CoolSNAP HQ2 camera from Photometrics. The whole system was controlled by the software Nikon NIS

element. In these experiments FlAsH is directly excited at 484 nm (15 nm bandwidth), and emitted light is detected at 517 nm (30 nm bandwidth). At this excitation wavelength CFP does not have any significant absorption.[30]

RESULTS

Transient Induction of MtrC. Our experimental design involves induction of a tagged MtrC protein (i.e., MtrC*) in the wild-type cellular background that retains the operon associated with the coordinate expression of the metal reductase proteins that come together to form the supramolecular complex associated with biological activity.[1] *Shewanella oneidensis* MR-1 mutants were constructed that express the gene encoding MtrC (SO1778) with an appended C-terminal 52-amino acid sequence consisting of (in the following order) a tetracysteine tag (AREACCPGCCK) adjacent to a V5 epitope (KGGRADPAFLYKVVINSKLEGK-PIPNPLLGL) and a His_6 sequence, as previously described.[8] This genetic construct encoding the tagged MtrC (i.e., MtrC*) was cloned into the pBAD202/D-TOPO (Invitrogen) expression system which contains a promoter of the *araBAD* (arabinose) operon, which allows control of protein expression through its responsiveness to arabinose. In the absence of added arabinose there is no detectable expression of MtrC* (see Figure 7B), and MtrC represents ~0.02% of total cellular protein (see Figure S3 in Supporting Information). Using an antibody made against MtrC,[8] a major broad band that migrates with an apparent molecular mass centered near 74 kDa is apparent in lysates prepared from *Shewanella oneidensis* MR-1 that is consistent with the presence of both MtrC* and the smaller 71 kDa endogenous MtrC (Figure 2B). A minor band with an apparent molecular mass near 30−35 kDa is observed that is consistent with a proteolytic fragment of MtrC that may be indicative of an endogenous degradative mechanism associated with protein turnover.

Following arabinose induction of MtrC* for 2 h under aerobic conditions, cultures were split and incubated for further growth in either open (aerobic) or sealed (suboxic) tubes. No anaerobic electron acceptor is provided under suboxic growth conditions, as these latter conditions limit growth and simplify turnover measurements as growth dilution of long-lived proteins is minimized. Under aerobic conditions there is a 5-fold increase in biomass (i.e., total protein) in comparison with that observed under suboxic growth conditions, which reflects the increased metabolic efficiencies of fully aerobic growth (Figure 2A). Irrespective of incubation conditions, there are substantial amounts of wild-type (endogenous) MtrC that are detected using immunoblots. Total MtrC protein levels after 4 days in culture (following MtrC* induction) are between 54 ± 4% (aerobic) and 74 ± 4% (suboxic) of that immediately following MtrC* induction by arabinose (Figure 2C), indicating that under our expression system transient induction of MtrC* results in a modest increase in the abundance of total MtrC proteins (i.e., $MtrC_{wt}$ + MtrC*) of between 35% (suboxic) and 85% (aerobic) above wild-type expression levels. The larger abundance of total MtrC proteins at long times following transient induction of MtrC*, apparent under suboxic conditions in comparison to aerobic growth conditions, is consistent with the known induction of the Mtr genes (both MtrC and OmcA) under reduced O_2 and anaerobic conditions.[3] Differences in the apparent rates of re-equilibration of total MtrC abundance levels (i.e., $MtrC_{wt}$ + MtrC*) following induction (i.e., 0.14 ± 0.05 h^{-1} under aerobic conditions and 0.07 ± 0.02 h^{-1} under suboxic conditions) are largely the result of differences in growth rates and the associated dilution of expressed MtrC* proteins over time. Independent measurements of the abundance of the recombinant (tagged) MtrC* by immunoblotting using an antibody directed against the V5 epitope portion of the C-terminal tag sequence on MtrC* permits direct measurements of the rate of the decay of the transiently induced MtrC* which, upon correcting for dilution following growth, involve turnover times of 16 ± 4 and 12 ± 6 h for MtrC* under suboxic or aerobic growth conditions, respectively (Figure 3). Measurements

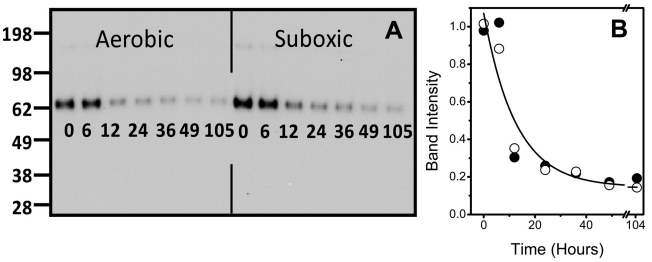

Figure 3. Recombinant MtrC* turnover rates. Immunoblots using antibodies directed against the V5-tag engineered into recombinant MtrC* (panel A) and associated densitometric abundance changes (panel B) following 2 h induction by arabinose (1 mM) prior to growth under under aerobic (●) or suboxic (○) conditions, as defined in Materials and Methods. Line represents nonlinear least-squares fit to an exponential decay, with a rate constant of 13 ± 3 h. Correcting for changes in biomass, individual rates of MtrC turnover are 16 ± 4 h (suboxic) or 12 ± 6 h (aerobic). In all cases 2 μg of lysate proteins was applied to each lane of the SDS-PAGE gel.

made under suboxic conditions, when there is minimal growth dilution of expressed proteins, result in similar turnover rates irrespective of whether measurements use antibodies against $MtrC_{wt}$ (Figure 2) or a V5-tag on the construct (Figure 3), which are respectively 12 ± 6 h (0.08 ± 0.03 h^{-1}) and 16 ± 4 h (0.06 ± 0.01 h^{-1}). These latter results indicate that $MtrC_{wt}$ and MtrC* have similar turnover kinetics, suggesting that they are recognized in equivalent manners by cellular machinery associated with protein maturation, trafficking, and turnover. A primary surprise from these measurements relates to the very slow turnover rates of the MtrC protein (irrespective of whether probes were used against wild-type MtrC ($MtrC_{wt}$) or transiently expressed tagged MtrC (i.e., MtrC*).

Thus, rates of MtrC degradation are very slow in comparison with the rapid changes in message levels, which occur on the time scale of minutes for this protein in response to environmental growth conditions.[31] While increased oxygen levels result in a 50% reduction in the abundance of MtrC, this is not a result of oxidative stress, as evidenced by unaltered levels of methioinine sulfoxide, a common oxidative modification linked to excess reactive oxygen species (ROS) formation.[32−34] Furthermore, MtrC itself is not appreciably oxidized in comparison with other abundant heme-containing proteins (e.g., the periplasmic protein fumarate reductase is extensively oxidized), suggesting that MtrC turnover will involve normal cellular mechanisms that are unrelated to stress response pathways[35] (Figure 2D). The observation that MtrC abundance is maintained in cultures growing aerobically, where this pathway is likely to be nonfunctional, is consistent with prior suggestions that many essential microbial proteins associated with specific metabolic pathways are maintained

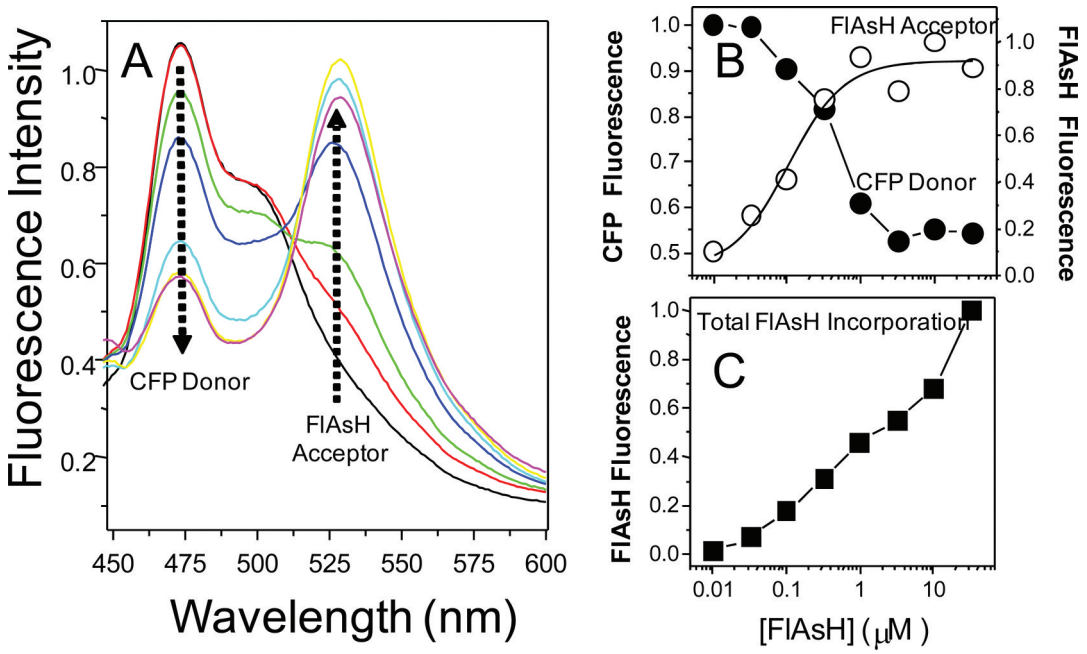

Figure 4. Quantitative labeling of cytosolic proteins by biarsenical probes. Fluorescence emission spectra (panel A) and associated fluorescence intensity changes (panel B) for CFP donor (engineered with a tetracysteine tag; ●) and FlAsH (acceptor; ○) (stimulated emission) of live *S. oneidensis* MR-1 incubated with variable concentrations of FlAsH-EDT_2, as indicated by the colored lines: 10 nM (black), 33 nM (red), 100 nM (green), 333 nM (blue), 1.0 μM (aqua), 3.3 μM (yellow), and 10 μM (magenta), where λ_{ex} = 410 nm. Total FlAsH incorporation into *Shewanella* (panel C) was quantified from direct excitation of FlAsH (λ_{ex} = 490 nm) and measurements of fluorescence at 529 nm. Under all tested conditions there is no loss in cell viability.

under a range of environmental conditions to enhance the organism's ability to adapt to changing and unpredictable environments.[36]

Selective and Quantitative Labeling of Tagged Proteins. It has been estimated that as much as 40% of the total MtrC protein content may exist in immature forms localized in the periplasm or cytosolic cellular compartments,[15,23] suggesting the need to differentiate immature from mature protein following its maturation in the periplasm and translocation to the outer membrane. To accomplish this, we have synthesized biarsenical probes with varying physical properties that are expected to modify their ability to cross biological membranes (Figure 1).[25,26,37] Specifically, in comparison to FlAsH-EDT_2 the incorporation of an additional carboxyl group in carboxy-FlAsH (CrAsH) is expected to prevent entry into the cell, thus permitting specific labeling of MtrC* exposed on the cell surface. To be useful, CrAsH must selectively label MtrC* localized on the outer membrane, permitting us to distinguish these proteins from the total population of cellular protein sensitive to FlAsH labeling to permit live-cell pulse labeling of cells to monitor the turnover of MtrC. Furthermore, the labeling efficiencies of FlAsH and CrAsH should not depend on the cellular location of the tagged protein to allow for quantitative measurements of protein localization and turnover. In these measurements the small size of the tetracysteine tagging sequence offers a distinct advantage over more conventional fusions (e.g., 27 kDa fluorescent proteins) for MtrC, as these large fusions prevent trafficking through both inner and outer cell membranes via type II secretory systems before final folding and assembly of MtrC with the Mtr complex associated with the bacterial outer membrane.[1,15] Moreover, biarsenical probes bind with high affinity to the tetracysteine sequence; the additional advantage is the low fluorescence in EDT-bound forms prior to binding, which is greatly enhanced (up to 35-fold) by binding through disulfide exchange to the tetracysteine sequence engineered into the target protein.[8,10,25,38]

This class of probe-tag pairs, known as multiuse affinity probes (MAPs), provides an important tool in determining abundance, location, size, binding interfaces, and function of tagged protein in living cells.[8,10,25,26,28,39−47] While the MAPs work well for live-cell imaging in eukaryotic cells, their application has been limited to the detection of highly overexpressed proteins. Further, current protocols do not permit the stoichiometric labeling of tagged proteins, limiting their quantitative applications. As a first step to solve this latter hurdle in the application of MAPs in prokaryotes, we have systematically investigated conditions that permit the specific FlAsH labeling of tagged cytosolic proteins at near equimolar stoichiometries with minimal background signal from nonspecific labeling. Critical for monitoring specific fluorophore labeling was the expression of a tetracysteine tagged-cyanofluorescent protein (CFP-C4), whose cytosolic location requires the delivery of the biarsenical fluorophore (in this case FlAsH-EDT_2) across both outer and inner membranes, which is monitored as the fluorescence resonance energy transfer (FRET) efficiency between the CFP donor and FlAsH acceptor (Figure 4). When live bacteria are incubated with increasing concentrations of FlAsH-EDT_2, a progressive decrease in CFP donor fluorescence at 475 nm is observed, with a concomitant increase in FlAsH fluorescence centered near 530 nm (sensitized emission) characteristic of the occurrence of FRET. These progressive increases in FRET saturate near 50% energy transfer at an added FlAsH concentration of 1.0 μM, which is similar to prior measurements that report between 50 and 80% energy transfer efficiencies between these two

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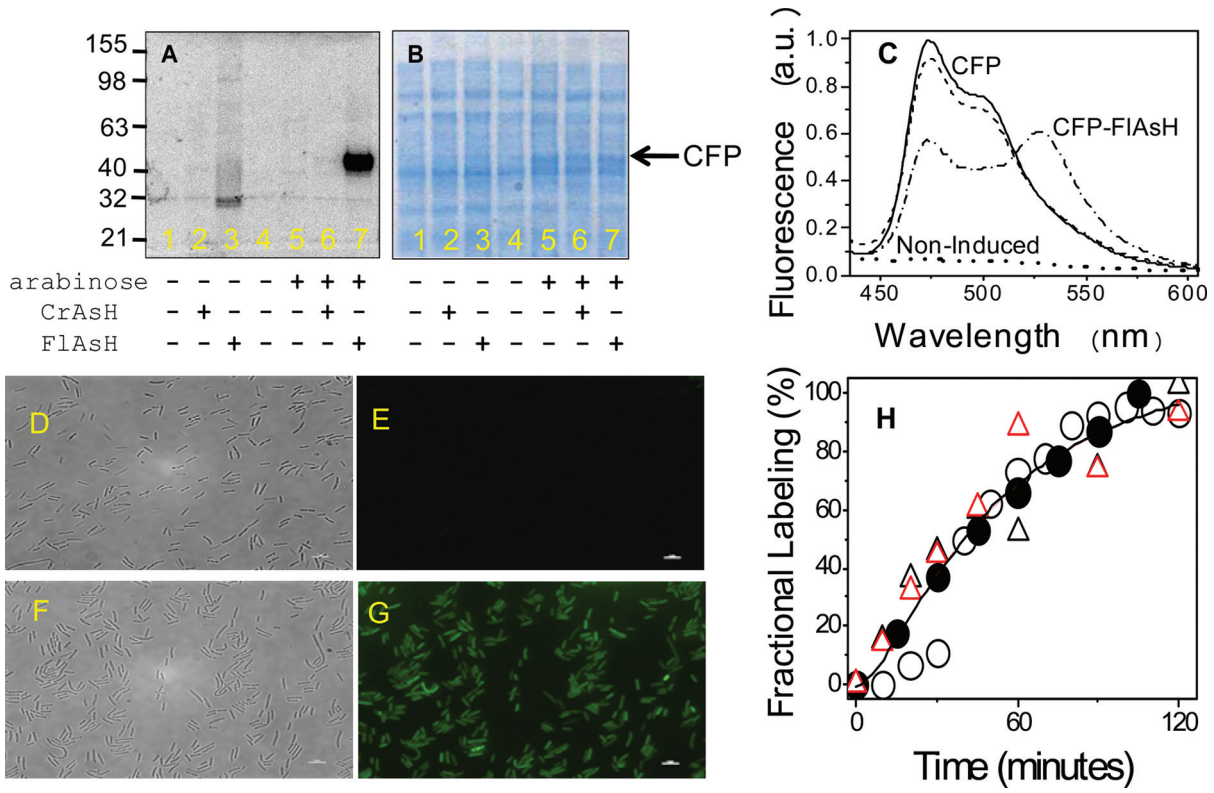

Figure 5. Cell impermeability of CrAsH-EDT$_2$ is responsible for the inability to label cytosolic proteins in comparison to FlAsH-EDT$_2$. Fluorescence (panel A) and Coomassie blue stained (panel B) electrophoresis gels of cell lysates following incubation of live *S. oneidensis* MR-1 cells with either CrAsH-EDT$_2$ (lanes 2 and 6) or FlAsH-EDT$_2$ (lanes 3 and 7) prior to (lanes 1−4) or following (lanes 5−7) induction of CFP-C4 expression. Fluorescence of CrAsH or FlAsH on SDS-PAGE was monitored prior to staining with Coomassie blue (λ_{ex} = 465 nm, λ_{em} = 530 nm). Positions of molecular mass markers are indicated on the left. Fluorescence emission spectra (panel C) of live *Shewanella oneidensis* MR-1 cells expressing CFP* (engineered to contain a tetracysteine tag) prior to (solid line) or following incubation with either CrAsH-EDT$_2$ (dashed line) or FlAsH-EDT$_2$ (dot-dashed line) in comparison to noninduced cells (dotted line), where λ_{ex} = 410 nm. Live cell bright-field (panels D and F) and fluorescence images (panels E and G) following incubation of FlAsH-EDT$_2$ (0.5 μM FlAsH-EDT$_2$) with live *S. oneidensis* MR-1 cells for 2 h prior to (panels D and E) or following (panel F and G) induction of CFP* (engineered to contain a tetracysteine tag), where λ_{ex} = 484 nm (15 nm bandwidth) and λ_{em} = 517 nm (30 nm bandwidth). Labeling kinetics for different tetracyteine-tagged proteins by biarsenical fluorophores (1.0 μM) (panel H) measured using CrAsH-EDT$_2$ (black triangles, red triangles) or FlAsH-EDT$_2$ (○) for tetracysteine-tagged MtrC* (black triangles) or CFP (○) proteins expressed in live *S. oneidensis* MR-1 in comparison to a purified protein (i.e., calmodulin; 1.0 μM) engineered to contain a tetracysteine tag (●), where the extent of labeling was determined following protein separation by SDS-PAGE and the protein−associated fluorescence signal, which is plotted as a function of time. Labeling of MtrC* by CrAsH-EDT$_2$ was done 15 min (red triangles) or 2 h (black triangles) after inhibition of protein synthesis by cycloheximide (0.5 mM).

chromophores following *in vivo* labeling of a similar tagged CFP construct in mammalian cell culture[48,49] (Figure 4B). These results emphasize that the FlAsH-EDT$_2$ labeling procedure works well for labeling cytosolic bacterial proteins. Higher concentrations of added FlAsH-EDT$_2$ result in nonspecific labeling as indicated by the continued increase in the fluorescence of total bound FlAsH monitored by its direct excitation at 490 nm (Figure 4C), emphasizing the need to avoid high concentrations of FlAsH to achieve specific labeling of CFP-C4 and other cellular proteins. As parallel *in vitro* measurements indicate that equimolar labeling of CFP-C4 results in ∼80% FRET (Figure S6 in Supporting Information), our results indicate that substoichiometric labeling is necessary to maintain optimal labeling specificity.

Use of optimal conditions for CFP-C4 labeling results in bright fluorescence delineating *Shewanella* cells in a microscope field without significant fluorescence associated with nontagged proteins (i.e., nonspecific labeling) (Figure 5E,G), indicating an ability to use biarsenical probes for live cell measurements of protein turnover without inherent complications associated with bleach chase or measurements that require cell disruption.[50] Cellular labeling is highly uniform, as is apparent in a direct comparison between the fluorescence and bright-field images of the Shewanella cells (Figure 5F,G). To verify that the observed FRET is the result of FlAsH labeling of CFP-C4 rather than of other nonspecific, but proximal sites, we monitored FlAsH fluorescence associated with CFP after electrophoretic separation of cellular proteins. Under optimal labeling conditions, we find that the CFP-C4 in *Shewanella* cells is selectively labeled by FlAsH as evidenced by the single fluorescent band migrating at the predicted molecular mass of CFP-C4 (Figure 5A). A small background signal is observed with an apparent mass near 32 kDa as indicated by additional fluorescent bands in the empty-vector control; however, importantly, this nonspecific labeling is reduced following expression of CFP-C4, which accounts for about 1% of the total cellular protein (Figure 5B). Thus, the tetracysteine tag sequence on CFP-C4 successfully competes with nonspecific sites for FlAsH. Moreover, this result highlights that the empty vector overestimates the extent of nonspecific binding that occurs in the presence of a tetracysteine-tagged protein. Thus, we have demonstrated conditions that permit specific labeling

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of tetracysteine-tagged intracellular protein (i.e., CFP-C4) within a living bacterial cell without requiring high levels of CFP overexpression (i.e., above 1−2% of total cellular protein). Moreover the CFP-C4 construct will provide a standard reference for comparison of cell surface labeling by CrAsH, as described below.

Impermeability of *Shewanella* Cells to CrAsH. To explicitly test the predicted cell impermeability of CrAsH-EDT_2, we made a direct comparison of its ability to label intracellular CFP tagged with the tetracysteine labeling sequence (i.e., CFP-C4) as compared with that of FlAsH-EDT_2. Following its induction, CFP-C4 represents about 1% of total cellular protein (Figure 5B). We find that, in contrast to the case of FlAsH-labeling under the same conditions, CrAsH-labeling exhibits no detectable FRET with CFP-C4 and no fluorescence associated with the 45 kDa CFP-C4 protein band on SDS-PAGE (Figure 5A,C). Further, unlike FlAsH, there is no evidence of nonspecific CrAsH binding to other lysate proteins; neither does direct excitation of CrAsH in living cells exhibit fluorescence emission that would indicate nonspecific binding.

Binding Kinetics Are Insensitive to Protein Location. Complete labeling of MtrC* by CrAsH in living *Shewanella* required up to 2 h as measured from the amount of fluorescence associated with the Mtr-C4 protein band after electrophoretic separation of cell proteins, which is independent of the time following inhibition of protein synthesis using cycloheximide (Figure 5H). These kinetics are identical to those of FlAsH modification of both CFP-C4 in living *Shewanella* and isolated tetracysteine tagged-calmodulin (CaM*), a nonmembrane associated cytoplasmic protein. Maximal incorporation of biarsenical fluorophore corresponds to stoichiometric (1:1) labeling of each tetracysteine-tagged protein as compared with modification of CaM*, assessed by spectrometric measurements of the CrAsH-labeled CaM associated fluorescence.[28] Notably, unlike CFP-C4 and MtrC*, for which the tetracyseine tag sequence is added to the C-terminus, the calmodulin tagging sequence is incorporated by site-specific mutations within a native protein helix. Despite different tag and protein locals, labeling kinetics are the same, providing strong evidence that these biarsenical probes bind to tagged proteins independently of their cellular localization; in other words, membrane partitioning is not rate-limiting. Rather, diffusion of the biarsenical probe into the cell is fast relative to the rate of displacement of the EDT caps present on the FlAsH-EDT_2 or CrAsH-EDT_2 by cysteines within the engineered tag. These results suggest that 2 h of incubation is sufficient to label tagged proteins in different locations, permitting quantitative measurements of labeling specificity. Moreover, as misfolding or mistranslocation of recombinant MtrC* might restrict the accessibility of cysteines to the arsenic groups, these results indicate that the majority of MtrC* properly folds following its translation.

Complementation of Metal Reductase Function by MtrC*. To ensure that the tagged MtrC protein (i.e., MtrC*) remains functionally intact and is able to effectively traffic to the outer membrane and form a functional complex capable of metal reduction, we have used a knockout mutant lacking MtrC (i.e., ΔMtrC) and assessed our ability to regain full metal reductase activity upon complementation using a vector encoding either wild-type MtrC (i.e., $MtrC_{wt}$) or MtrC* containing the tetracysteine and V5 tagging sequence. In comparison to wild-type Shewanella, the ΔMtrC mutant displays about one-half of the normal metal reductase activity (Figure 6). This latter observation is consistent with the presence of OmcA, which acts synergistically with MtrC in wild-type *Shewanella* to maintain optimal metal reductase activity.[8] Following complementation with either $MtrC_{wt}$ or MtrC* the metal reductase activity is fully restored. These results indicate that MtrC*, like $MtrC_{wt}$, is able to associate with MtrB upon trafficking to the outer membrane to couple intracellular metabolism to the reduction of iron oxides.

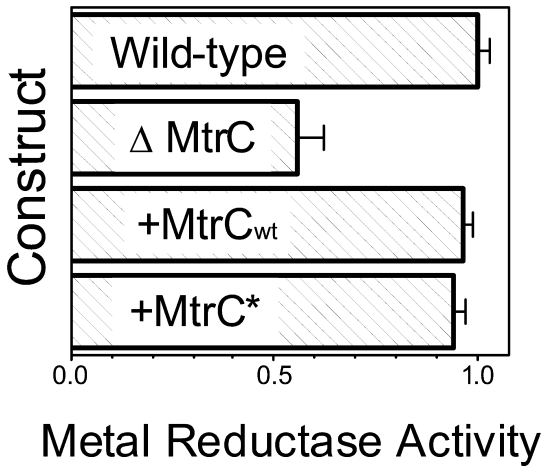

Figure 6. Functional complementation following MtrC expression. Reduction of iron by wild-type *S. oneidensis*, Δ*mtrC* mutant, and following introduction into the Δ*mtrC* mutant of pBAD202/D TOPO vectors encoding genes for $MtrC_{wt}$ or MtrC*. Experimental conditions involved assays of 0.5 N HCl extractable Fe(II) formation measured following incubation of *Shewanella* with 10 mM ferrihydrite in 30 mM NaH_2CO_3 and 5 mM lactate for 120 h, essentially as previously described.[68] Standard deviations represent errors associated with triplicate measurements. All values are normalized relative to wild-type *Shewanella oneidensis* MR-1 (i.e., 2.39 ± 0.07 mM Fe(II)).

Selective Labeling of Surface Expressed MtrC and Efficient Proteolysis of Immature MtrC. Knowing that the kinetics of selective labeling of cellular proteins by biarsenical probes (i.e., CrAsH-EDT_2 or FlAsH-EDT_2) occurs in 2 h (Figure 5H), irrespective of their cellular location of the tagged protein, allows us to take advantage of differences in the cellular permeability of these probes to assess the location of MtrC* in the cell (Figure 7). Using CrAsH-EDT_2, we can monitor the population of MtrC* following trafficking to the outer cell membrane. Using FlAsH-EDT_2, we can monitor the entire population of MtrC*, including proteolytic fragments located within the cell (lane 6 in Figure 7A). In view of the requirement of the T2S system for the trafficking of MtrC to the outer membrane,[15] we examined the effects of deletion of two different T2S genes on the ability to label MtrC with CrAsH. Three *S. oneidensis* mutant strains which express MtrC* were compared that incorporate either a deletion of T2S genes, (i) *gspD* or (ii) *gspG*, or (iii) have an intact T2S system. A fourth (empty vector) strain (iv) was examined which lacked recombinant MtrC but contained the expression plasmid (without an inserted gene) and contained an intact T2S system.

CrAsH-EDT_2 or FlAsH-EDT_2 labeling of MtrC* in live cells results in the appearance of a single fluorescent band migrating with an electrophoretic mobility matching that of both purified and immunoreactive MtrC* (Figure 7). There is no full-length MtrC* protein detected using immunoblots against the V5-tag

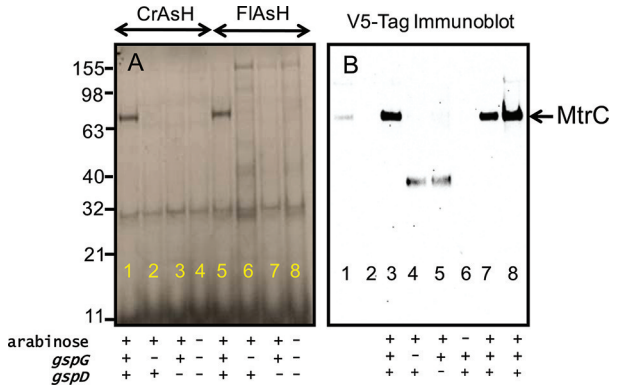

Figure 7. Targeting and maturation of MtrC requires a functional Type 2 secretion system. Fluorescent gel imaging following live-cell labeling using either CrAsH-EDT$_2$ (lanes 1−4) or FlAsH-EDT$_2$ (lanes 5−8) (panel A) and complementary immunoblots directed against the V5-epitope of MtrC* (panel B) for lysates prepared using recombinant *S. oneidensis* containing an arabinose-inducible vector encoding MtrC* with an intact Type 2 secretion system (T2S) containing expressed proteins GspG or GspD (indicated by plus signs) or following deletion of T2S genes *gspD* or *gspG* (indicated by minus signs). For comparison, an authentic MtrC* standard is run in lane 1 (panel B). Cells were grown, labeled, and lysed as described in Materials and Methods before fluorescent gel (30 μg) or immunoblot (5 μg) analysis. Positions of molecular mass markers are indicated on the left.

upon deletion of T2S genes for constructs that knock out either *gspG*, involved in transport of proteins across the inner membrane, or *gspD*, an outer membrane secretin.[4,15,51] Instead, a 35 kDa fragment of immunoreactive MtrC* appears when the T2S system is rendered nonfunctional. Under these conditions involving deletion of T2S genes there are no corresponding fluorescent bands for cells incubated with CrAsH-EDT$_2$, indicating that CrAsH-EDT$_2$ labeling of intact or proteolytically digested MtrC* only occurs in the presence of an intact T2S system. In comparison, using the cell permeable FlAsH-EDT$_2$, one observes a large number of additional fluorescently labeled protein bands, particularly pronounced upon deletion of *gspG*, that are indicative of intracellular proteolytic fragments of MtrC*. The absence of a detectable population of intact FlAsH-labeled MtrC* following deletion of either *gspG* or *gspD* suggests the presence of an efficient quality control mechanism for internal degradation of newly synthesized MtrC when its secretion is impaired, which effectively minimizes accumulation of immature MtrC in the periplasmic space and the potential disruption of efficient electron transfer processes associated with the inner membrane and periplasm.

An additional fluorescent band, migrating at an apparent molecular mass of 30 kDa, is present in all strains including both the empty vector and wild-type in the absence of added CrAsH. Further characterization has shown that this is an endogenous trypsin-sensitive nonheme protein of *Shewanella* that copurifies with membranes (see Supporting Information). This 30 kDa fluorescent protein can be extracted using the detergent CHAPS, but not with high salt concentrations,

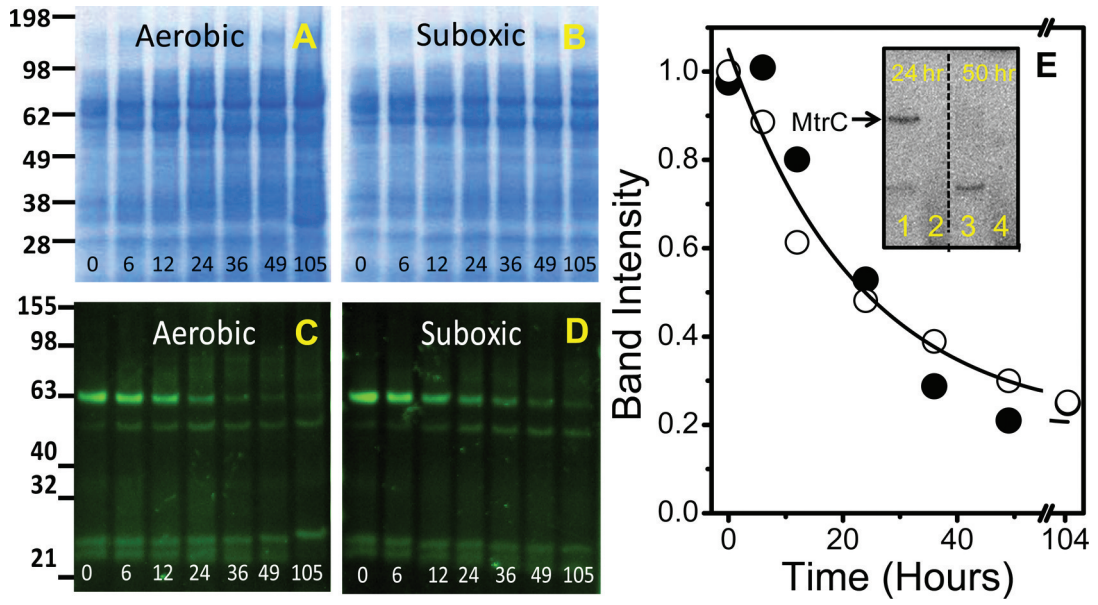

Figure 8. *In vivo* protein turnover of CrAsH-labeled MtrC*. Coomassie blue stained (panels A, B) and corresponding fluorescence (panels C, D) following electrophoretic separation of lysates from cells following induction and pulse-labeling of MtrC* in live *Shewanella* with CrAsH and subsequent growth under aerobic (panels A, C) or suboxic (panels B, D) conditions; aliquots of cells were lysed at indicated times (in hours). Cell lysates were applied for electrophoretic separation, loading each lane with the same protein amount (Coomassie stained gels) or identical volumes to compensate for fluorescence dilution due to cell growth (fluorescence gels). Quantitative changes in fluorescence intensity associated with the intact 74 kDa MtrC* under aerobic (●) or suboxic (○) conditions were measured by densitometry and plotted as a function of time in culture (panel E); the line corresponds to the nonlinear least-squared fit to all data points to an exponential decay model. Independent fitting of the separate data sets yields best fits corresponding to 24 ± 5 h (aerobic) or 21 ± 5 h (suboxic); residual protein degraded on slower time scales was 20 ± 6%. Inset (panel E) shows fluorescence associated with CrAsH-labeled MtrC (and 38 kDa large fragment) after 24 h (lanes 1 and 2) or 49 h (lanes 3 and 4) following pulse-labeling live cells for pelleted cells (6000*g*; 10 min) following lysis (lanes 1 and 3) or extracellular medium concentrated to the same volume as the cell lysate, using a Microcon YM50 centrifugal device (Millipore, Inc.) (lanes 2 and 4). In all cases 10 μg of lysate proteins was applied to each lane of the SDS-PAGE gel.

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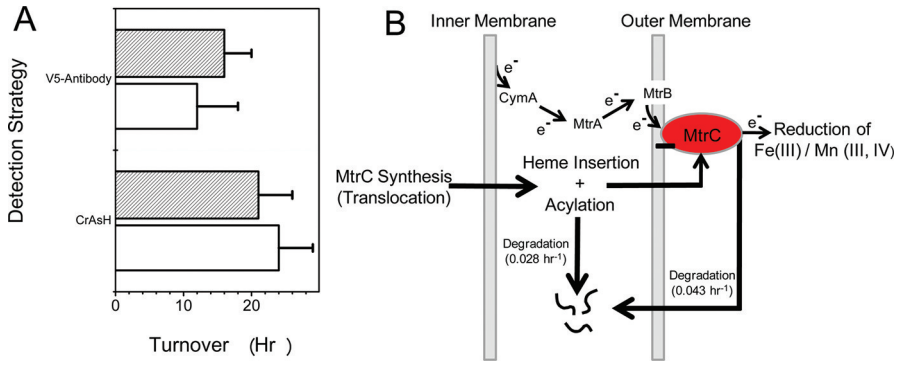

Figure 9. Long-term stability and inefficient maturation of MtrC following trafficking to outer membrane. (Panel A) Summary of turnover rates for MtrC in *Shewanella oneidensis* MR-1 grown under suboxic (hatched) or aerobic (open) conditions using complementary assays involving antibodies against the V5-tag on expressed total MtrC* (i.e., both during maturation and following assembly on the outer membrane surface; Figure 3) or CrAsH that selectively labels mature MtrC* following trafficking to the outer membrane (Figure 8). (Panel B) Model summarizing trafficking pathway of MtrC, which involves (i) translocation of message in the cytosol, (ii) heme insertion and acylation in the periplasm, and (iii) subunit assembly with OmcA (not shown) and MrB to form outer membrane metal reducing complex necessary for extracellular electron transfer to solid minerals (e.g., Fe(III)/Mn(III,IV)) to facilitate anaerobic metabolism. Transport requires the functional T2S system (see Figure 7). Highlighted is the electron transfer pathway, which involves transfer from quinols in the inner membrane to the outer membrane metal reducing complex through intermediary proteins CymA, MtrA, and MtrB.[1]

indicating that the 30 kDa protein is tightly associated with the bilayer and is not a peripheral membrane protein. Such a native fluorescent protein may have an important sensory function, and while not further studied here, fluorescent sensor proteins stable following SDS-PAGE may have useful technological applications and may warrant identification and further study.

Stability of Outer Membrane MtrC. In order to directly examine the fate of MtrC on the outer membrane of *Shewanella*, we measured its protein turnover rate from the disappearance of fluorescence associated with MtrC* after pulse labeling with CrAsH-EDT$_2$. For these measurements, 1 mM arabinose was included in the medium of the recombinant *Shewanella* for 2 h to induce expression of MtrC*, thus providing a bolus of newly synthesized MtrC* for subsequent CrAsH labeling. After 2 h of labeling under aerobic conditions, the cells were washed prior to their division into two separate cultures, incubating in fresh medium under either suboxic or aerobic conditions. During aerobic or suboxic growth, aliquots of cells (constant volume) were harvested at designated times for analysis of fluorescence associated with CrAsH-MtrC* after electrophoretic separation of lysate proteins (Figure 8). As indicated, measurements of fluorescence used a constant volume of added cells, thereby avoiding artifacts associated with changes in biomass that can dilute tagged proteins and prevent accurate measurements of turnover. In the absence of further induction by arabinose, the time course of the decrease in fluorescence provides a reliable estimate of outer membrane-associated MtrC degradation, exclusive of any contribution from protein synthesis. Comparisons of total protein composition examined by Coomassie blue stained gels applied a uniform amount of added protein to facilitate detection of any large changes in protein composition induced by alterations in growth conditions.

We observe a progressive time-dependent decrease in the fluorescence intensity associated with the intact MtrC* without marked changes in the abundance of cellular proteins. In all cases, a number of additional lower molecular mass CrAsH-labeled proteins are apparent, which include some large fragments whose relative abundance changes during the time course of the experiment. Identified protein fragments include a predominant 35 kDa fragment (Figure 8E, inset), whose migration in SDS-PAGE is similar to that observed during normal growth (Figure 2B) or following deletion of the functional T2S system (Figures 6). No CrAsH fluorescence was detected in the external cell medium following its concentration using a Microcon centrifugal filter to an equivalent volume as that of the cell pellet. This result suggest that the turnover of outer membrane localized MtrC is not accompanied by release outside the cell, as would be expected if turnover involved formation of small membrane vesicles that "bud" off from the cells.[52] Rather, degradation of MtrC appears to occur in a targeted manner, suggestive of a highly regulated cellular reuptake mechanism.

The data of time-dependent changes in fluorescence from cells cultured under either aerobic or suboxic conditions overlay each other, fitting well to an exponential decay time associated with MtrC* following trafficking to the outer membrane (k_{ext}) of 23 ± 5 h (i.e., $1/k_{ext}$ = 0.043 h^{-1}). Independent fits of the data under suboxic or aerobic growth conditions respectively indicate turnover times of 21 ± 5 and 24 ± 5 h. This slow rate of protein turnover highlights the stability of MtrC following its localization on the outer membrane. Notably, culture conditions previously reported to promote the appearance or disappearance of MtrC-containing surface protrusions (i.e., the absence or presence of terminal electron acceptors such as O_2), are not associated with altered rates or extents of MtrC degradation, suggesting instead that changes in synthesis rates are responsible for changes in MtrC content in response to varying O_2 concentrations. These results are consistent with prior suggestions that essential proteins are often long-lived and maintained against rapid changes in environmental conditions to enhance viability.[36,53]

Moreover, under conditions where there is minimal growth dilulation (i.e., under suboxic conditions), turnover measurements made using antibodies against MtrC$_{wt}$ (Figure 2) or a V5-tag on the MtrC* construct (Figure 3) identify similar turnover rates (i.e., 12 ± 6 h vs 16 ± 4 h, respectively), which suggest both MtrC$_{wt}$ and MtrC* behave in a similar manner and that the tagging sequence in MtrC* does not impair the ability of MtrC to associate with the native supramolecular complex. These latter results are strongly supported by earlier experiments that demonstrated (i) copurification of a high-

affinity complex between OmcA and MtrC following introduction of the same tagging sequence on OmcA and (ii) high-affinity binding between purified FlAsH-labeled MtrC* and OmcA (K_d < 500 nM).[8] From these measurements it is apparent that MtrC* turnover is substantially faster when total cellular protein is monitored in comparison to outer membrane localized MtrC* (Figure 9A). Indeed, rates of degradation of MtrC* measured using the V5 antibody correspond to the sum of degradation rates linked to cellular trafficking to the outer membrane (i.e., k_{int}) and subsequent degradation and reuptake following formation of the outer membrane protein complex (i.e., k_{ext}), where $1/k_{total} = 1/k_{ext} + 1/k_{int} = 0.071\ h^{-1}$. As we know the turnover rate of CrAsH-labeled MtrC* ($1/k_{ext} = 0.043\ h^{-1}$), these results indicate a substantial rate of intracellular degradation of MtrC* (i.e., $1/k_{int} = 0.028\ h^{-1}$), suggesting that as much as 40% of MtrC* is degraded prior to trafficking to the outer cellular membrane (Figure 9B).

DISCUSSION

MtrC Dynamics and Insensitivity to Molecular Oxygen. Using a newly developed cell-impermeable biarsenical probe (i.e., CrAsH), we have selectively labeled outer-membrane localized MtrC* using an engineered MtrC, containing a C-terminus tetracysteine tagging sequence expressed in *Shewanella* oneidensis MR-1 and measured the rate of MtrC* protein turnover. Mature MtrC* is a long-lived protein that undergoes turnover on a slow time scale (i.e., 0.043 h^{-1}) that is relatively insensitive to O_2 concentration (Figure 8). However, MtrC* maturation is inefficient, with ~40% of the total protein undergoing degradation during its maturation pathway (Figure 9). These latter results are consistent with recent proteome analyses of subcellular fractions which indicate that up to 40% of cellular MtrC resides within the cytoplasm, inner membrane, or periplasmic space of *Shewanella*.[23] Disruption of the T2S system necessary for MtrC transport to the outer membrane results in the appearance of protein fragments associated with degradative pathways in the periplasm, which includes a large immunoreactive 35 kDa fragment (Figure 7). A similar large molecular mass protein fragment derived from CrAsH-labeled MtrC* is observed in lysates prepared from growing *Shewanella* cells following cellular isolation and removal of membrane fractions (Figure 8E), indicating that a stepwise degradation of MtrC in the periplasm is likely part of the normal turnover mechanism.

The absence of detectable CrAsH-labeled MtrC* in the growth medium suggests that turnover of outer membrane (mature) MtrC involves an active process involving protein reuptake (salvage) and degradation. Our results do not support models of diffusive mechanisms involving a nonspecific association between released MtrC and extracellular polymeric substance by, for example, a membrane associated lipase or protease that may release bound MtrC.[3,19−21,54] Rather, these results suggest that the extracellular localization of MtrC (i.e., with extracellular polymeric substance and/or pilin) involves a controlled process associated with extracellular assembly and subsequent reuptake and degradation.

Maintenance of mature MtrC localized within supramolecular metal reductase complexes on the outer membrane of *S. oneidensis* permits cells to rapidly respond to changing environmental conditions to exploit a range of extracellular terminal electron acceptors. These findings are consistent with prior work establishing the adaptive value of maintaining long-lived proteins that are useful under unfavorable environmental conditions in microbial populations, acting to maximize their ability to respond to environmental change.[53] Moreover, microbial exoenzymes can encounter extremes in environmental conditions, i.e., pH and ionic strength, compared to the relatively constant environment of the cytoplasm and need to be robust to maintain integrity and function. Importantly, the ability to maintain active Mtr complexes on the cell surface under a range of environmental conditions is essential for optimal function of extracellular electron transport for biofuel and biosensor applications.[20,22]

Utility of Cell Impermeable Biarsenical Probe CrAsH To Monitor Abundance of Mature MtrC in Metal Reductase Complex. In the case of MtrC*, we have demonstrated labeling conditions with CrAsH that provides stoichiometric modification without the requirement of extensive overexpression of the recombinant MtrC. Thus, CrAsH permits a pulse-labeling of the newly synthesized cell surface population of MtrC for subsequent measurements that are specific for the degradation of MtrC*. These measurements demonstrate the utility of the biarsenic fluorophore, CrAsH, in combination with a tetracysteine tagging sequence to monitor the fate of outer membrane decaheme cytochrome MtrC in living *Shewanella* cells. The small size of the tetracysteine tag permits normal processing of this protein, which undergoes multiple steps of cellular trafficking involving secretion across the cell membrane, protein folding, heme insertion, acylation, and translocation by T2S prior to assembly in complex with other subunits of the metal reductase supramolecular complex, including the trans-outer membrane spanning beta-barrel protein MtrB that acts as a central scaffold and conduit to mediate electron transport across the outer membrane.[7,12] Like other biarsenic fluorophores (e.g., FlAsH-EDT_2), we demonstrate that CrAsH-EDT_2 provides high affinity and specific binding to the tetracysteine tag sequence (i.e., CCPGCC) engineered onto a protein of interest with the added advantages of both cell impermeability and minimal nonspecific binding due to the increased negative charge conferred by the 5-carboxyl group.[25] Thus, in addition to the ability to monitor molecular events inside cells with cell permeable biarsenics (i.e., FlAsH-EDT_2), CrAsH-EDT_2 extends the applications of these affinity probes for monitoring cell surface and exocellular proteins. In particular, the biarsenic affinity probes provide distinct advantages in their smaller size and rapid binding as compared with the larger (~27 kDa) fluorescent proteins which require many hours for folding into the three-dimensional structure necessary for formation of the emitting fluorophore, and which commonly prohibit secretion through the T2S system. Further, biarsenical probes are useful under suboxic conditions, as they avoid the oxygen requirement of fluorescent proteins for fluorophore formation that make these probes less desirable for monitoring the effects of changing environmental redox conditions.[55−57]

MtrC and Metal Reductase Supramolecular Complex. Well-understood electron transfer pathways between highly conserved inner membrane supramolecular protein complexes (required for optimal energy generation) and newly identified proteins present in the periplasm and outer membranes in metal reducing bacteria (that provide electron conduits across the outer membrane to permit the reduction of extracellular Fe(III) or Mn(III,IV) metal oxides that act as terminal electron acceptors) have important implications with respect to understanding terrestrial ecology and the colonization of niches deficient in commonly used terminal electron

acceptors, such as molecular oxygen. These metal reductase pathways result in the appearance of extracellular polymeric filaments (nanowires) that enhance the association of *Shewanella* with solid metal oxides, through an active process termed electrokinesis, which is dependent on the expression of MtrC and OmcA.[20] MtrC represents a terminal element in an electron conduit (along with OmcA) that form a high-affinity supramolecular complex, which upon association with the outer membrane scaffold MtrB accepts electrons from MtrA (and via CymA with quinones in the inner membrane) (Figure 9B).[8,12] The conductivity of isolated nanowires is dependent on the presence of MtrC,[19] consistent with observations that isolated outer membrane decaheme cytochromes (i.e., MtrC and OmcA) directly bind and transfer electrons to mineral oxides.[10,58] Further, the directed mobility (electrokinesis) of *Shewanella* is dependent on the expression of MtrC, and strain-dependent efficiencies in the metal reductase pathway correlate with the utility of different *Shewanella* strains to form biofuel cells.[20] Expression of MtrC in conjunction with the MtrA and MtrB proteins in *E. coli* results in a functional pathway (albeit with relatively low conductance), in which these engineered cells are able to mediate extracellular electron transfer.[22] An understanding of the mechanisms responsible for the efficient maturation of MtrC (and MtrA and MtrB) is necessary to enhance the efficient expression of this metal reductase pathway, which offers a means to create robust biosensors that are electronically addressable for a range of biosensing and bioenergy applications.

Proteolytic Degradative Pathways. Bacteria rely on multiple proteases for efficient removal of misfolded and dysfunctional proteins as a quality control mechanism to ensure maintenance of optimal function of the proteome.[59] In this respect, the protease DegP has been demonstrated to play a role in the degradation of MtrB in the outer membrane of Shewanella.[60] The important role of proteases is highlighted by inspection of the *Shewanella* genome, which encodes 77 functional proteases that are localized among multiple cellular compartments of the organism. For example, cytoplasmic proteolysis may be performed by any of the several expressed forms of ATP-dependent proteases: ClpP (caseinolytic protease), Lon/La, and HslV (heat shock locus gene V). These proteases have tertiary structures consisting of hept- or hexameric rings that form an enclosed chamber for the controlled proteolysis of substrates.[61] Separate ATPase subunits (ClpA, ClpX, HslU), or domains, in the case of the single polypeptide Lon, are required for the recognition, unfolding, and translocation of protein substrates into the proteolytic compartment. Also associated with cytoplasmic quality control is the membrane bound protease FtsH.[61−63] In view of the apparent redundancy of these heat shock and stress response proteases, little is known in *Shewanella* regarding either the precise conditions under which each is active or their specific substrates. The periplasm in which outer membrane cytochromes undergo folding, in particular, requires effective quality control mechanisms in view of its potential exposure to more highly oxidizing environments in facultative organisms such as *Shewanella*.[64] For example, accumulation in the periplasm of immature redox active proteins such as the decaheme MtrC in *Shewanella*, whose hemes titrate over a broad potential ranging from +100 to −500 mV, have the potential to accept electrons from supramolecular complexes in the inner membrane to disrupt cellular metabolism.[3] In this respect, the ATP-independent serine protease DegP/Htr represents an important quality control system in the periplasm of many Gram-negative bacteria. Several forms of the DegS gene are present in *Shewanella*; notably, DegS performs a critical function in the activation of the σ^E transcriptional stress response.[65] Thus, genes encode for different proteases that are tailored to each cellular compartment in *Shewanella* and are consistent with our observations regarding the substantial rate of degradation for MtrC during biosynthesis (0.028 h^{-1}) (Figures 6 and 8) or following reuptake for CrAsH-labeled MtrC* (0.043 h^{-1}) (Figure 8E). In this latter respect, the inefficient maturation of MtrC is consistent with examples from eukaryotic systems where the maturation and assembly process of a number of receptor proteins localized on the membrane have been studied more extensively. For example, in the case of the cystic fibrosis transmembrane conductance regulator (CFTR) or nicotinic acetylcholine receptor less than 30% of newly synthesized proteins undergo maturation, or more than 70% of proteins are normally degraded during maturation due to quality control apparatus, which involves dozens of maturation proteins.[66,67]

Conclusions and Future Directions. The cell impermeable affinity fluorophore, CrAsH, in combination with a genetically engineered C-terminal tag sequence appended to MtrC, has permitted the selective labeling of the mature MtrC following its trafficking to the cell membrane. Moreover, it has allowed measurement of rates of protein turnover and cell mediated degradative pathways that are indicative of a targeted pathway consistent with models that suggest an active cell-mediated trafficking of Mtr proteins to the cell exterior. Mature decaheme MtrC localized on the outer membrane is highly stable and relatively insensitive to O_2 despite the high abundance of covalently bound hemes. The environmental stability and existence of cellular quality control mechanisms that mediate trafficking and degradation of extracellular MtrC all suggest the utility of this extracellular electron transfer pathway in synthetic biology applications in which cells can be reengineered to enhance rates of extracellular electron transfer for applications involving bioenergy and biosensing. However, prior to robust applications, it will be essential to identify the machinery associated with both the maturation and degradative pathways that optimize the efficient targeting, self-assembly, and regulated turnover of MtrC in complex with the extracellular Mtr supramolecular complex.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

A single file containing figures corresponding to the characterization of an endogenous 30 kDa fluorescence protein expressed in *Shewanella oneidensis* (Figure S1) and summarizing its association with membranes (Figure S2), evidence summarizing that there is a linear relationship between detected signals and protein abundance using either immunoblots against wild-type MtrC (Figure S3), the V5-epitope on MtrC* (Figure S4), or fluorescence signals for CrAsH-labeled MtrC* (Figure S5), and the fluorescence spectrum of a purified CFP-C4 protein before and after labeling with $FlAsH\text{-}EDT_2$ (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

β-ME, 2-mercaptoethanol; CaM, calmodulin; CrAsH, carboxy-FlAsH; EDT, ethanedithiol; FlAsH-EDT$_2$, 4′,5′-bis(1,3,2-dithoarsolan-2-yl)fluorescein-(1,2-ethanedithiol)$_2$; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MAP, multiuse affinity probe; MtrC*, expressed recombinant MtrC protein containing tetracysteine and V5 engineered tags; MtrC$_{wt}$, wild-type MtrC; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCEP, tris-(carboxyethyl)phosphine.

■ REFERENCES

(1) Shi, L., Squier, T. C., Zachara, J. M., and Fredrickson, J. K. (2007) Respiration of metal (hydr)oxides by Shewanella and Geobacter: a key role for multihaem c-type cytochromes. *Mol. Microbiol. 65*, 12−20.

(2) Coursolle, D., and Grainick, J. (2010) Modularity of the Mtr respiratory pathway of Shewanella oneidensis strain MR-1. *Mol. Microbiol. 77*, 995−1008.

(3) Fredrickson, J. K., and Zachara, J. M. (2008) Electron transfer at the microbe-mineral interface: a grand challenge in biogeochemistry. *Geobiology 6*, 245−253.

(4) Cianciotto, N. P. (2005) Type II secretion: a protein secretion system for all seasons. *Trends Microbiol 13*, 581−588.

(5) Myers, C. R., and Myers, J. M. (2004) The outer membrane cytochromes of Shewanella oneidensis MR-1 are lipoproteins. *Lett Appl Microbiol 39*, 466−470.

(6) Richardson, D. J. (2000) Bacterial respiration: a flexible process for a changing environment. *Microbiology 146* (Pt 3), 551−571.

(7) Hartshorne, R. S., Jepson, B. N., Clarke, T. A., Field, S. J., Fredrickson, J., Zachara, J., Shi, L., Butt, J. N., and Richardson, D. J. (2007) Characterization of Shewanella oneidensis MtrC: a cell-surface decaheme cytochrome involved in respiratory electron transport to extracellular electron acceptors. *J. Biol. Inorg. Chem. 12*, 1083−1094.

(8) Shi, L., Chen, B., Wang, Z., Elias, D. A., Mayer, M. U., Gorby, Y. A., Ni, S., Lower, B. H., Kennedy, D. W., Wunschel, D. S., Mottaz, H. M., Marshall, M. J., Hill, E. A., Beliaev, A. S., Zachara, J. M., Fredrickson, J. K., and Squier, T. C. (2006) Isolation of a high-affinity functional protein complex between OmcA and MtrC: Two outer membrane decaheme c-type cytochromes of Shewanella oneidensis MR-1. *J. Bacteriol. 188*, 4705−4714.

(9) Clarke, T. A., Edwards, M. J., Gates, A. J., Hall, A., White, G. F., Bradley, J., Reardon, C. L., Shi, L., Beliaev, A. S., Marshall, M. J., Wang, Z., Watmough, N. J., Fredrickson, J. K., Zachara, J. M., Butt, J. N., and Richardson, D. J. (2011) Structure of a bacterial cell surface decaheme electron conduit. *Proc. Natl. Acad. Sci. U. S. A. 108*, 9384−9389.

(10) Xiong, Y., Shi, L., Chen, B., Mayer, M. U., Lower, B. H., Londer, Y., Bose, S., Hochella, M. F., Fredrickson, J. K., and Squier, T. C. (2006) High-affinity binding and direct electron transfer to solid metals by the Shewanella oneidensis MR-1 outer membrane c-type cytochrome OmcA. *J. Am. Chem. Soc. 128*, 13978−13979.

(11) Myers, J. M., and Myers, C. R. (2001) Role for outer membrane cytochromes OmcA and OmcB of Shewanella putrefaciens MR-1 in reduction of manganese dioxide. *Appl. Environ. Microbiol. 67*, 260−269.

(12) Hartshorne, R. S., Reardon, C. L., Ross, D., Nuester, J., Clarke, T. A., Gates, A. J., Mills, P. C., Fredrickson, J. K., Zachara, J. M., Shi, L., Beliaev, A. S., Marshall, M. J., Tien, M., Brantley, S., Butt, J. N., and Richardson, D. J. (2009) Characterization of an electron conduit between bacteria and the extracellular environment. *Proc. Natl. Acad. Sci. U. S. A. 106*, 22169−22174.

(13) Zhang, H., Tang, X., Munske, G. R., Zakharova, N., Yang, L., Zheng, C., Wolff, M. A., Tolic, N., Anderson, G. A., Shi, L., Marshall, M. J., Fredrickson, J. K., and Bruce, J. E. (2008) In vivo identification of the outer membrane protein OmcA-MtrC interaction network in Shewanella oneidensis MR-1 cells using novel hydrophobic chemical cross-linkers. *J. Proteome Res. 7*, 1712−1720.

(14) Ross, D. E., Ruebush, S. S., Brantley, S. L., Hartshorne, R. S., Clarke, T. A., Richardson, D. J., and Tien, M. (2007) Characterization of protein-protein interactions involved in iron reduction by Shewanella oneidensis MR-1. *Appl. Environ. Microbiol. 73*, 5797−5808.

(15) Shi, L., Deng, S., Marshall, M. J., Wang, Z., Kennedy, D. W., Dohnalkova, A. C., Mottaz, H. M., Hill, E. A., Gorby, Y. A., Beliaev, A. S., Richardson, D. J., Zachara, J. M., and Fredrickson, J. K. (2008) Direct involvement of type II secretion system in extracellular translocation of Shewanella oneidensis outer membrane cytochromes MtrC and OmcA. *J. Bacteriol. 190*, 5512−5516.

(16) DiChristina, T. J., Moore, C. M., and Haller, C. A. (2002) Dissimilatory Fe(III) and Mn(IV) reduction by Shewanella putrefaciens requires ferE, a homolog of the pulE (gspE) type II protein secretion gene. *J. Bacteriol. 184*, 142−151.

(17) Biju, V., Pan, D., Gorby, Y. A., Fredrickson, J., McLean, J., Saffarini, D., and Lu, H. P. (2007) Combined spectroscopic and topographic characterization of nanoscale domains and their distributions of a redox protein on bacterial cell surfaces. *Langmuir 23*, 1333−1338.

(18) Marshall, M. J., Beliaev, A. S., Dohnalkova, A. C., Kennedy, D. W., Shi, L., Wang, Z., Boyanov, M. I., Lai, B., Kemner, K. M., McLean, J. S., Reed, S. B., Culley, D. E., Bailey, V. L., Simonson, C. J., Saffarini, D. A., Romine, M. F., Zachara, J. M., and Fredrickson, J. K. (2006) c-Type cytochrome-dependent formation of U(IV) nanoparticles by Shewanella oneidensis. *PLoS Biol. 4*, e268.

(19) El-Naggar, M. Y., Wanger, G., Leung, K. M., Yuzvinsky, T. D., Southam, G., Yang, J., Lau, W. M., Nealson, K. H., and Gorby, Y. A. (2010) Electrical transport along bacterial nanowires from Shewanella oneidensis MR-1. *Proc. Natl. Acad. Sci. U. S. A. 107*, 18127−18131.

(20) Harris, H. W., El-Naggar, M. Y., Bretschger, O., Ward, M. J., Romine, M. F., Obraztsova, A. Y., and Nealson, K. H. (2010) Electrokinesis is a microbial behavior that requires extracellular electron transport. *Proc. Natl. Acad. Sci. U. S. A. 107*, 326−331.

(21) Gorby, Y. A., Yanina, S., McLean, J. S., Rosso, K. M., Moyles, D., Dohnalkova, A., Beveridge, T. J., Chang, I. S., Kim, B. H., Kim, K. S., Culley, D. E., Reed, S. B., Romine, M. F., Saffarini, D. A., Hill, E. A., Shi, L., Elias, D. A., Kennedy, D. W., Pinchuk, G., Watanabe, K., Ishii, S., Logan, B., Nealson, K. H., and Fredrickson, J. K. (2006) Electrically conductive bacterial nanowires produced by Shewanella oneidensis strain MR-1 and other microorganisms. *Proc. Natl. Acad. Sci. U. S. A. 103*, 11358−11363.

(22) Jensen, H. M., Albers, A. E., Malley, K. R., Londer, Y. Y., Cohen, B. E., Helms, B. A., Weigele, P., Groves, J. T., and Ajo-Franklin, C. M. (2010) Engineering of a synthetic electron conduit in living cells. *Proc. Natl. Acad. Sci. U. S. A. 107*, 19213−19218.

(23) Brown, R. N., Romine, M. F., Schepmoes, A. A., Smith, R. D., and Lipton, M. S. (2010) Mapping the subcellular proteome of Shewanella oneidensis MR-1 using sarkosyl-based fractionation and LC-MS/MS protein identification. *J. Proteome Res. 9*, 4454−4463.

(24) Sanders, C., Turkarslan, S., Lee, D. W., and Daldal, F. (2010) Cytochrome c biogenesis: the Ccm system. *Trends Microbiol. 18*, 266−274.

(25) Cao, H., Chen, B., Squier, T. C., and Mayer, M. U. (2006) CrAsH: a biarsenical multi-use affinity probe with low non-specific fluorescence. *Chem. Commun. (Cambridge, U. K.)*, 2601−2603.

(26) Adams, S. R., Campbell, R. E., Gross, L. A., Martin, B. R., Walkup, G. K., Yao, Y., Llopis, J., and Tsien, R. Y. (2002) New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. *J. Am. Chem. Soc. 124*, 6063−6076.

(27) Shi, L., Lin, J. T., Markillie, L. M., Squier, T. C., and Hooker, B. S. (2005) Overexpression of multi-heme C-type cytochromes. *Biotechniques 38*, 297−299.
(28) Chen, B., Mayer, M. U., Markillie, L. M., Stenoien, D. L., and Squier, T. C. (2005) Dynamic motion of helix A in the amino-terminal domain of calmodulin is stabilized upon calcium activation. *Biochemistry 44*, 905−914.
(29) Lower, B. H., Yongsunthon, R., Shi, L., Wildling, L., Gruber, H. J., Wigginton, N. S., Reardon, C. L., Pinchuk, G. E., Droubay, T. C., Boily, J. F., and Lower, S. K. (2009) Antibody recognition force microscopy shows that outer membrane cytochromes OmcA and MtrC are expressed on the exterior surface of Shewanella oneidensis MR-1. *Appl. Environ. Microbiol. 75*, 2931−2935.
(30) Patterson, G., Day, R. N., and Piston, D. (2001) Fluorescent protein spectra. *J. Cell Sci. 114*, 837−838.
(31) Yang, Y., Harris, D. P., Luo, F., Xiong, W., Joachimiak, M., Wu, L., Dehal, P., Jacobsen, J., Yang, Z., Palumbo, A. V., Arkin, A. P., and Zhou, J. (2009) Snapshot of iron response in Shewanella oneidensis by gene network reconstruction. *BMC Genomics 10*, 131.
(32) Bigelow, D. J., and Squier, T. C. (2005) Redox modulation of cellular signaling and metabolism through reversible oxidation of methionine sensors in calcium regulatory proteins. *Biochim. Biophys. Acta 1703*, 121−134.
(33) Schoneich, C. (2005) Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease. *Biochim. Biophys. Acta 1703*, 111−119.
(34) Levine, R. L., Mosoni, L., Berlett, B. S., and Stadtman, E. R. (1996) Methionine residues as endogenous antioxidants in proteins. *Proc. Natl. Acad. Sci. U. S. A. 93*, 15036−15040.
(35) Levine, R. L., Oliver, C. N., Fulks, R. M., and Stadtman, E. R. (1981) Turnover of bacterial glutamine synthetase: oxidative inactivation precedes proteolysis. *Proc. Natl. Acad. Sci. U. S. A. 78*, 2120−2124.
(36) Eldar, A., and Elowitz, M. B. (2010) Functional roles for noise in genetic circuits. *Nature 467*, 167−173.
(37) Genin, E., Carion, O., Mahler, B., Dubertret, B., Arhel, N., Charneau, P., Doris, E., and Mioskowski, C. (2008) CrAsH-quantum dot nanohybrids for smart targeting of proteins. *J. Am. Chem. Soc. 130*, 8596−8597.
(38) Chen, B., Cao, H., Yan, P., Mayer, M. U., and Squier, T. C. (2007) Identification of an orthogonal peptide binding motif for biarsenical multiuse affinity probes. *Bioconjugate Chem. 18*, 1259−1265.
(39) Griffin, B. A., Adams, S. R., Jones, J., and Tsien, R. Y. (2000) Fluorescent labeling of recombinant proteins in living cells with FlAsH. *Methods Enzymol. 327*, 565−578.
(40) Hoffmann, C., Gaietta, G., Bunemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilardaga, J. P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. (2005) A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nature Methods 2*, 171−176.
(41) Martin, B. R., Giepmans, B. N., Adams, S. R., and Tsien, R. Y. (2005) Mammalian cell-based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity. *Nature Biotechnol. 23*, 1308−1314.
(42) Sosinsky, G. E., Gaietta, G. M., Hand, G., Deerinck, T. J., Han, A., Mackey, M., Adams, S. R., Bouwer, J., Tsien, R. Y., and Ellisman, M. H. (2003) Tetracysteine genetic tags complexed with biarsenical ligands as a tool for investigating gap junction structure and dynamics. *Cell Commun. Adhes. 10*, 181−186.
(43) Tour, O., Adams, S. R., Kerr, R. A., Meijer, R. M., Sejnowski, T. J., Tsien, R. W., and Tsien, R. Y. (2007) Calcium Green FlAsH as a genetically targeted small-molecule calcium indicator. *Nature Chem. Biol. 3*, 423−431.
(44) Chen, B., Markillie, L. M., Xiong, Y., Mayer, M. U., and Squier, T. C. (2007) Increased catalytic efficiency following gene fusion of bifunctional methionine sulfoxide reductase enzymes from Shewanella oneidensis. *Biochemistry 46*, 14153−14161.
(45) Stenoien, D. L., Knyushko, T. V., Londono, M. P., Opresko, L. K., Mayer, M. U., Brady, S. T., Squier, T. C., and Bigelow, D. J. (2007) Cellular trafficking of phospholamban and formation of functional sarcoplasmic reticulum during myocyte differentiation. *Am. J. Physiol. 292*, C2084−2094.
(46) Verma, S., Xiong, Y., Mayer, M. U., and Squier, T. C. (2007) Remodeling of the bacterial RNA polymerase supramolecular complex in response to environmental conditions. *Biochemistry 46*, 3023−3035.
(47) Yan, P., Xiong, Y., Chen, B., Negash, S., Squier, T. C., and Mayer, M. U. (2006) Fluorophore-assisted light inactivation of calmodulin involves singlet-oxygen mediated cross-linking and methionine oxidation. *Biochemistry 45*, 4736−4748.
(48) Hoffmann, C., Gaietta, G., Zurn, A., Adams, S. R., Terrillon, S., Ellisman, M. H., Tsien, R. Y., and Lohse, M. J. (2010) Fluorescent labeling of tetracysteine-tagged proteins in intact cells. *Nature Protoc. 5*, 1666−1677.
(49) Domingo, B., Sabariegos, R., Picazo, F., and Llopis, J. (2007) Imaging FRET standards by steady-state fluorescence and lifetime methods. *Microsc. Res. Tech. 70*, 1010−1021.
(50) Eden, E., Geva-Zatorsky, N., Issaeva, I., Cohen, A., Dekel, E., Danon, T., Cohen, L., Mayo, A., and Alon, U. (2011) Proteome half-life dynamics in living human cells. *Science 331*, 764−768.
(51) Py, B., Loiseau, L., and Barras, F. (2001) An inner membrane platform in the type II secretion machinery of Gram-negative bacteria. *EMBO Rep. 2*, 244−248.
(52) Gorby, Y., McLean, J., Korenevsky, A., Rosso, K., El-Naggar, M. Y., and Beveridge, T. J. (2008) Redox-reactive membrane vesicles produced by Shewanella. *Geobiology 6*, 232−241.
(53) Plotkin, J. B. (2011) Cell biology. The lives of proteins. *Science 331*, 683−684.
(54) Cao, B., Shi, L., Brown, R. N., Xiong, Y., Fredrickson, J. K., Romine, M. F., Marshall, M. J., Lipton, M. S., and Beyenal, H. (2011) Extracellular polymeric substances from Shewanella sp. HRCR-1 biofilms: characterization by infrared spectroscopy and proteomics. *Environ. Microbiol.*, DOI: 10.1111/j.1462-2929.2010.02407.x.
(55) Elowitz, M. B., Surette, M. G., Wolf, P. E., Stock, J., and Leibler, S. (1997) Photoactivation turns green fluorescent protein red. *Curr. Biol. 7*, 809−812.
(56) Miyawaki, A., Nagai, T., and Mizuno, H. (2003) Mechanisms of protein fluorophore formation and engineering. *Curr. Opin. Chem. Biol. 7*, 557−562.
(57) Remington, S. J. (2006) Fluorescent proteins: maturation, photochemistry and photophysics. *Curr. Opin. Struct. Biol. 16*, 714−721.
(58) Lower, B. H., Shi, L., Yongsunthon, R., Droubay, T. C., McCready, D. E., and Lower, S. K. (2007) Specific bonds between an iron oxide surface and outer membrane cytochromes MtrC and OmcA from Shewanella oneidensis MR-1. *J. Bacteriol. 189*, 4944−4952.
(59) Wang, K. H., Oakes, E. S., Sauer, R. T., and Baker, T. A. (2008) Tuning the strength of a bacterial N-end rule degradation signal. *J. Biol. Chem. 283*, 24600−24607.
(60) Schicklberger, M., Bucking, C., Schuetz, B., Heide, H., and Gescher, J. (2011) Involvement of the Shewanella oneidensis decaheme cytochrome MtrA in the periplasmic stability of the beta-barrel protein MtrB. *Appl. Environ. Microbiol. 77*, 1520−1523.
(61) Butler, S. M., Festa, R. A., Pearce, M. J., and Darwin, K. H. (2006) Self-compartmentalized bacterial proteases and pathogenesis. *Mol. Microbiol. 60*, 553−562.
(62) Urban, S. (2009) Making the cut: central roles of intramembrane proteolysis in pathogenic microorganisms. *Nature Rev. 7*, 411−423.
(63) Langer, T. (2000) AAA proteases: cellular machines for degrading membrane proteins. *Trends Biochem. Sci. 25*, 247−251.
(64) Subrini, O., and Betton, J. M. (2009) Assemblies of DegP underlie its dual chaperone and protease function. *FEMS Microbiol. Lett. 296*, 143−148.
(65) Ades, S. E. (2008) Regulation by destruction: design of the sigmaE envelope stress response. *Curr. Opin. Microbiol. 11*, 535−540.

(66) Kopito, R. R. (1999) Biosynthesis and degradation of CFTR. *Physiol. Rev. 79*, S167−173.
(67) Amaral, M. D. (2005) Processing of CFTR: traversing the cellular maze--how much CFTR needs to go through to avoid cystic fibrosis? *Pediatr. Pulmonol. 39*, 479−491.
(68) Reardon, C. L., Dohnalkova, A. C., Nachimuthu, P., Kennedy, D. W., Saffarini, D. A., Arey, B. W., Shi, L., Wang, Z., Moore, D., McLean, J. S., Moyles, D., Marshall, M. J., Zachara, J. M., Fredrickson, J. K., and Beliaev, A. S. (2010) Role of outer-membrane cytochromes MtrC and OmcA in the biomineralization of ferrihydrite by Shewanella oneidensis MR-1. *Geobiology 8*, 56−68.