

Light-Driven H₂ Evolution and C=C or C=O Bond Hydrogenation by *Shewanella oneidensis*: A Versatile Strategy for Photocatalysis by Nonphotosynthetic Microorganisms

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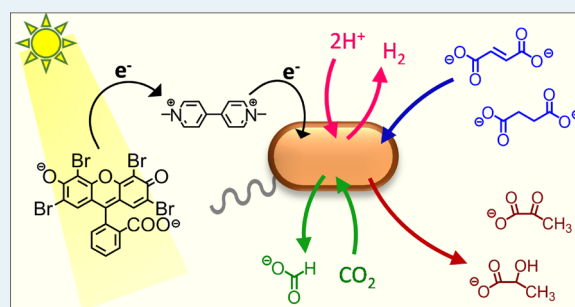
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Supporting Information

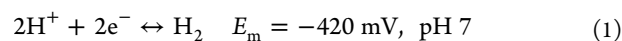
ABSTRACT: Photocatalytic chemical synthesis by coupling abiotic photosensitizers to purified enzymes provides an effective way to overcome the low conversion efficiencies of natural photosynthesis while exploiting the high catalytic rates and selectivity of enzymes as renewable, earth-abundant electrocatalysts. However, the selective synthesis of multiple products requires more versatile approaches and should avoid the time-consuming and costly processes of enzyme purification. Here we demonstrate a cell-based strategy supporting light-driven H₂ evolution or the hydrogenation of C=C and C=O bonds in a nonphotosynthetic microorganism. Methylviologen shuttles photoenergized electrons from water-soluble photosensitizers to enzymes that catalyze H₂ evolution and the reduction of fumarate, pyruvate, and CO₂ in *Shewanella oneidensis*. The predominant reaction is selected by the experimental conditions, and the results allow rational development of cell-based strategies to harness nature's intrinsic catalytic diversity for selective light-driven synthesis of a wide range of products.

KEYWORDS: proton reduction, CO₂ reduction, photocatalysis, hydrogenase, formate dehydrogenase, visible light



INTRODUCTION

Photovoltaics provide scalable and cost-efficient conversion of solar energy to electricity and the inspiration for developing equally effective routes to solar chemicals for a sustainable society. An example for such processes is natural photosynthesis, but as it is primarily designed to convert CO₂ to sugars, this process presents several challenges for controlled generation of additional products. An example of contemporary interest is solar H₂ production through water splitting.^{1–4} Certain algae and cyanobacteria can deliver photoenergized electrons from oxygenic, plantlike photosynthesis to hydrogenase enzymes that catalyze H₂ evolution from water (eq 1; all potentials are given vs SHE).



However, scalable solutions to biological H₂ production by this route have yet to be demonstrated, due to typical

requirements of anaerobicity, sulfur deprivation, and the absence of CO₂. Furthermore, the process shares drawbacks with other routes to developing natural photosynthesis for solar chemicals production such as inefficient energy conversion⁵ and reducing equivalents partitioned across competing metabolic pathways.⁶

To address these challenges, it is attractive to consider the oxidative and reductive half-reactions in isolation and the possible advantages of combining biotic and abiotic materials for photocatalysis. For example, abiotic photosensitizers can improve on Photosystems I and II as light-harvesting components by offering increased absorption across the incident solar spectrum, effective charge separation, and

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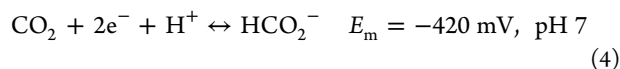
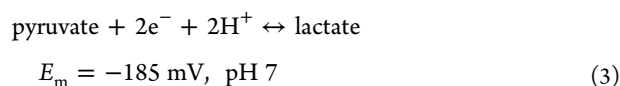
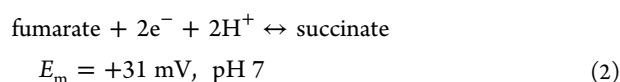
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photosensitizer longevity.^{7–10} However, enzymes remain attractive renewable electrocatalysts^{11–13} and often deliver performances that match, or exceed, those of their best abiotic counterparts.^{14–17} As a consequence, molecular dyes, (nano-structured) semiconductors, and quantum dots have been widely employed to deliver photoenergized electrons to purified enzymes for photocatalysis.^{12,18–23} Nevertheless, there are significant bottlenecks to delivering scalable solar-driven synthesis by such approaches. Enzyme purification can be costly and time consuming, whereas methods to overcome the limited stability of the purified materials, for example through their covalent attachment to solid materials,^{24,25} may be necessary. In seeking to harness nature's wide-ranging catalytic diversity to meet societal needs, there are many opportunities to evaluate alternate and ideally more versatile approaches.

Protein purification is avoided through the use of cell-based systems. For example, acetate was produced from CO₂ and sunlight using *Moorella thermoacetica*^{26,27} with self-generated CdS nanoparticles as photosensitizers. Similarly, catalytic monooxygenation was achieved by delivering photoenergized electrons from the organic dye eosin Y to a P450 heme domain introduced into *Escherichia coli*.²⁸ Motivated by these examples, we reasoned that it should be possible to develop cell-based strategies affording selective access to a wide product range. A single microorganism would serve as a multifaceted catalyst with a repertoire defined by its intrinsic, or genetically enhanced, complement of enzymes and the light-driven reaction selected by user-defined conditions.

Here we demonstrate this concept for the nonphotosynthetic γ -proteobacterium *Shewanella oneidensis* MR-1 (MR-1) (Figure 1a). Methylviologen (MV, $E_m \approx -440$ mV,²⁹ pH 7) shuttles electrons from water-compatible photosensitizers to enzymes in MR-1 that catalyze four transformations of current interest: namely, H₂ evolution (eq 1), fumarate reduction as an example of C=C bond hydrogenation (eq 2), and the reductions of pyruvate and CO₂ as examples of C=O bond hydrogenation (eqs 3 and 4).



Notably, photocatalysis is not dependent on the presence of outer membrane spanning porin:cytochrome (Mtr) complexes of MR-1 that can facilitate electron exchange between extra- and intracellular redox couples.^{30–32} Instead, light-driven chemistry is underpinned by the well-described^{16,33–35} abilities of MV to permeate cells and deliver electrons to numerous enzymes such that we envisage this strategy providing a versatile route to accessing a diversity of products from enzymes in numerous microbial species and genetically engineered strains. Our quantitative assessment of the determinants of light-driven synthesis provides a framework for the rational development of this strategy to access a selectable product range with control of product distribution.

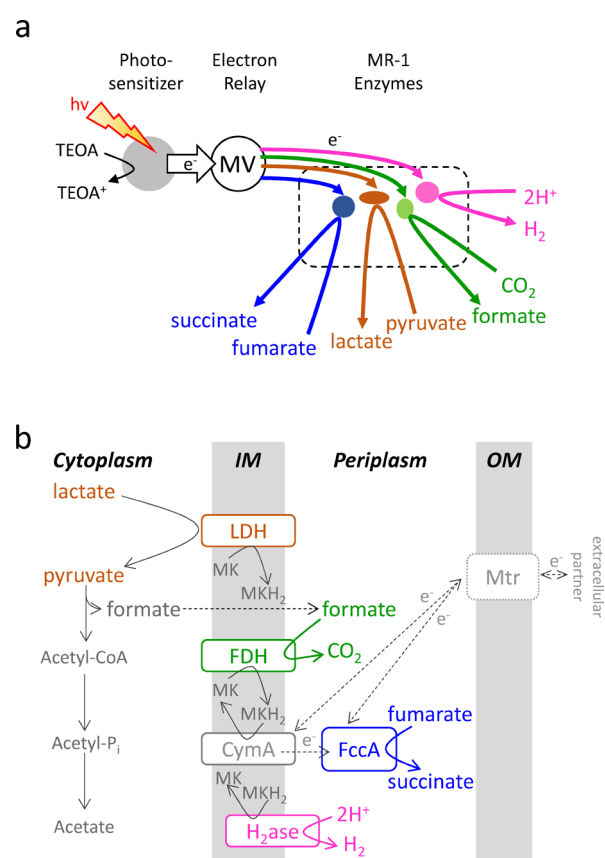


Figure 1. Cell-based photocatalysis by *S. oneidensis* MR-1. (a) Schematic of the strategy for MR-1-dependent photocatalytic reduction of protons, CO₂, fumarate, or pyruvate. Methylviologen (MV) relays electrons to MR-1 enzymes from abiotic photosensitizers with triethanolamine (TEOA) as a sacrificial electron donor. (b) Key MR-1 enzymes relevant to this study and anaerobic respiration by lactate oxidation coupled to the reduction of fumarate or protons (see text for details). Enzymes are lactate dehydrogenase (LDH), formate dehydrogenase (FDH), fumarate reductase (FccA), the [NiFe]- and [FeFe]-hydrogenases (H₂ase), and porin:cytochrome complexes (Mtr). IM denotes the inner membrane, OM the outer membrane, and MK menaquinone.

RESULTS AND DISCUSSION

Our findings are presented in the following order. First we demonstrate MR-1 can be cultured to support the four targeted transformations (eqs 1–4). Then we identify photosensitizers and sacrificial electron donors that allow photoreduction of MV²⁺ under conditions compatible with MR-1 catalysis. Finally we present quantitative analysis of MV-dependent light-driven chemical transformations by MR-1. Unless stated otherwise, experiments were performed in anaerobic solutions of 50 mM HEPES, 50 mM NaCl at pH 7. Reduction of CO₂ was investigated in solutions of dissolved sodium carbonate, and for simplicity the term “CO₂” is used to include the HCO₃⁻, H₂CO₃, and dissolved CO₂ present in the equilibrated solutions. Quantification of product (substrate) is given in nanomoles to aid comparison across experiments designed to probe dissolved and gaseous species as described; full details can be found in the [Supporting Information](#).

MR-1 Supports Chemically Driven Reduction of Protons, Fumarate, Pyruvate, and CO₂. Our approach to delivering cell-based photocatalytic production of multiple chemicals was informed by the metabolic capabilities of MR-1

(Figure 1b). This bacterium has [NiFe]- and [FeFe]-hydrogenases^{36–38} and catalyzes pyruvate reduction by the action of lactate dehydrogenase,^{39,40} fumarate reduction by flavocytochrome *c*₃,⁴¹ and formate oxidation by formate dehydrogenases.⁴² While the reverse of the latter reaction, CO₂ reduction, has not to our knowledge been reported for MR-1, there is precedent from the behavior of homologous enzymes^{16,43,44} that this chemistry may be possible.

As outlined in Figure 1b, MR-1 can grow anaerobically when oxidation of the electron donor lactate to acetate and CO₂ is coupled to reduction of the terminal electron acceptor fumarate to succinate.^{39,42} Lactate oxidation becomes coupled to proton reduction upon depletion of fumarate during acceptor-limited growth.^{36–38} As a consequence, and with the aim to produce MR-1 containing enzymes active toward the four desired transformations, an inoculum (2% v/v) of MR-1 was introduced to Hungate tubes that contained M72 medium (see Methods in the Supporting Information) with 37.5 mM lactate as the electron donor and 18.8 mM fumarate as the electron acceptor (10 mL media, 7 mL headspace of N₂). Growth of the cultures was monitored spectrophotometrically at 590 nm (OD_{590 nm}), and headspace H₂ was analyzed by gas chromatography (Figure 2a). There was no evidence for H₂ production in parallel experiments that omitted MR-1 or used a strain⁴⁵ (HydA⁻/HyaB⁻) without functional hydrogenases (Figure 2a). It was concluded that MR-1 contained active hydrogenases after culture for at least 18 h under the selected conditions.

Sodium dithionite, a chemical reductant that readily reduces MV²⁺ to MV⁺, was used to assess whether MV⁺ would reduce the MR-1 hydrogenases. Sodium dithionite was added to samples containing MV and MR-1 grown for 24 h as described above, harvested anaerobically, and resuspended in the desired solution to OD_{590 nm} ≈ 0.25 (equivalent to ~0.17 mg of protein mL⁻¹). A H₂-sensing electrode in the sample reported an accumulation of 725 ± 97 nmol of H₂ over 30 min (Figure 2b). Without MV there was 60-fold less H₂ (Figure 2b), revealing MV to be an effective facilitator of electron transfer to MR-1 hydrogenases.

The activity of MR-1 toward fumarate reduction was assessed by 30 min incubation of cells (OD_{590 nm} ≈ 0.25, 1 mL), MV (0.5 mM), and sodium dithionite (0.8 mg mL⁻¹) with sodium fumarate (~4700 nmol). After the cells had been pelleted by centrifugation, the supernatant was removed and analyzed by ¹H NMR spectroscopy. The presence of succinate at ~3800 nmol and fumarate at ~600 nmol revealed that the desired reaction had occurred (Table S1 and Figure S1 in the Supporting Information). An equivalent experiment with pyruvate (~2200 nmol) in place of fumarate demonstrated formation of lactate (~900 nmol) with pyruvate consumption (Table S1). Formate (~1500 nmol) was produced when MR-1 was incubated with CO₂ (~5000 nmol) (Table S1). Thus, MR-1 cultured under our chosen conditions catalyzed all four reactions of interest: namely, the reduction of protons, fumarate, pyruvate, and CO₂ to, respectively, H₂, succinate, lactate, and formate.

Light-Driven Production of MV⁺ in Conditions Compatible with MR-1 Biocatalysis. Our strategy for cell-based photocatalysis requires effective transfer of photoenergized electrons to a number of MR-1 enzymes (Figure 1a). The ability of MV to permeate bacterial cells and deliver electrons from chemical reductants to numerous enzymes is well-documented and the basis of many biochemical

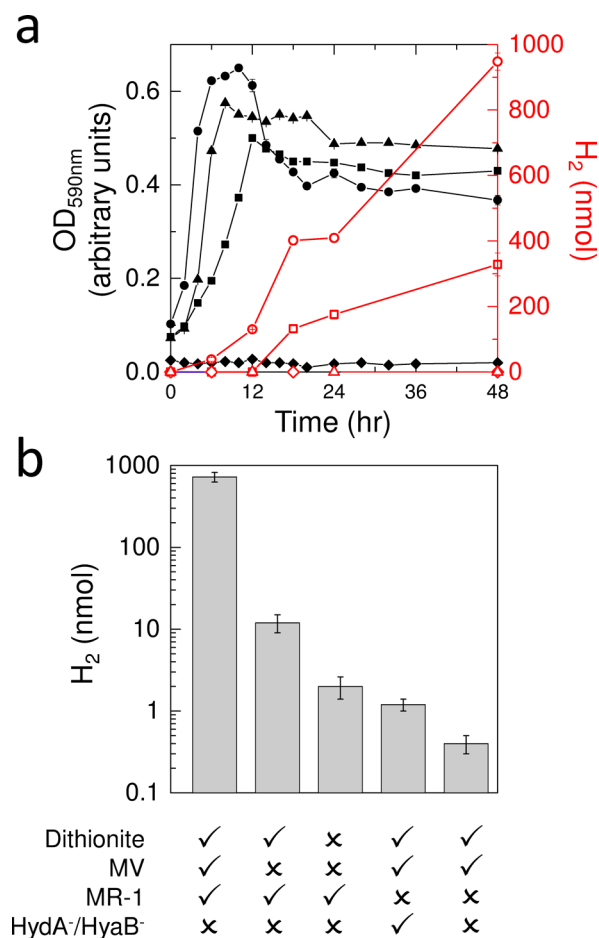


Figure 2. Hydrogenase activity of MR-1. (a) Growth curves (black) and headspace H₂ (red) for MR-1 (squares), HydA⁻/HyaB⁻ (triangles), Mtr⁻ (circles), or no bacteria (diamonds). Inoculation was at 0 h of media (10 mL) with 37.5 mM lactate and 18.8 mM fumarate. Samples had 7 mL headspace (100% N₂ at inoculation). Optical densities (OD_{590 nm}) are mean values (*n* = 4), and the majority of error bars with standard error are too small to resolve. Headspace H₂ mean values (*n* = 2) are given; error bars indicate maximum and minimum. Lines serve as a guide to the eye. (b) Dissolved H₂ in anaerobic suspensions of MR-1 or HydA⁻/HyaB⁻ (OD_{590 nm} ≈ 0.25 for 1.29 mL volume with negligible headspace) 30 min after addition of dithionite (0.3 mg mL⁻¹) and/or MV (0.3 mM) as indicated in 50 mM HEPES, 50 mM NaCl, pH 7. Mean values with standard error from five biological replicates for MR-1, dithionite, and MV are given. Other values are means from two biological replicates; error bars indicate maximum and minimum.

assays.^{16,33–35} This property of MV mediates electron exchange between bacteria and electrodes in microbial fuel cells.^{46,47} In addition, water-compatible dyes^{34,35,48–51} are reported to photoreduce MV²⁺ to MV⁺, and MV has been shown to support photocatalytic H₂ production by relaying electrons from TiO₂ and Bi₂O₃ semiconductor particles to hydrogenases inside *E. coli*,^{52–54} *Rhodospseudomonas capsulate*,⁵⁰ and *Clostridium butyricum*.⁵⁵ Here, we confirmed the photocatalytic reduction of MV²⁺ under conditions where MR-1 performed the four desired chemical transformations by representatives of three classes of photosensitizer: an acridine dye proflavine,⁴⁸ the xanthene dyes eosin Y⁴⁹ and fluorescein,⁵⁰ and the inorganic complexes Ru(bpy)₃²⁺ (bpy = 2,2'-bipyridine)⁵¹ and Ru(bpy)₂(4,4'-(PO₃H₂)₂bpy)²⁺ (RuP).^{56,57} Structures and

spectral properties of these molecules are presented in Figure S2 and Table S2 in the Supporting Information.

The ability of the selected dyes to photoreduce colorless MV²⁺ to blue MV⁺ was assessed by electronic absorption spectroscopy after 10 min exposure to visible light (0.7 kW m⁻², see Methods and Figure S3 in the Supporting Information) in the presence of triethanolamine (TEOA, E_m ≈ +820 mV,⁵⁸ pH 7) as a sacrificial electron donor (Figure 3).

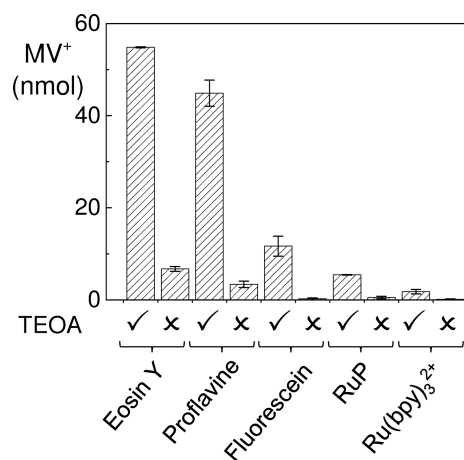


Figure 3. Photoreduction of MV by xanthene, acridine, and Ru(II) dyes. MV⁺ was quantified after 10 min irradiation (0.7 kW m⁻²) of MV²⁺ (0.061 mM) with the indicated photosensitizer (0.02 mM) in the presence or absence of TEOA (12 mM) as a sacrificial electron donor. Anaerobic samples (1 mL volume) with 50 mM HEPES, 50 mM NaCl, pH 7. The path length was 1 cm. Mean values (*n* = 2) are given; error bars indicate maximum and minimum.

The rate of MV⁺ formation was dependent on photosensitizer and decreased in the order eosin Y > proflavine > fluorescein > RuP > Ru(bpy)₃²⁺ (Figure 3). Replacing TEOA by other established sacrificial electron donors⁵⁹ photogenerated less MV⁺ (Figure S3). As a consequence and unless stated otherwise, TEOA was included in the experiments described below that quantified photocatalysis by MR-1.

Quenching of a photosensitizer excited state (PS*) can occur by two pathways (Figure S4a in the Supporting Information). For reductive quenching PS* oxidizes TEOA; this forms PS⁻, which can then reduce MV²⁺ to MV⁺ and in the process regenerate PS⁰. For oxidative quenching PS* reduces MV²⁺ to MV⁺; this forms PS⁺, which can then oxidize TEOA to re-form PS⁰. To gain insight into the mechanism(s) operating under our conditions, fluorescence intensity was assessed as a function of the concentration of TEOA or MV²⁺ (Figure S4b). For all photosensitizers the Stern–Volmer plots revealed MV²⁺ to be a more effective quencher than TEOA. Why then is photo-production of MV⁺ (Figure 3) greater in the presence than in the absence of TEOA? Making the reasonable assumption that fluorescence quenching arises from electron transfer and noting that the PS⁺⁰ couples are >+900 mV (Table S2 in the Supporting Information), we can explain this observation if PS⁺, generated by oxidative quenching with MV⁺ formation, is returned to PS⁰ by oxidation of excess TEOA rather than MV⁺ (Figure S4a). The Stern–Volmer plots fail to provide immediate insight into the relative extents of MV⁺ photo-production by the PS studied, for which a full description requires resolution of multiple electron transfer rates (Figure

S4a) and appropriate consideration of the spectral properties of each photosensitizer and the incident light.

Light-Driven H₂ Evolution by MR-1. Visible-light-driven H₂ production by MR-1 was quantified by a H₂-sensing electrode placed in a sealed, anaerobic, and transparent (wavelengths >400 nm) glass vessel filled with an anaerobic cell suspension (1.7 mL, OD_{590 nm} ≈ 0.25): i.e., there was negligible headspace. H₂ accumulated in irradiated suspensions containing MV and a photosensitizer (Figure 4). As is usual for

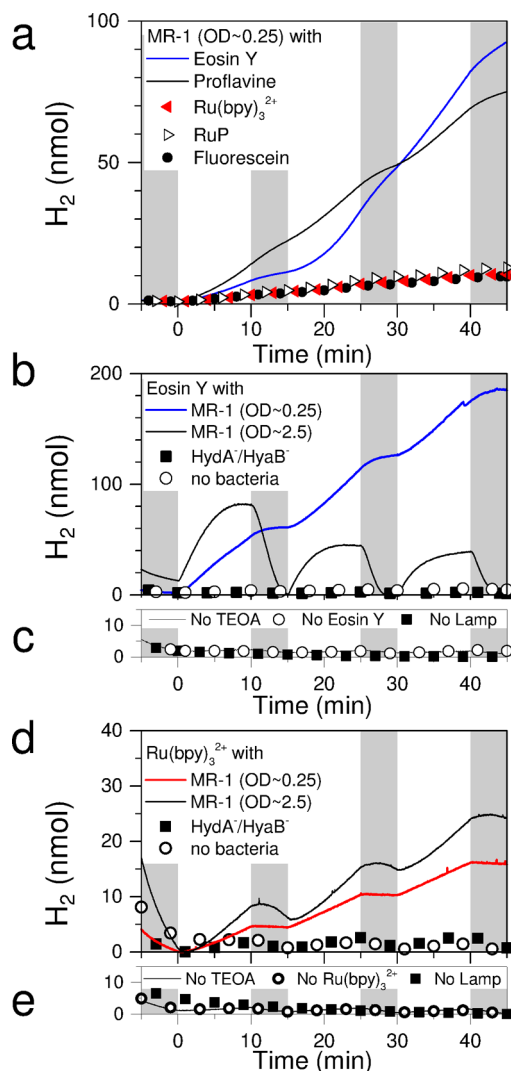


Figure 4. Light-driven H₂ production by MR-1. Samples experienced ambient light throughout and irradiation by visible light at 0.7 kW m⁻² between 0 and 10, 15 and 25, and 30 and 40 min (white panels). (a) MR-1 suspensions (OD_{590 nm} ≈ 0.25) with the indicated photosensitizers (0.11 mM), TEOA (60 mM), and MV (0.3 mM). (b) Suspensions of MR-1 (OD_{590 nm} ≈ 0.25 or 2.5 as indicated) and HydA⁻/HyaB⁻ (OD_{590 nm} ≈ 0.25) with eosin Y (0.11 mM), TEOA (60 mM), and MV (0.3 mM). (c) As for the blue line in (b) except for the omission of TEOA, eosin Y, or irradiation at 0.7 kW m⁻² as indicated. (d) Suspensions of MR-1 (OD_{590 nm} ≈ 0.25 or 2.5 as indicated) and HydA⁻/HyaB⁻ (OD_{590 nm} ≈ 0.25) with Ru(bpy)₃²⁺ (0.11 mM), TEOA (60 mM), and MV (0.3 mM). (e) As for the red line in (d) except for the omission of TEOA, Ru(bpy)₃²⁺, or irradiation at 0.7 kW m⁻² as indicated. Anaerobic samples (1.7 mL volume, negligible headspace) contained 50 mM HEPES, 50 mM NaCl at pH 7 and 23 °C. Different MR-1 cultures were aliquoted for the experiments of (a), of (b, c), and of (d, e) (see text for details).

studies with biological samples, the use of cells from separate cultures produced some variation (<3-fold) in the absolute amount of product for a given condition (e.g. Figure S5 in the Supporting Information). Nevertheless, and most significantly for the work presented here, the H₂ generated varied in a reproducible manner with changes in experimental parameters as described below.

Effect of Photosensitizer Identity. The nature of the photosensitizer was found to have a decisive effect on the amount of H₂ produced (e.g., Figure 4a). Eosin Y and proflavine supported most H₂ production. Fluorescein, Ru(bpy)₃²⁺, and RuP generated 7-fold less H₂, in accord with the relative effectiveness of these photosensitizers for MV²⁺ photoreduction (Figure 3). No detectable H₂ was produced when photosensitizer, irradiation, or TEOA was omitted (e.g. Figure 4c,e) or when MR-1 was omitted or replaced by the HydA⁻/HyaB⁻ strain (e.g. Figure 4b,d). Omission of MV, discussed further below, also led to no detectable H₂ production (e.g., Figure 5). Taken together, these observations demonstrate light-driven H₂ evolution by MR-1 hydrogenases using the selected photosensitizers with MV as electron relay and TEOA as sacrificial electron donor (Figure 1a).

Effect of MR-1 Cell Density. Further insight into rate-defining features of light-driven H₂ production was gained with eosin Y and Ru(bpy)₃²⁺ as representative photosensitizers. The first series of experiments assessed the effect of a 10-fold increase of MR-1 cell density in samples exposed to periodic irradiation at 0.7 kW m⁻² (Figure 4b,d). As discussed below, over the 45 min experiment H₂ accumulated in all of the samples except that of the higher density MR-1 suspension with eosin Y (Figure 4b,d).

For both photosensitizers the 10-fold increase in cell density led to a 1.5-fold increase in the rate of H₂ evolution during the first 10 min of irradiation (Figure 4b,d, 0 to 10 min). This is significantly less than the 10-fold increase expected if hydrogenase activity contributed to the rate-defining step(s) of H₂ evolution, a point we discuss further in the next section. When the light source was removed, the dissolved H₂ decreased for the higher density cell suspensions (Figure 4b,d, 10 to 15 min), and this was particularly pronounced with eosin Y, where the dissolved H₂ fell below a detectable level. As hydrogenases are reversible enzymes,⁶⁰ this behavior is most likely due to H₂ oxidation coupled to the reduction of MR-1 enzymes and metabolites. Why this effect was more pronounced with eosin Y than with Ru(bpy)₃²⁺ is not apparent, but these patterns were repeated during two further cycles of exposure to irradiation at 0.7 kW m⁻². Over successive cycles of irradiation it was also noted that less H₂ was produced with eosin Y in the higher density cell suspension (Figure 4b). This may be an indication that this photosensitizer is more susceptible to photodamage in these conditions (see below).

Rate-Defining Events in H₂ Production. In view of the results above, the H₂ produced in response to changes in light intensity, pH, or MV concentration was investigated with MR-1 suspensions of OD_{590 nm} ≈ 0.25 irradiated continuously for 30 min (Figure 5). Lower intensity light produced less H₂. The pH dependence of H₂ production with eosin Y was opposite to that with Ru(bpy)₃²⁺. For both dyes the changes were small despite hydrogenases typically⁶⁰ evolving H₂ more rapidly at pH 6 than at pH 8 and neither dye changing protonation state⁶¹ in this pH range. H₂ production did not scale linearly with MV concentration. The highest MV concentration will favor reduction of PS⁺ by oxidation of MV⁺ rather than TEOA

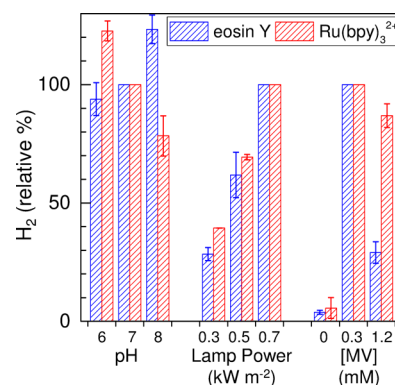


Figure 5. Effect of pH, lamp power, and MV concentration on light-driven H₂ production by MR-1. Dissolved H₂ was determined after 30 min irradiation of gently stirred MR-1 suspensions (OD_{590 nm} ≈ 0.25) with eosin Y or Ru(bpy)₃²⁺ (0.11 mM). The light intensity was 0.7 kW m⁻², at pH 7 with MV present at 0.3 mM unless stated otherwise. Mean values are given for two biological replicates; error bars indicate maximum and minimum. Values are normalized to the mean with 0.3 mM MV, pH 7 irradiated at 0.7 kW m⁻² for each photosensitizer where dissolved H₂ was 33 ± 5 nmol with Ru(bpy)₃²⁺ (*n* = 5) and 242 ± 24 nmol with eosin Y (*n* = 6). Anaerobic samples (1.65 mL volume, negligible headspace) were used with TEOA (60 mM) in 50 mM HEPES, 50 mM NaCl at 23 °C.

(see above), without leading to H₂ production. Very little H₂ was produced in the absence of the MV electron shuttle. These observations, in agreement with those presented above, point to the rate-limiting event(s) of H₂ production involving photosensitizer and MV. Given the previously reported ability of eosin Y to act as an intracellular photosensitizer in *E. coli*²⁸ and to deliver photoenergized electrons directly to *Desulfomicrobium baculatum* [NiFeSe]-hydrogenase,⁶² the requirement for MV to support eosin Y dependent light-driven chemistry in our system is notable. Clearly the requirements for electron delivery to the MR-1 hydrogenases in this cell-based system are more complex than might be inferred from those previous reports.

It is well-established that MV can enter Gram-negative bacteria⁶³ such as MR-1. However, electrons can also enter MR-1 through porin:cytochrome complexes^{30–32} that span the bacterial outer membrane (Figure 1b). The most prevalent of these complexes, MtrCAB, was revealed in our samples when proteins were resolved by SDS-PAGE and visualized by immunoblotting (Figure S6 in the Supporting Information). An MR-1 strain⁶⁴ (Mtr⁻) lacking MtrCAB and the paralog MtrDEF was studied to assess whether these complexes were critical for light-driven H₂ production. The Mtr⁻ strain grew faster and produced more H₂ than MR-1 (Figure 2a), and immunoblots failed to detect MtrCAB in harvested Mtr⁻ (Figure S6a). The enhanced H₂ production may be due to Mtr⁻ having approximately twice the hydrogenase activity of MR-1 (Figure S6b), although increased electron delivery to the hydrogenases in the absence of a reservoir for electrons provided by the hemes of the Mtr complexes is also possible. Nevertheless, light-driven H₂ production with both eosin Y and Ru(bpy)₃²⁺ was comparable to that by MR-1 (Figure S6c). Thus, light-driven H₂ evolution was not dependent on the presence of MtrCAB or MtrDEF complexes in the MR-1 outer membrane and it is likely that MV enters the periplasm to pass electrons to hydrogenases (Figure 1a).

Sustained H₂ Production. The conditions of our studies were chosen for their experimental tractability rather than MR-1 viability: for example, no substrates for growth or cell maintenance reactions were included. Indeed, cells pelleted after 30 min irradiation (0.7 kW m⁻²) with TEOA, MV, and eosin Y or Ru(bpy)₃²⁺ contained respectively no detectable and 77 colony-forming units (CFU) mL⁻¹. Equivalent MR-1 suspensions incubated in the dark contained $\sim 6 \times 10^4$ CFU mL⁻¹, whereas $\sim 7 \times 10^7$ CFU mL⁻¹ was recovered from suspensions irradiated in the absence of photosensitizers, MV, and TEOA. Thus, it appears that electron flow due to irradiation enhances the toxicity of the chemicals required for photocatalysis, which may relate to methylviologen (paraquat) induced stress response(s) in MR-1⁶⁵ that can lead to cell death. Nevertheless, it was of interest to build on the results presented above with the aim to improve photocatalytic H₂ production. To this end a N₂-filled headspace (3.35 mL) was introduced into which H₂ could escape from the cell suspension (1.65 mL) and headspace H₂ was quantified over a period of 4 days of irradiation (Figure 6a). Equivalent irradiation (0.02 kW m⁻²) of the necessary number of samples was ensured by use of a photosynthetic growth chamber (see Methods in the Supporting Information).

Eosin Y supported significantly more H₂ production in the first 24 h irradiation than Ru(bpy)₃²⁺ (Figure 6a), in accord with the behavior described above (Figure 3). When illumination of the MR-1 suspensions was continued beyond

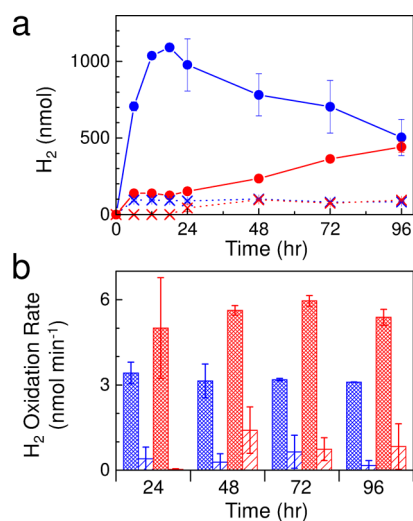


Figure 6. Properties of MR-1 suspensions over 4 days of irradiation by visible light (0.02 kW m⁻²) with eosin Y (blue) or Ru(bpy)₃²⁺ (red). (a) Headspace H₂ (circles) for anaerobic suspensions (OD_{590 nm} \approx 0.25 in 1.65 mL volume with 3.35 mL headspace) containing photosensitizer (0.11 mM), MV (0.3 mM), and TEOA (60 mM) in 50 mM HEPES, 50 mM NaCl, pH 7 at 25 °C. Mean values (symbols) with lines given as a guide to the eye; for 6–18 h $n = 2$ (technical replicates), error bars indicate maximum and minimum; for 0 and 24–96 h mean values ($n \geq 5$ with ≥ 3 biological replicates) are given with standard error. Headspace H₂ (crosses) values are given for parallel experiments without MR-1 (mean values from technical duplicates, error bars indicate maximum and minimum). (b) Initial rates of H₂ oxidation coupled to benzylviologen reduction by cell pellets (darker bars) and supernatants (lighter bars) recovered after centrifugation of MR-1 suspensions irradiated with eosin Y (blue) or Ru(bpy)₃²⁺ (red) as described in (a). Mean values are given for two biological replicates; error bars indicate maximum and minimum.

24 h, the H₂ content rose steadily with Ru(bpy)₃²⁺ but diminished with eosin Y and after 4 days both samples contained equivalent levels of H₂. Hydrogenase activity quantified for the resuspended cellular materials collected after centrifugation, and the supernatants, showed that active enzyme was primarily associated with the cellular material and varied little over the course of the experiment (Figure 6b). It is evident that the hydrogenases remain active during the 4 days of irradiation, and this matches the longest-lived system we are aware of with purified hydrogenase⁶⁶ aside from cases where longevity has been increased by covalent attachment to an electrode.^{24,25} The sustained H₂ production seen with Ru(bpy)₃²⁺ is most likely due to its being less susceptible to photodamage than eosin Y.⁶⁷ Photodamage of eosin Y will remove the driving force for H₂ evolution such that the loss of H₂ after 24 h irradiation of that sample may be due to slow leakage of H₂ from the vessel or H₂ oxidation coupled to reduction of cellular enzymes and metabolites as described above.

The apparent quantum efficiency for H₂ production was defined under irradiation (0.01 kW m⁻²) as $[2 \times (\text{number of H}_2 \text{ molecules produced})]/(\text{number of incident photons})$. MR-1 suspensions were irradiated at 500 or 450 nm with eosin Y or Ru(bpy)₃²⁺, respectively, and the headspaces were sampled after 1 h (see Methods in the Supporting Information). Under these conditions the apparent quantum efficiencies for eosin Y and Ru(bpy)₃²⁺ were $0.6 \pm 0.1\%$ and $0.5 \pm 0.1\%$, respectively, and we note that these values represent lower limits for photocatalysis, due to the assumption that all of the light is harvested by the photosensitizer-containing MR-1 suspension. Photoproduction of MV⁺ in the absence of MR-1 but otherwise equivalent conditions occurred with an apparent quantum efficiency of $2.1 \pm 0.2\%$ for eosin Y and $1.0 \pm 0.1\%$ for Ru(bpy)₃²⁺, where the efficiency is defined as (number of molecules of MV⁺ produced)/(number of incident photons). It can be concluded that for both photosensitizers the primary bottleneck to more efficient photocatalytic H₂ production is transfer of photoexcited electrons to MV²⁺, rather than the delivery of electrons from MV⁺ to the hydrogenases. We note that, during irradiation with white light, significantly greater photoproduction of H₂ with eosin Y than with Ru(bpy)₃²⁺ can be explained by the former having greater extinction coefficients at wavelengths for which the incident light has greater intensity (Figure S2 and Table S2 in the Supporting Information).

Light-Driven C=C and C=O Bond Hydrogenation by MR-1. Given the efficiency with which MV⁺ transferred photogenerated electrons to hydrogenases, it was of interest to assess whether these electrons could be delivered to enzymes that catalyze the reduction of C-based substrates. The superior H₂ production achieved with 0.11 mM eosin Y, 0.3 mM MV, and an MR-1 suspension of OD_{590 nm} \approx 0.25 made these the conditions of choice for such investigations. H₂ production over 30 min irradiation (0.7 kW m⁻²) was significantly diminished by the presence of fumarate, pyruvate, or CO₂ (Figure 7a), consistent with the diversion of photoenergized electrons to enzymes catalyzing the reduction of the C-based substrates. Confirmation of, and greater insight into, this behavior was afforded by NMR analysis of replicate samples that were best obtained from 24 h irradiation (0.02 kW m⁻²) in a photosynthetic growth chamber (see above and Methods in the Supporting Information).

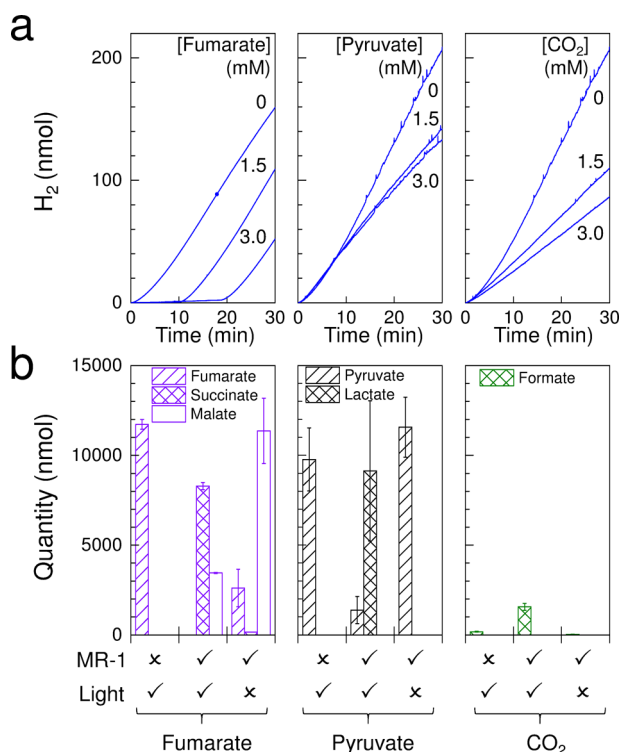


Figure 7. Light-driven reduction of fumarate, pyruvate, and CO₂ by MR-1. (a) Dissolved H₂ in gently stirred MR-1 suspensions (OD_{590 nm} ≈ 0.25 for 1.29 mL volume with negligible headspace) with eosin Y (0.11 mM), TEOA (60 mM), MV (0.3 mM), and the indicated concentrations of fumarate (left), pyruvate (middle), or CO₂ (right). Irradiation was at 0.7 kW m⁻² throughout. All samples were anaerobic in 50 mM HEPES, 50 mM NaCl, pH 7 at 23 °C. (b) Composition of supernatants recovered from samples incubated for 24 h with eosin Y (0.08 mM), MV (0.5 mM), TEOA (50 mM), and 10 mM fumarate (left), pyruvate (middle), or CO₂ (right). Assays were performed with or without MR-1 (OD_{590 nm} ≈ 0.25) in the dark or irradiated (~0.02 kW m⁻²). Mean values are given from technical duplicates; error bars indicate maximum and minimum. All samples were anaerobic in 50 mM HEPES, 50 mM NaCl, pH 7 at 25 °C.

¹H NMR data for MR-1 irradiated in the presence of ~9800 nmol of pyruvate revealed almost complete conversion to lactate (~9100 nmol, 93% yield) with no evidence for generation of additional products or dark reactions (Figure 7b and Table S3 and Figure S7 in the Supporting Information). Irradiation in the presence of ~11700 nmol of fumarate produced succinate (~8300 nmol, 70% yield) as the major product and malate (~3500 nmol, 30% yield) as the minor product. Dark incubated MR-1 hydrolyzed fumarate to malate (~11400 nmol, 100% yield) in a reaction catalyzed by fumarase (SO_2222). That significantly less malate is present after 24 h irradiation illustrates the scope of light-driven chemistry to exert temporal control over the progress of a reaction through the imposition of a persistent driving force to ensure the availability of reducing equivalents. It is also of note that the time courses (Figure 7a) for H₂ evolution with 1.5 and 3 mM fumarate show no detectable H₂ production for the first 10 or 20 min of irradiation, respectively. This makes it reasonable to assume that the majority of fumarate is consumed prior to H₂ production such that the Coulombic efficiency for fumarate reduction need not be sacrificed by using MV to deliver photoenergized electrons to microorganisms containing hydrogenases. The average rate of fumarate reduction is on the order

of 250 nmol min⁻¹, whereas in the absence of carbon substrates, H₂ is produced at a rate of 4 nmol min⁻¹. We anticipate that an appropriate choice of conditions would secure complete conversion of fumarate to succinate: i.e., without malate production and with high Coulombic efficiency.

The ¹H NMR data for MR-1 irradiated with ~10000 nmol of CO₂ revealed ~1600 nmol of formate (16% yield) (Figure 7b and Table S3 and Figure S7 in the Supporting Information). ¹³C NMR analysis of equivalent experiments with ¹³C-CO₂ revealed similar levels of ¹³C-formate (~1790 nmol, 18% yield), and parallel samples kept in the dark contained no detectable ¹³C-formate (Figure S8 in the Supporting Information). Given that the time courses for H₂ production over 30 min (Figure 7a) revealed CO₂ reduction to be as effective as pyruvate reduction in competing with hydrogenases for photoenergized electrons, these yields of formate were lower than expected. Dissolution of CO₂ or the compulsory involvement of H₂ as an intermediate in CO₂ reduction, such as would occur with a formate–hydrogen lyase complex, is unlikely to cause this behavior, as only 3-fold more formate was observed after minimizing the sample headspace or replacing MR-1 with the HydA⁻/HyaB⁻ strain. The lower than anticipated amount of formate may indicate that enzymatic conversion to other products occurs or that the pathway leading to CO₂ reduction is less stable under prolonged irradiation than those reducing protons, fumarate, and pyruvate.

Finally we note that the H₂ evolution time courses of Figure 7a reveal that light-driven fumarate reduction is significantly faster than reduction of protons, pyruvate, and CO₂. This may reflect the high cellular abundance of the soluble, periplasmic fumarate reductase that has surface-exposed redox cofactors.⁶⁸ The hydrogenases,^{36–38} formate dehydrogenases,⁴² and lactate dehydrogenases^{39,40} are membrane associated and exchange electrons primarily with the Q-pool such that they may receive electrons from MV⁺ via the electron transfer properties of intermediary enzymes.

Prospects for Whole-Cell Photocatalytic Production of Multiple, Selected Chemicals. The results presented here demonstrate the ability of MV to transfer photogenerated electrons to multiple enzymes for cell-based photocatalytic production of a selectable product range. The apparent quantum efficiencies are low (~0.5%) and primarily limited by photoreduction of MV²⁺, and similar behavior was noted⁵³ for MV-dependent H₂ production by *E. coli* irradiated with anatase TiO₂ as a photosensitizer. While the present work was under review, Honda et al. reported⁵⁴ that replacing the anatase with P-25 TiO₂ improved the quantum efficiency for H₂ production to ~25%. As a consequence, given the promiscuity of MV as an electron relay to intracellular enzymes, we envisage similarly impressive quantum efficiencies for the production of multiple chemicals when MV is used in combination with photosensitizers optimized for MV²⁺ reduction.

For more sustainable whole-cell photocatalysis it is desirable to increase the longevity and decrease the complexity of the present systems. Ideally, the repair and regeneration machinery of living cells would be retained. Inclusion of cellular energy sources could help to achieve this but would increase the chemical complexity of the system. More benefit is likely to be gained by changing and/or simplifying the components required for sustaining the supply of photoenergized electrons. For example, TEOA has proved to be a successful sacrificial electron donor for the reductive half-reactions studied here but its one-electron-oxidation product is a potentially harmful

radical species. Using a cathode or recycling oxidized ascorbate⁶⁹ with tris(carboxyethyl)phosphine would avoid generating such species.

Ultimately a system that delivers photoenergized electrons to enzymes inside bacteria without a requirement for MV would be preferable, since its substitution would avoid using a toxic and relatively costly, although regenerated, electron relay. Such a relay may be naturally produced³⁰ or be a membrane-permeable ferrocene such as DFSo+.⁷⁰ Alternatively, the photosensitizer could deliver electrons directly to a naturally occurring outer membrane spanning electron conduits; in this context it is significant that eosin Y, proflavine, and fluorescein were recently shown⁷¹ to catalyze complete photoreduction of the purified outer membrane spanning the MtrCAB complex from MR-1. The present work illustrates how engaging these photosensitizers for direct light-driven catalysis by MR-1 via Mtr complexes is more challenging than predicted from the behavior of these dyes with purified MtrCAB. In the cellular context the targeted transformations may go undetected due to slow intracellular electron transfer from photoreduced Mtr complexes or the photosensitizers may be unable to reduce the extracellular cytochromes due to hindrance by lipopolysaccharides or nonproductive quenching of their excited states. These possibilities form the focus of continued experimentation in our laboratories, with the aim of developing cell-based systems as sustainable routes to access a wide product range.

CONCLUSIONS

In summary we have demonstrated light-driven H₂ evolution and the hydrogenation of the C=C bond in fumarate or the C=O bonds in pyruvate and CO₂ by a nonphotosynthetic microorganism. These transformations are achieved without enzyme purification by using MV to transfer photoenergized electrons from water-soluble dyes to the corresponding enzymes in a cell-based assay. Given the promiscuity of MV as a redox partner of enzymes in numerous microorganisms, our approach provides a versatile route to accessing diverse and selective visible-light-driven chemical syntheses without the need for costly enzyme purification.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.7b02736.

Materials and methods, representative NMR spectra, NMR-derived quantification of carbon substrates, photosensitizer structures, and absorbance maxima, spectral properties of irradiation sources and photosensitizers, methylviologen photoreduction with different photosensitizers and sacrificial electron donors, and hydrogenase activity and MtrCAB content of *Shewanella* strains (PDF)

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ABBREVIATIONS

bpy, 2,2'-bipyridine; CFU, colony forming units; FDH, formate dehydrogenase; IM, inner membrane; LDH, lactate dehydrogenase; MK, menaquinone; MR-1, *Shewanella oneidensis* MR-1; MV, methyl viologen; OD_{590 nm}, optical density at 590 nm; OM, outer membrane; RuP, Ru(bpy)₂(4,4'-(PO₃H₂)₂bpy)²⁺; TEOA, triethanolamine

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