A Synthetic Plasmid Toolkit for Shewanella oneidensis MR-1

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Shewanella oneidensis MR-1 is a platform microorganism for understanding extracellular electron transfer (EET) with a fully sequenced and annotated genome. In comparison to other model microorganisms such as Escherichia coli, the available plasmid parts (such as promoters and replicons) are not sufficient to conveniently and quickly fine-tune the expression of multiple genes in S. oneidensis MR-1. Here, we constructed and characterized a plasmid toolkit that contains a set of expression vectors with a combination of promoters, replicons, antibiotic resistance genes, and an RK2 origin of transfer (oriT) cassette, in which each element can be easily changed by fixed restriction enzyme sites. The expression cassette is also compatible with BioBrick synthetic biology standards. Using green fluorescent protein (GFP) as a reporter, we tested and quantified the strength of promoters. The copy number of different replicons was also measured by real-time quantitative PCR. We further transformed two compatible plasmids with different antibiotic resistance genes into the recombinant S. oneidensis MR-1, enabling control over the expression of two different fluorescent proteins. This plasmid toolkit was further used for overexpression of the MtrCAB porin-c-type cytochrome complex in the S. oneidensis ΔmtRA strain. Tungsten trioxide (WO₃) reduction and microbial fuel cell (MFC) assays revealed that the EET efficiency was improved most significantly when MtrCAB was expressed at a moderate level, thus demonstrating the utility of the plasmid toolkit in the EET regulation in S. oneidensis. The plasmid toolkit developed in this study is useful for rapid and convenient fine-tuning of gene expression and enhances the ability to genetically manipulate S. oneidensis MR-1.

Keywords: Shewanella oneidensis MR-1, plasmid toolkit, BioBrick, c-type cytochrome, fine-tuning, synthetic biology

INTRODUCTION

Shewanella oneidensis MR-1, a Gram-negative exoelectrogen, is regarded as an important model microorganism with extracellular electron transfer (EET) pathways for bio-electrochemical applications in environmental and energy fields, including bioelectricity generation and production of chemicals (Hou et al., 2009, 2011; Kouzuma et al., 2015; Shi et al., 2016; Kumar et al., 2017; Li et al., 2018b). After fully sequencing and annotating its genome (Venkateswaran et al., 1999; Heidelberg et al., 2002; Daraselia et al., 2003), a number of efforts have been made to engineer EET in S. oneidensis. For example, disruption of the putative cell surface polysaccharide...
biosynthesis gene SO3177 (Kouzuma et al., 2010) or overexpression of the c-type cytochrome gene mtrC (Bretschger et al., 2008) were undertaken to generate higher current output. With the advancement of synthetic biology, multiple genes were simultaneously manipulated to facilitate EET in Shewanella. Goltisch et al. (2013) used an arabinose-inducible promoter pBAD to upregulate the expression of an operon composed of the genes mtrF, mtrA, and mtrB, which had previously been deleted from the genome in S. oneidensis, showing that modulation of the operon expression levels is an efficient strategy in enhancing ferric iron reduction capacity and anode reduction performance. West et al. (2017) characterized a native inducible expression system induced by trimethylamine N-oxide (TMAO) in S. oneidensis to control EET, which was then successfully used to regulate EET by inducing mtrCAB gene expression with TMAO. The ability to induce this pathway was assessed by measuring iron reduction over time and by analyzing anodic current produced by cells grown in bioreactors. Yang et al. (2015) heterologously expressed a synthetic flavin biosynthesis pathway from Bacillus subtilis in the BioBrick compatible vector pYYDT, derived from the plasmid pHG101, to enhance the rate of EET in engineered S. oneidensis MR-1. By using this plasmid, Li et al. (2018a) assembled four genes that are mostly responsible for increasing NADH regeneration in S. oneidensis MR-1.

Improved genetic engineering strategies could provide additional opportunities to modulate the metabolism of microorganisms (Lin et al., 2018). Although manipulation of multiple genes has been employed to engineer S. oneidensis, the availability of gene expression tools is still not as abundant as other well-studied platform microorganisms, such as Escherichia coli or Saccharomyces cerevisiae (Keasling, 2012; Chae et al., 2017). In E. coli, a large number of expression and regulatory elements make the fine-tuning of gene expression quick and convenient. Researchers have combined various biological parts in E. coli, such as promoters with different strengths (e.g., pBAD, pTrc, pT5, pT7, placUV5, pLtetO-1, and double tac), replication origins (e.g., p15A, ColE, pBBR1, pSC101, and pBR322), antibiotic resistance markers [e.g., chloramphenicol (CmR), kanamycin (Kan), carbenicillin (Cb), ampicillin (Amp), and spectinomycin (Spect)], to fine-tune gene expression, which led to significant increases in the production of taxadiene (Ajikumar et al., 2010), n-butanol (Bond-Watts et al., 2011), L-tyrosine (Juminaga et al., 2012), and amorphadiene (Anthony et al., 2009). Heterologous expression of MtrCAB under the control of the T7 promoter in E. coli could create an artificial electrogenic cell (Jensen et al., 2010), but high MtrCAB expression in the presence of the Isopropyl β-D-Thiogalactoside (IPTG) inducer may impair EET efficiency in E. coli. Goldbeck et al. (2013) used E. coli with a more tunable induction system and a panel of constitutive promoters to express the MtrCAB pathway. They found that the efficiency of MtrC and MtrA synthesis decreased when controlled by strong promoters, leading to a significant decrease in EET efficiency. To improve the ability of manipulating S. oneidensis MR-1, more vectors were developed. Hajimorad and Gralnick (2016) demonstrated that different replication origins (e.g., p15A, pMB1, and pBBR1), selection markers (e.g., Kan and CmR), and an RK2 oriT cassette could be used in S. oneidensis MR-1, which provided a useful tool in engineering S. oneidensis. Continued development of synthetic biology parts, coupled with quantitative characterization, will enable more delicate or complicated engineering of S. oneidensis.

Here, we report the development of a synthetic plasmid toolkit for S. oneidensis MR-1. A number of available biological parts, such as promoters, replication origins, a conjugal transfer shuttle cassette, and antibiotic resistance genes were tested to design and construct easily available and compatible vectors for Shewanella (Figure 1). Firstly, we evaluated and characterized the strength of various promoters in E. coli and S. oneidensis MR-1 using the green fluorescent protein (GFP) as a reporter. Furthermore, we characterized the strength of inducible promoters by regulating inducer concentrations and induction times. Secondly, we quantified the copy numbers of different replicons in S. oneidensis MR-1 by real-time quantitative PCR (RT-qPCR) and found that copy number was directly correlated with the fluorescence intensity of GFP. Additionally, we simultaneously transformed two compatible plasmids into recombinant S. oneidensis MR-1, thus achieving fine control of the expression of two different fluorescent proteins [GFP and blue fluorescent protein (BFP)/superfolder cyan fluorescent protein (CFP)] through modulation of inducer concentration or the use of promoters with different strengths. Lastly, we took the porin-cytochrome complex MtrCAB for an example to demonstrate that EET efficiency can be fine-tuned in recombinant S. oneidensisΔmtrA strain by the plasmid toolkit we developed in this study. The plasmid toolkit developed in this study enabled rapid and convenient fine-tuning of gene expression with greater controllability and predictability, which will accelerate future genetic manipulations of S. oneidensis MR-1.

## MATERIALS AND METHODS

### Strains and Growth Conditions

All bacterial strains used in this study are listed in Table 1. For genetic manipulation, E. coli Trans1-T1, BL21, MG1655, and WM3064 were cultivated in Luria-Bertani (LB) medium at 37°C. The wild-type and recombinant S. oneidensis MR-1 strains were cultured in LB medium at 30°C. Unless otherwise specified, genetic manipulation antibiotics were used at the following concentrations: kanamycin at 50 μg/ml and chloramphenicol at 34 μg/ml. The E. coli WM3064 culture was supplemented with 0.3 mM 2,6-Diaminopimelic acid (DAP).

### Plasmid Constructions

All the plasmids used in this study are listed in Table 1. Plasmid construction processes were performed in E. coli Trans1-T1 and E. coli BL21. The plasmid is composed of four elements: replication origin, antibiotic resistance gene, conjugal transfer shuttle cassette, and expression cassette, with fixed restriction enzyme sites between each part as shown in Figure 1 and Supplementary Figure S1. The sequences of each biological element and the corresponding restriction enzyme sites were synthesized by Genewiz (China), which are listed in Supplementary Table S2. Specifically, plasmid pHG12 was
FIGURE 1 | Schematic diagram of the assembled shuttle vectors in S. oneidensis MR-1. The cutting sites of the restriction enzymes are shown in red. Sa, SacI; E, EcoRI; Xb, XbaI; A, AvrII; Nt, NdeI; Nh, NheI; Xh, XhoI; Sp, SpeI; P, PstI; K, KpnI; H, HindIII. High-strength promoters (i.e., pBAD, pXyl, pCI, pJ23119, and pTac) are shown in orange; medium-strength promoters (pTet and pTrc+) are shown in aqua; and low-strength promoters (placUV5 and pLlacO1) are shown in olive.

constructed from plasmid pHG11 (Cao et al., 2017) by site-directed mutagenesis of the NcoI restriction site and insertion of the SacI in front of EcoRI restriction site. The primers used are presented in Supplementary Table S1.

The sequences of green, blue, and superfolder cyan fluorescent protein gens (gfp, bfp, and cfp) were codon-optimized and synthesized by Genewiz (China), which were listed in Supplementary Table S2. They were digested with NdeI/XhoI and inserted into the corresponding sites of the expression cassette in relative plasmid. The MtrCAB clusters were amplified from the S. oneidensis MR-1 genome using MtrCAB-F/MtrCAB-R primer pairs, listed in Supplementary Table S1, which were then digested with NdeI/XhoI and inserted into the corresponding sites of the plasmids carrying the promoter pBAD, pTet, and placUV5, respectively, resulting in three mtrCAB expression vectors.

Conjugation Assay
Transformed into S. oneidensis MR-1 or S. oneidensis ΔmtrA strains (Cao et al., 2017), plasmids were firstly transformed into the plasmid donor cell E. coli WM3064 (a DAP auxotroph) and then transferred into S. oneidensis MR-1 or S. oneidensis ΔmtrA by conjugation. For the growth of E. coli WM3064 that has RP4 Tra function integrated into the chromosome, 0.3 mM 2,6-Diaminopimelic acid (DAP) was needed in the culture medium. In brief, 500 µl donor cells and 500 µl recipient cells were mixed and then the mixture was collected by centrifugation (5000 rpm for 10 min). Cells were suspended with 1 ml LB medium containing DAP and incubated for 1 h at 30°C. The appropriate volume of cells was next spread on LB plates with appropriate antibiotics and placed overnight in 30°C incubator to allow conjugation. To arrive at multiple-plasmid Shewanella strains, S. oneidensis MR-1 cells harboring a single-plasmid served as the recipient cell in the above steps.

Fluorescence Assay
Green, blue, and superfolder cyan fluorescent proteins (GFP, BFP, and CFP) were used to characterize the expression intensity. A total of 0.1 mM IPTG, 10 mM arabinose, 1 g/l xylose, or 100 ng/ml anhydrotetracycline hydrochloride (aTc) was used as the normal concentration for inducing the corresponding promoters. For concentration-response and time-response assays, GFP intensities in S. oneidensis MR-1 were tested with a series of inducer concentrations. IPTG, arabinose, and
TABLE 1 | Strains and plasmids used in the study.

| Strain | Description | Source |
|--------|-------------|--------|
| E. coli MG1655 | Wild type | Lab stock |
| S. oneidensis MR-1 | Wild type | Lab stock |
| E. coli WM3064 | A DPA auxotroph of E. coli could transfer plasmid into S. oneidensis MR-1 by conjugation | Lab stock |
| E. coli Trans1 T1 | Cloning strain | TransGen |
| E. coli BL21(DE3) | Cloning strain | TransGen |
| S-ΔmtrA | A mtrA deletion mutant of S. oneidensis MR-1 | TransGen |
| L-mtrCAB | S-ΔmtrA carrying pHG13-placUV5-CoIE-MtrCAB | This study |
| M-mtrCAB | S-ΔmtrA carrying pHG13-pTet-CoIE-MtrCAB | This study |
| H-mtrCAB | S-ΔmtrA carrying pHG13-pTrc-MtrCAB | This study |

| Plasmid | Description | Source |
|---------|-------------|--------|
| pHG11 | rep<sup>PBBR1</sup>, KanR without PstI restriction site, Mob without Bsal restriction site | Lab stock |
| pHG12 | rep<sup>PBBR1</sup>, KanR without PstI and Ncol restriction sites, Mob without Bsal restriction site, with insertion of SacI in front of EcoRI restriction site | This study |
| pHG12-pBAD | pBAD, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-pXYl | pXYl, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-pTet | pTet, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-pTrc<sup>+</sup> | pTrc<sup>+</sup>, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-pTac | pTac, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-pLacO1 | pLacO1, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-placUV5 | placUV5, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-pCl | pCl, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-pJ23119 | pJ23119, rep<sup>PBBR1</sup>, KanR, Mob | This study |
| pHG12-pBAD-GFP | pBAD, rep<sup>PBBR1</sup>, KanR, Mob, gfp amplified from pLac-GFP | This study |
| pHG12-pXYl-GFP | pXYl, rep<sup>PBBR1</sup>, KanR, Mob, gfp | This study |
| pHG12-pTet-GFP | pTet, rep<sup>PBBR1</sup>, KanR, Mob, gfp | This study |
| pHG12-pTrc<sup>+</sup>-GFP | pTrc<sup>+</sup>, rep<sup>PBBR1</sup>, KanR, Mob, gfp | This study |
| pHG12-pTac-GFP | pTac, rep<sup>PBBR1</sup>, KanR, Mob, gfp | This study |
| pHG12-pLacO1-GFP | pLacO1, rep<sup>PBBR1</sup>, KanR, Mob, gfp | This study |
| pHG12-placUV5-GFP | placUV5, rep<sup>PBBR1</sup>, KanR, Mob, gfp | This study |
| pHG12-pCl-GFP | pCl, rep<sup>PBBR1</sup>, KanR, Mob, gfp | This study |
| pHG12-pJ23119-GFP | pJ23119, rep<sup>PBBR1</sup>, KanR, Mob, gfp | This study |
| pHG13-pTrc<sup>+</sup>-CoIE-GFP | pTrc<sup>+</sup>, rep<sup>CoIE</sup>, Cmr, gfp, ori<sup>T</sup> | This study |
| pHG13-pTrc<sup>+</sup>-p15A-GFP | pTrc<sup>+</sup>, rep<sup>p15A</sup>, Cmr, gfp, ori<sup>T</sup> | This study |
| pHG13-pTrc<sup>+</sup>-pSC101-GFP | pTrc<sup>+</sup>, rep<sup>pSC101</sup>, Cmr, gfp, ori<sup>T</sup> | This study |
| pHG13-pTrc<sup>+</sup>-CoIE-BFP | pTrc<sup>+</sup>, rep<sup>CoIE</sup>, Cmr, ori<sup>T</sup>, BFP | This study |
| pHG13-pBAD-CoIE-BFP | pBAD, rep<sup>CoIE</sup>, Cmr, ori<sup>T</sup>, BFP | This study |
| pHG13-pBAD-CoIE-GFP | pBAD, rep<sup>CoIE</sup>, Cmr, ori<sup>T</sup>, GFP | This study |
| pHG13-pBAD-BFP | pBAD, rep<sup>pSC101</sup>, Cmr, ori<sup>T</sup>, BFP | This study |
| pHG13-pLacO1-CoIE-BFP | placUV5, rep<sup>pSC101</sup>, Cmr, ori<sup>T</sup>, BFP | This study |
| pHG13-pBAD-CoIE-MtrCAB | pBAD, rep<sup>pSC101</sup>, Cmr, ori<sup>T</sup>, mtrA, mtrC, mtrB | This study |
| pHG13-pTet-CoIE-MtrCAB | pTet, rep<sup>CoIE</sup>, Cmr, ori<sup>T</sup>, mtrA, mtrC, mtrB | This study |
| pHG13-placUV5-CoIE-MtrCAB | placUV5, rep<sup>CoIE</sup>, Cmr, ori<sup>T</sup>, mtrA, mtrC, mtrB | This study |

xylose were diluted to 10⁻³–10⁻⁴ μM, 10⁻²–10⁻⁵ μM, and 10⁻⁵–10⁻⁶ g/l using 10-fold serial dilutions, respectively. Additionally, aTc was used at the following concentrations (ng/ml): 25, 50, 100, 250, 500, 1000, 1500, 2000, and 2500. To simultaneously fine-tune the expression of multiple fluorescent proteins in S. oneidensis MR-1, we adjusted inducer concentrations for IPTG-inducible pTac or arabinose-inducible pBAD promoters. IPTG was used at the following concentrations (μM): 1, 12.5, 15, and 100. Arabinose was used at the following concentrations (mM): 0.1, 0.25, 0.5, and 1.

For fluorescence intensity assays, each strain was inoculated from a freshly transformed single colony on an LB agar plate into 2 ml LB medium as a seed culture. When cell accumulation reached stationary phase (assessed spectrophotometrically at OD₆₀₀), 50 μl of seed culture was re-inoculated in test tubes containing 5 ml fresh LB medium supplemented with the appropriate antibiotic and the corresponding concentration of inducer. After 24 h of growth at 30°C with constant shaking (200 rpm), 200 μl suspensions from each test tube were centrifuged at 4000 rpm for 2 min to remove the supernatant, after which the culture was washed and resuspended in phosphate-buffered saline (PBS) and transferred to a 96-well polystyrene plate (black plate, clear bottom) (Corning Incorporated 3603, United States). Cell optical density and fluorescence intensity were detected by a SpectraMax M2 microplate reader. Optical density was measured at 600 nm. The excitation/emission wavelengths for GFP, BFP, and CFP were set at 485 nm/520 nm, 399 nm/456 nm, and 600 nm. The excitation/emission wavelengths for GFP, BFP, and CFP were set at 485 nm/520 nm, 399 nm/456 nm, and 600 nm. The excitation/emission wavelengths for GFP, BFP, and CFP were set at 485 nm/520 nm, 399 nm/456 nm, and 600 nm. The excitation/emission wavelengths for GFP, BFP, and CFP were set at 485 nm/520 nm, 399 nm/456 nm, and 600 nm.

Relative fluorescence was calculated as the fluorescence per OD₆₀₀ for each strain subtracted from that of the untransformed E. coli MG1655 or untransformed S. oneidensis MR-1 control:

\[
\text{Relative Fluorescence} = \frac{\text{Fluorescence (i)} - \text{Fluorescence (control)}}{\text{OD (i)} - \text{OD (control)}}
\]

Determination of Replicon Copy Number by Real-Time Quantitative PCR (RT-qPCR)

Preparation of Template DNA for qPCR

Recombinant S. oneidensis MR-1 isolates harboring different plasmids (e.g., pHG13-pTrc<sup>+</sup>-CoIE-GFP, pHG13-pTrc<sup>+</sup>-p15A-GFP, pHG13-pTrc<sup>+</sup>-pSC101-GFP, and pHG12-pTrc<sup>+</sup>-pSC101-GFP) were cultivated to exponential growth phase, after which total DNA was extracted using the Bacterial Genome DNA extraction kit (Kang Wei Century, China) according to the manufacturer's instructions. Template DNA extracted from the recombinant S. oneidensis MR-1 was normalized to 2 ng/μl for consistency in the RT-qPCR assay (Lee et al., 2006).

Construction of Standards for RT-qPCR

The sequences of the four replicons (CoIE, p15A, pSC101, and pBBR1) were amplified using primers listed in
Supplementary Table S1. The products were separately cloned into T vectors using a Puc-T TA cloning kit (Kang Wei Century, China). Each cloned plasmid was purified using the TIANquick Mini Purification kit (Tiangen, Beijing, China) and was used as a standard (Dhanasekaran et al., 2010). RT-qPCR was carried out using a real-time fluorescent quantitative PCR machine (Applied Biosystems) in white-walled PCR plates (96 wells). SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) and ROXplus (TaKaRa) were used to make a Master Mix, which also contained TaKaRa Ex Taq HS, a dNTP mixture, Mg²⁺, Tli RNaseH, TB Green, and ROX Reference Dye. Reactions were prepared in a total volume of 18 µl, containing 10 µM of each standard RT-qPCR primer, 2× Master Mix, sterile water and 2 µl cDNA. The cycle conditions were set as follows: initial template denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and combined primer annealing/elongation at 60°C for 40 s, with the fluorescence data acquired at 60°C and analyzed using 7500 Software V2.0.4 (Applied Biosystems).

Construction of Standard Curves and Absolute Quantification
A 10-fold serial dilution series of each standard plasmid, ranging from 1 × 10⁷ to 1 × 10⁰ copies/µl, was used to construct the standard curves. The concentration of the plasmid was measured using a spectrophotometer (Thermo Scientific), and the corresponding copy number was calculated using the following equation (Whelan et al., 2003):

\[
\text{DNA (copy) } = \frac{6.02 \times 10^{23} \text{ (copy/mol)} \times \text{DNA amount (g)}}{\text{DNA length (dp)} \times 660 \text{ (g/mol/dp)}}
\]

The threshold cycle (Cₜ) values for each dilution were measured in triplicate by RT-qPCR with the CoIE-, p15A-, pSC101-, and pBBRI-sets to generate the corresponding standard curves. Standard curves were obtained by plotting the threshold cycle (Cₜ) on the y-axis and the natural log of concentration (copies/µl) on the x-axis. Cₜ, slope, PCR efficiency, correlation coefficient (R²), and percentage of variance in copy numbers were calculated using the default settings of 7500 Software V2.0.4 (Applied Biosystems). Absolute quantification of the copy numbers of target replicon genes were obtained by comparing the value of each template DNA sample to a standard curve (Yu et al., 2005).

Tungsten Trioxide Reduction Assay
This assay was performed according to Yuan et al. (2014). The process for the tungsten trioxide (WO₃) nanorod assembly synthesis solution is as follows: Dilute 0.825 g of Na₂WO₄ · 2H₂O and 0.290 g of NaCl in 20 ml of sterile water. Add 3 M HCl slowly under stirring until the pH reaches 2.0. This solution needs to be freshly prepared. The process for the sodium lactate minimal salt medium is as follows: Per liter, dissolve 2.02 g of sodium lactate, 5.85 g of NaCl, 11.91 g of HEPES, 0.3 g of NaOH, 1.498 g of NH₄Cl, 0.097 g of KCl, 0.67 g of NaH₂PO₄ · 2H₂O and 1 ml per liter trace mineral stock solution (containing per liter: 1.5 g of Ca₃(PO₄)₂, 30 g of MgSO₄ · 7H₂O, 5 g of MnSO₄ · H₂O, 10 g of NaCl, 1 g of FeSO₄ · 7H₂O, 1 g of CaCl₂ · 2H₂O, 1 g of CoCl₂ · 6H₂O, 1.3 g of ZnCl₂, 0.1 g of CuSO₄ · 5H₂O, 0.1 g of AlK(SO₄)₂ · 12H₂O, 0.1 g of H₃BO₃, 0.25 g of Na₂MoO₄ · 2H₂O, 0.25 g of NiCl₂ · 6H₂O, and 0.25 g of Na₂WO₄ · 2H₂O) in sterile water and autoclave the mixture immediately. For the WO₃ nanorod assembly containing sodium lactate minimal salt suspension, the process is as follows: dissolve 5 g per liter of WO₃ nanorod assembly in sodium lactate minimal salt suspension and autoclave the resulting suspension immediately.

Overexpression of the c-type cytochromes, MtrCAB, in engineered S. oneidensis ΔmtrA was tested under the control of placUV5, pTet, and pBAD promoters. The parental and recombinant strains were named as S-ΔmtrA, L-mtrCAB, M-mtrCAB, and H-mtrCAB, respectively, according to the expression level of the gene (i.e., L, low; M, medium; H, high). They were activated overnight in LB medium with or without chloramphenicol. Then, 50 µl from each bacterial culture suspension from a 2 ml LB medium seed culture (originating from a freshly transformed single colony on a LB agar plate) was used to inoculate a test tube containing 5 ml fresh LB medium supplemented with or without chloramphenicol. The inducers for each promoter (i.e., 0.1 mM IPTG for placUV5, 1,000 ng/ml aTc for pTet, and 10 mM arabinose for pBAD) were added to express mtrCAB when OD₆₀₀ reached ~0.5. After 24 h of induction, cells were collected by centrifugation at 4000 rpm at 4°C for 5 min, after which the pellets were resuspended in sterile sodium lactate minimal salt medium, with appropriate dilution to achieve an OD₆₀₀ ~0.8. Then, 100 µl of the above resuspended bacterial solution and 80 µl of 5 g/l sterile WO₃ nanorod assembly containing sodium lactate minimal salt suspension were mixed completely before being added into a 96-well plate. After that, 80 µl of petroleum oil was added into each well immediately to ensure anaerobic conditions for the bioelectrochromic reaction, and the plate was incubated at 30°C in an incubator. We observed conspicuous color development in the plate after 30 min, but maintained incubation for 6 h, after which we transferred the plate into a scanner. As the plate was being scanned, an opaque box was used to shield it from external light. Then, the average color intensity of each well was determined by analyzing their ‘Density (mean)’ of the area of interest in the scanned photograph with the ImageJ Software V1.8.0. Culture medium without bacteria was used as a control to ensure that the WO₃ electrochromic reaction was not caused by the culture medium. Triplicate experiments were performed for each strain.

MFC Setup and Electrochemical Analysis
Overnight S-ΔmtrA, L-mtrCAB, M-mtrCAB, and H-mtrCAB culture suspensions were inoculated into 100 ml fresh LB broth at 30°C with shaking (200 rpm). After around 10 h culture, the amplification-cultured cells were subsequently centrifuged and collected at 4°C and underwent 6000 rpm for 10 min to remove the supernatant. The concentrations of cell suspensions were adjusted to the same level (OD₆₀₀ ≈ 0.5), and the suspensions were dispersed into the anode chambers. In this MFC, S-ΔmtrA was used as a control. Whenever needed, 34 µg/ml chloramphenicol was added in anolytes to ensure consistent culture condition. For recombinant S. oneidensisΔmtrA strain
harboring the plasmid under the control of the inducible promoter, the medium was supplemented with 0.1 mM IPTG for promoter placUV5, 1,000 ng/ml aTc for pTet, or 10 mM arabinose for pBAD. All MFCs were incubated in a 30°C incubator and each group was tripled for parallel experiments. Dual-chamber MFCs with a working volume of 110 mL separated by the Nafion 117 membrane (DuPont Inc., United States) were applied in this study. Carbon cloth was used as the electrodes for both anode (2.5 cm × 2.5 cm, i.e., the geometric area is 6.25 cm²) and cathode (2.5 cm × 3 cm). The anolyte consisted of M9 buffer (Na₂HPO₄, 6 g/l; KH₂PO₄, 3 g/l; NaCl, 0.5 g/l; NH₄Cl, 1 g/l; MgSO₄, 1 mM; CaCl₂, 0.1 mM), supplemented with 18 mM lactate and 5% (v/v) LB broth. The catholyte was made of 50 mM K₃[Fe (CN)₆] in 50 mM KH₂PO₄ and 50 mM K₂HPO₄ solution. To measure the voltage generation, a 2 kΩ external resistor was connected into the external circuit of MFCs, and the output voltages were recorded across the external loading resistor with a digital multimeter (DT9205A). Linear sweep voltammetry (LSV) analysis with a scan rate (0.1 mV/s) was conducted on a two-electrode mode, where the anode performed as the working electrode and the cathode as the reference as well as the counter electrode to obtain the polarization curves to estimate the maximum power density (the potential decreased to −0.3 V). Power density (P) was calculated as P = V (output voltage) × I (current density). Both I and P were normalized to the projected area of the anode surface.

RESULTS AND DISCUSSION

Characterization of Promoters in S. oneidensis MR-1

In this study, we designed a number of shuttle vectors, in which each element could be constructed or replaced using fixed restriction enzyme sites (Figure 1 and Supplementary Figure S1). Firstly, we characterized 9 promoters in S. oneidensis MR-1, which included pBAD, pLacO1, placUV5, pTet, pXyl, pCI, pTac, pJ23119, and pTrc*. These promoters could be classified into two categories: constitutive promoters (e.g., pCI, pJ23119, and pTrc*) and inducible promoters (e.g., IPTG-inducible pTac, IPTG-inducible pLacO1, IPTG-inducible placUV5, arabinose-inducible pBAD, xylose-inducible pXyl, and aTc-inducible pTet). The strength of these promoters was characterized using GFP as a reporter in E. coli MG1655 and S. oneidensis MR-1. 0.1 mM IPTG, 10 mM arabinose, 1 g/l xylose, or 100 ng/ml aTc was used to induce the corresponding promoters (Figure 2). In E. coli MG1655, the relative fluorescence showed a sigmoid-like distribution, which ranged from about 1,000 to more than 20,000 caused by the different expression strengths of the promoters (from low to high: pBAD, pLacO1, placUV5, pTet, pXyl, pCI, pTac, pJ23119, and pTrc*). However, the relative fluorescence intensities revealed only three expression levels in S. oneidensis MR-1. The pLacO1 and placUV5 promoters showed low expression levels (~1,000), pTet and pTrc* showed medium expression levels (~20,000), pBAD, pXyl, pCI, pJ23119, and pTac showed substantially higher expression levels (~40,000). The highest fluorescence level was 40-fold higher than the lowest fluorescence level for S. oneidensis MR-1. Furthermore, the highest fluorescence intensity observed in S. oneidensis MR-1 was ~2-fold higher than that in E. coli.

To further assess the levels of gene expression in S. oneidensis MR-1, the inducible promoters were characterized by adjusting inducer concentration and induction time (Figure 3). The distribution of relative fluorescence intensities was narrow in the early stage of induction but became broader with increasing inducer concentrations and longer induction times. With increasing inducer concentrations, GFP expression increased until a plateau level was reached for each promoter. All promoters resulted in a characteristic sigmoidal “S”-shaped curve. For all inducible promoters, the higher expression levels were achieved at the normal inducer concentrations. Almost full induction of the pTac, pLacO1, and placUV5 promoters occurred at IPTG concentrations of ~0.1, ~1, and ~1 mM, respectively (Figures 3A–C). pBAD and pXyl were fully induced at an arabinose or a xylose concentration of about 10 mM or 1 g/l, respectively (Figures 3D,E). The pTet promoter resulted in a higher expression at an aTc concentration of about 100 ng/ml (Figure 3F). The IPTG-inducible pTac, pLacO1, and placUV5 promoters and the arabinose-inducible pBAD promoter could tightly control gene expression, since GFP fluorescence was basically not observed at low inducer concentrations. This feature is particularly important for the expression of genes whose products may be toxic, such that expression can occur at very low levels. In contrast, we found that the xylose-inducible pXyl and the aTc-inducible pTet promoters were susceptible to leaky expression. In addition, the inducible promoters were not only regulated by inducer concentration, but were also influenced by induction time. At the beginning, the fluorescence intensities were enhanced with longer induction times, but GFP fluorescence eventually reached a higher and more stable level by 24 h. The relative fluorescence intensities of all promoters at various inducer concentrations were compared
FIGURE 3 | Characterization of the inducible promoters by regulating inducer concentration and induction time in S. oneidensis MR-1. (A–F) GFP expression under the control of inducible promoters was measured at 6, 10, 24, 32, and 48 h after induction with a series of different inducer concentrations. (A–C) IPTG-inducible pTac, pLacO1, and placUV5; (D) arabinose-inducible pBAD; (E) xylose-inducible pXyl; (F) aTc-inducible pTet. (G) GFP expression was measured at 24 h after induction with different inducer concentrations. The error bars (mean ± SD) were derived from triplicate experiments for each strain.

after 24 h of incubation (Figure 3G), allowing for easy selection of promoters and inducer concentrations depending on the experimental needs.

It is worth mentioning that for expression cassettes, we designed and constructed biological parts that conformed to BioBrick standards (Canton et al., 2008; Shetty et al., 2008). BioBrick vectors feature four isocaudomer pairs (AvrII, NdeI, XbaI, and SpeI), can form the monocistron or polycistron, and support the modular assembly of numbers of molecular components and multigene pathways, collectively contributing to convenient and rapid gene combination and plasmid construction (Supplementary Figure S2).

Characterization of Antibiotic Resistance Genes, Replication Origins, and Coexistence of Two Plasmids in S. oneidensis

For fine-tuning the expression of multiple genes, genes should be expressed from two separate plasmids. As such, knowledge of compatible replication origins, different antibiotic resistances, and the shuttle component involved in conjugation and transformation in S. oneidensis MR-1 are required.

We first assessed the ability of S. oneidensis MR-1 to resist different antibiotics to identify suitable resistance markers for maintaining multiple plasmids. Sensitivities of the wild-type S. oneidensis MR-1 to three antibiotics (kanamycin at 50 or 100 µg/ml, chloramphenicol at 15 or 34 µg/ml, and ampicillin at 100 µg/ml) were tested in LB broths and on LB agar plates (Supplementary Figure S3). We found that the wild-type S. oneidensis MR-1 could grow on media containing ampicillin, indicating natural resistance to this antibiotic. Based on antibiotic sensitivity tests, two different genes conferring resistance to chloramphenicol and kanamycin were selected.

Next, we addressed the use of the shuttle element oriT, an alternative to the mob gene, since transformation of S. oneidensis MR-1 relies on conjugation. Bacterial conjugation machinery is composed of RK2-oriT modules containing the traJ gene (Smillie et al., 2010), which is needed within a plasmid to enable its uptake by the recipient cell through conjugation. We introduced the RK2-oriT cassette, which is smaller than the mob gene, into the vector backbone and demonstrated successful
transformation from *E. coli* to *S. oneidensis* MR-1. Using this efficient transfer system, longer genes can be accommodated and expressed in the vectors.

In order to be compatible with the commonly used vector pHG101, we used the chloramphenicol resistance (CmR) gene and the shuttle oriT gene to test various replication origins. We chose ColE, p15A, and pSC101, which are commonly used in *E. coli*, and repB, which was used as a replication origin in the shuttle vector pSX33 from *Shewanella xiamenensis* BC01 (Zhou and Ng, 2016). We determined that shuttle vectors carrying ColE, p15A, or pSC101 could be efficiently imported into *S. oneidensis* MR-1. However, *S. oneidensis* MR-1 carrying repB could not grow due to failed transformation into *E. coli* and *S. oneidensis* MR-1. When considering and assessing replication origins, plasmid copy number is a key parameter. Hajimorad and Gralnick (2016) showed that different replication origins (p15A, pMB1, and pBBR1) could be maintained in *Shewanella* by counting colony forming unites (CFU). Here, we determined the copy number of different replicons using real-time quantitative PCR (RT-qPCR). As depicted in Figure 4A, the results indicated that the ColE replication origin had a copy number of 54, which was higher than the other replication origins: pSC101 (40), p15A (33), and pBBR1 (23). Meanwhile, we also measured the corresponding fluorescence of strains carrying these replication origins (Figure 4B), demonstrating that fluorescence was in accordance with copy number.

While screening the replication origins, we also tested whether the origins ColE, p15A, and pSC101 were compatible with the commonly used replication origin, pBBR1, in *S. oneidensis* MR-1. We co-transformed *S. oneidensis* MR-1 with two vectors carrying different replication origins: pBBR1/ColE, pBBR1/p15A, and pBBR1/pSC101. Plasmids carrying the pBBR1 origin confer kanamycin resistance, while plasmids carrying the ColE, p15A, or pSC101 origin confer chloramphenicol resistance. Transfer and maintenance of either a single plasmid or two plasmids (containing two replication origins) in *S. oneidensis* MR-1 were verified by colony PCR (Figure 5) using primers listed in Supplementary Table S1. The expected lengths of PCR products were 840 bp, 1,145 bp, 1,132 bp, and 2,535 bp for pBBR1, ColE, p15A, and pSC101, respectively. Agarose gel electrophoresis analysis confirmed band sizes, as shown from Lane 1 to Lane 4 in Figure 5. Lanes 5–10 depict three groups of two-plasmid containing replication origins: pBBR1/ColE (Lanes 5 and 6), pBBR1/p15A (Lanes 7 and 8), and pBBR1/pSC101 (Lanes 9 and 10). The results revealed compatibility of ColE/pBBR1, p15A/pBBR1, and pSC101/pBBR1 as well as successful co-transformation and maintenance of multiple plasmids in *S. oneidensis* MR-1.

**Fine-Tuning the Expression of Multiple Fluorescent Proteins in *S. oneidensis***

The above results demonstrated the utility of the developed plasmid toolkit, which enabled precise control of gene expression using different strength promoters, regulating the inducer concentration or induction time for inducible promoters, and using various replication origins with different copy numbers. Fine-tuning gene expression can be achieved by adopting suitable
elements with different levels of expression according to the actual experimental needs. The next step was to test the ability of the modular plasmids to modulate the simultaneous expression of multiple genes in *S. oneidensis* MR-1. For this purpose, three fluorescent proteins (i.e., GFP, BFP, and CFP) were chosen and expressed in separated plasmids.

Two manipulation strategies were applied. Firstly, two different fluorescent genes were expressed using different types of promoters (e.g., IPTG-inducible or arabinose-inducible) with two-gene expression fine-tuned by adjusting the inducer concentrations. GFP was expressed using vectors with the IPTG-inducible promoter pTac, the pBBR1 replication origin, and the kanamycin resistance (Kan) gene. The following IPTG concentrations were assessed: 1, 12.5, 15, and 100 µM. GFP expression level was relatively low (Supplementary Figure S5). In all, these results demonstrated that the expression of different genes in *S. oneidensis* MR-1 could be obtained by adjusting inducer concentrations for the inducible promoters.

Secondly, fine-tuning of gene expression was achieved by utilizing different strength promoters (low, medium, and high) at their normal induction concentrations. GFP was expressed under the control of the high-strength pBAD promoter and BFP was expressed under the control of the low-strength placUV5 promoter, high GFP and low BFP were detected. Similarly, when the BFP was expressed under the control of the high-strength pBAD promoter and GFP was expressed under the control of the low-strength placUV5 promoter, low GFP and high BFP were detected. In all, different levels of gene expression could also be achieved by using promoters of different strengths. Overall, the plasmid toolkit developed here can be used to delicately fine-tune the simultaneous expression of multiple genes using different strategies in *S. oneidensis*.

### Fine-Tuning MtrCAB Expression in the *S. oneidensis ΔmtrA* Strain

The EET pathway of *S. oneidensis* MR-1 is comprised of c-type cytochromes that shuttle electrons from oxidizing enzymes in the cytoplasm and inner membrane to the outside of the cell.

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**FIGURE 6 |** Fine-tuning multiple fluorescent protein expression in *S. oneidensis* MR-1 harboring two plasmids with replicons pBBR1 and CoIE. (A) GFP and BFP expression were regulated by the IPTG-inducible pTac promoter (IPTG concentrations: 1, 12.5, 15, and 100 µM) and the arabinose-inducible pBAD promoter (arabinose concentrations: 0.1, 0.25, 0.5, and 1 mM), respectively. (B) GFP and CFP expression were adjusted by the promoters pTac and pBAD at the above concentrations, respectively. (C) GFP and BFP expression were under the control of the high-strength pBAD promoter and placUV5, pTet, or pBAD, and placUV5 or pBAD, respectively. The error bars (mean ± SD) were derived from triplicate experiments for each strain.
FIGURE 7 | Fine-tuning MtrCAB expression to affect EET efficiency determined by WO$_3$ reduction assay. (A) Color development of the WO$_3$ reduction assay. Culture medium without bacteria was used as a control to eliminate disturbances caused by abiotic factors. The mtrCAB gene was overexpressed in the S. oneidensis mtrA strain under the control of placUV5, pTet, and pBAD promoters, which displayed low, medium, and high levels of gene expression, respectively. The parental and recombinant strains were named as S-ΔmtrA, L-mtrCAB, M-mtrCAB, and H-mtrCAB, respectively. Firstly, we performed a tungsten trioxide (WO$_3$) reduction assay (Yuan et al., 2014) to assess the fine-tuning effect of mtrCAB on EET. Culture medium without bacteria was used as a control to eliminate disturbances caused by abiotic factors. EET from S-ΔmtrA, L-mtrCAB, M-mtrCAB, or H-mtrCAB to the WO$_3$ nanorod assembly probe was accompanied by a bioelectrochromic reaction causing conspicuous color development in the 96-well plate, which enabled us to evaluate EET either qualitatively with the naked eye or quantitatively through image analysis (Figure 7A). For the latter, the color intensity of each well was determined by analyzing the ‘Density (mean)‘ (Wang et al., 2009; Tatro et al., 2013) of the area of interest with ImageJ Software V.1.8.0 (Figure 7B). The results showed that the EET capacity of MtrCAB under the control of the low-, medium-, and high-strength promoters was increased by 22.3, 133.5, and 65.3% over that of the parental strain, suggesting that overexpression of MtrCAB at a medium level improved EET most significantly in the S. oneidensis ΔmtrA strain.

To more quantitatively investigate EET efficiency upon fine-tuning the overexpression of MtrCAB, bio-electrochemical analyses were conducted in microbial fuel cells (MFCs).
parental and engineered strains were inoculated into the anodic chamber of MFCs, respectively, with a 2 kΩ external resistor (Figure 8). Experimental results showed that MtrCAB expression in the mutant S. oneidensis could increase the voltage output, with significant differences among the recombinant strains. The maximum voltage increased 140.3, 595.8, and 443.2%, when the mtrCAB complex was expressed at low, medium and high levels, respectively, compared to the parental S. oneidensis ΔmtrA strain (Figure 8A). Bioelectrochemical analyses were further conducted to study EET efficiency of these recombinant strains in MFCs (Figure 8B). These MFC results demonstrated that EET efficiency was improved most significantly when mtrCAB was expressed using a moderate promoter in the mutant S. oneidensis, which was consistent with the WO complex was expressed at low, medium and high levels, respectively.

**CONCLUSION**

We constructed and characterized a synthetic plasmid toolkit for S. oneidensis MR-1, which contains a set of shuttle vectors with a combination of promoters, replication origins, antibiotic resistance genes, and a shuttle component. We quantified the strength of promoters and the copy number of different replicons. We further transformed two plasmids conferring different antibiotic resistances into recombinant S. oneidensis MR-1 strains, enabling the expression of multiple genes (e.g., fluorescent reporters) to be fine-tuned. The synthetic plasmid toolkit was then successfully used to modulate the expression of MtrCAB and achieve improved EET efficiency. Moderate overexpression level of MtrCAB improved the EET efficiency most significantly in the S. oneidensis ΔmtrA strain. The plasmid toolkit developed in this study allows researchers to rapidly and conveniently fine-tune gene expressions with greater controllability and predictability, thus accelerating the development and application of genetic manipulations in S. oneidensis.

**DATA AVAILABILITY**

This manuscript contains previously unpublished data. The name of the repository and accession number are not available.

**AUTHOR CONTRIBUTIONS**

Y-XC designed the experiments. M-YS and Y-XC performed all the experiments. Y-RC, Y-YC, JX, and QD performed a part of MFC experiments. FL, C-FL, and XL helped with revising the manuscript. M-YS, Y-XC, and HS wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00410/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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