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ARTICLE

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Promoting bidirectional extracellular electron transfer of *Shewanella oneidensis* MR-1 for hexavalent chromium reduction via elevating intracellular cAMP level

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Abstract

The bioreduction capacity of Cr(VI) by *Shewanella* is mainly governed by its bidirectional extracellular electron transfer (EET). However, the low bidirectional EET efficiency restricts its wider applications in remediation of the environments contaminated by Cr(VI). Cyclic adenosine 3′,5′-monophosphate (cAMP) commonly exists in *Shewanella* strains and cAMP–cyclic adenosine 3′,5′-monophosphate receptor protein (CRP) system regulates multiple bidirectional EET-related pathways. This inspires us to strengthen the bidirectional EET through elevating the intracellular cAMP level in *Shewanella* strains. In this study, an exogenous gene encoding adenylate cyclase from the soil bacterium *Beggiatoa* sp. PS is functionally expressed in *Shewanella oneidensis* MR-1 (the strain MR-1/pbPAC) and a MR-1 mutant lacking all endogenous adenylate cyclase encoding genes (the strain Δca/pbPAC). The engineered strains exhibit the enhanced bidirectional EET capacities in microbial electrochemical systems compared with their counterparts. Meanwhile, a three times more rapid reduction rate of Cr(VI) is achieved by the strain MR-1/pbPAC than the control in batch experiments. Furthermore, a higher Cr(VI) reduction efficiency is also achieved by the strain MR-1/pbPAC in the Cr(VI)-reducing biocathode experiments. Such a bidirectional enhancement is attributed to the improved production of cAMP–CRP complex, which upregulates the expression levels of the genes encoding the c-type cytochromes and flavins synthetic pathways. Specially, this strategy could be used as a broad-spectrum approach for the other *Shewanella* strains. Our results demonstrate that elevating the intracellular cAMP levels could be an efficient strategy to enhance the bidirectional EET of *Shewanella* strains and improve their pollutant transformation capacity.

KEYWORDS

bidirectional extracellular electron transfer, biocathode, cAMP, Cr(VI) reduction, Shewanella

Zhou-Hua Cheng and Jia-Rui Xiong contributed equally to this study.

1 | INTRODUCTION

Hexavalent chromium-Cr(VI) is widely prevalent in natural environments and highly toxic to living organisms (Besser, Brumbaugh, Kemble, May, & Ingersoll, 2004; Costa, 1997; Xiao et al., 2013; Zhitkovich, 2011). Bioreduction of Cr(VI) using dissimilatory metal-reducing bacteria (DMRB) like *Shewanella* has recently attracted increasing interests because of its environmentally friendly feature (Belchik et al., 2011; Bencheikh-Latmani, Obraztsova, Mackey, Ellisman, & Tebo, 2007; Viamajala, Peyton, Apel, & Petersen, 2002; Xafenias, Zhang, & Banks, 2013). However, the bioreduction capacity of Cr(VI) by *Shewanella* is mainly governed by its bidirectional extracellular electron transfer (EET), which connects its intracellular metabolism to external electron donors/acceptors (Belchik et al., 2011; Viamajala et al., 2002; Xafenias et al., 2013). Extensive efforts have been made to elucidate the bidirectional EET mechanisms in such a strain (Kumar et al., 2017; Shi et al., 2016; White et al., 2016). The electrons released by MR-1 must be transferred from interior of cells to the outer membrane through a network of redox proteins, also known as the metal reducing and respiratory pathway (the Mtr respiratory pathway; Shi, Rosso, Zachara, & Fredrickson, 2012). The Mtr respiratory pathway includes CymA (the electron-transfer hub), MtrA (periplasmic c-type cytochrome), MtrB (β-barrel protein), MtrC and OmcA (two outer membrane c-type cytochromes). Interestingly, the Mtr respiratory pathway is also functionally reversible (Ross, Flynn, Baron, Gralnick, & Bond, 2011). Cathodic electrons can flow from outer membrane cytochromes into the quinone pool and back to periplasmic fumarate reductase or enter the electron transport chain when fumarate or oxygen is used as the terminal electron acceptor respectively (Ross et al., 2011; Rowe et al., 2018). Moreover, *Shewanella oneidensis* MR-1 can also secrete flavins to mediate EET (Marsili et al., 2008; von Canstein, Ogawa, Shimizu, & Lloyd, 2008). Flavin electron shuttling accounts for ~75% of EET to insoluble electron acceptors (Kotloski & Gralnick, 2013). However, bidirectional EET of *S. oneidensis* MR-1 is typically characterized by poor controllability and limited efficiency (TerAvest & Ajo-Franklin, 2016). Thus, efforts should be made to develop new approaches to improve the bidirectional EET in MR-1.

In *S. oneidensis* MR-1, cyclic adenosine 3′,5′-monophosphate (cAMP)–cyclic adenosine 3′,5′-monophosphate receptor protein (CRP) regulatory system plays a central role in regulation of metabolism (Charania et al., 2009; Dong et al., 2012; Fu et al., 2013; Saffarini, Schultz, & Beliaev, 2003; Yin, Meng, Fu, & Gao, 2016). CRP mutation in MR-1 caused defects in utilizing multiple electron acceptors, such as Mn^{4+}, Fe^{3+}, fumarate, nitrate, nitrite, and dimethyl sulfoxide (Saffarini et al., 2003). Elevated cAMP levels drove cells into a low-energetic status and inhibited aerobic respiration (Yin et al., 2016). *S. oneidensis* MR-1 could use cAMP–CRP system to coordinate relationships between EET and carbon catabolism by regulating the expression levels of D-lactate dehydrogenase (Kasai, Tomioka, Kouzuma, & Watanabe, 2019). Moreover, the cAMP–CRP system could regulate the EET directly by activating the expression of the metal-reducing conduit (MtrCAB and OmcA; Kasai et al., 2019). Thus, it is reasonable to assume that an increase in cAMP level and/or CRP level might enhance the bidirectional EET efficiency of MR-1. Although overexpression of an endogenous gene with respect to cAMP synthesis facilitated the outward EET by *S. oneidensis* MR-1 (Kasai et al., 2019), the impacts of the elevated intracellular cAMP level on the bidirectional EET efficiency of MR-1 remain unrevealed yet. Moreover, the molecular mechanisms underlying the regulation of the bidirectional EET by cAMP–CRP complex in MR-1 have not been fully elucidated.

Therefore, this study aims to evaluate the impacts of the elevated intracellular cAMP level on the bidirectional EET efficiency and pollutant reduction capacity by MR-1 and explore the underlying mechanisms. For this purpose, first an exogenous gene encoding adenylate cyclase from the soil bacterium *Beggiatoa* sp. PS, which showed a low cyclase activity in the dark but increased 300-fold in light (Stierl et al., 2011), was functionally expressed in MR-1 and a MR-1 mutant lacking all endogenous adenylate cyclase encoding genes. Then, the bidirectional EET performances of the engineered strains in bioelectrochemical systems and the transcription levels of the cAMP–CRP complex regulated genes were evaluated. Finally, the engineered strains were applied to reduce a typical heavy metal pollutant-hexavalent chromium-Cr(VI). In this way, the feasibility of elevating the bidirectional EET in *S. oneidensis* MR-1 by strengthening the intracellular cAMP levels was validated.

2 | MATERIALS AND METHODS

2.1 | Strains, plasmids, and culture conditions

The strains, plasmids, and primers used in this study are listed in Table S1. MR-1 and Δca grew aerobically in Luria-Broth (LB) medium at 30 °C. MR-1/pbPAC (the wild type containing the pbPAC plasmid), MR-1/pYYDT (the wild type containing the control plasmid), Δca/pbPAC (the Δca containing the pbPAC plasmid) and Δca/pYYDT (the Δca containing the control plasmid) grew in LB medium supplemented with 50 μg/ml kanamycin. *E. coli* WM3064 grew aerobically in LB medium supplemented with 100 μg/ml diaminopimelic acid at 37 °C. The growths of MR-1/pbPAC, MR-1/pYYDT, Δca/pbPAC and Δca/pYYDT in LB medium under aerobic conditions were measured at 600 nm.

2.2 | Strains construction

In-frame deletions of *cyaA*, *cyaB*, and *cyaC* in *S. oneidenisis* MR-1 were constructed through a two-step homologous recombination method with the suicide plasmid pRE112 as described previously (D. F. Liu et al., 2017). The expression plasmid pbPAC was constructed in *Escherichia coli* WM3064. In brief, the *bPAC* sequence was extracted from NCBI databases (https://www.ncbi.nlm.nih.gov/) and adapted for optimal expression in *S. oneidensis* MR-1 (Grote et al., 2005). The resulting sequence was synthesized and cloned into pYYDT (Min et al., 2017). An arabinose-inducible promoter was used to drive the expression of *bPAC*. The expression plasmid pbPAC was then introduced into Δca and MR-1 by electroporation as follows. The strains

Δca and MR-1 were cultured in 1 ml LB medium for 16 hr and then centrifuged at 7000*g* for 2 min. The pellets were immediately washed twice and resuspended with 100 μL sucrose solution (0.3 M). Five hundred-nanogram expression plasmid was added into the resuspend bacterial pellets and then electroporated using 0.1-cm cuvettes and a BioRad Gene PulserTM with pulse controller.

2.3 | Intracellular cAMP concentration determination and real-time quantitative reverse transcription PCR (qRT-PCR) analysis

CRP positively regulats the expression of *omcA* (Kasai et al., 2019). The *omcA* promoter was amplified from *S. oneidensis* MR-1 and used to drive the expression of *sfGFP*. Meanwhile, *cyOFP* was placed under the control of a constitutive promoter-J23102. Therefore, the relative levels of intracellular cAMP concentrations in the engineered strains could be monitored by using the fluorescent intensity ratio of sfGFP and CyOFP. These components were cloned into pYYDT and pbPAC via Gibson assembly (Gibson et al., 2009). The resulting cAMP reporter plasmids were transferred into the strain Δca by electroporation.

Bacterial solution of 5 μl was pipetted out and loaded on a pad containing 2% (wt/vol) agarose and FAB medium (Pu, Yang, Xia, & Jin, 2018). The pad was then flipped onto a 0.15 mm cover glass. Four images of each sample were snapped using confocal microscopy (IX-81, Olympus), equipped with a ×100 oil objective and an EMCCD camera (Andor iXon 897). In each image field, two images could be acquired, including one sfGFP image and one CyOFP image. Note, a 488 nm laser was used to excite both sfGFP and CyOFP and two emission filters, sized at 524 ± 25 nm and 607 ± 25 nm, were necessary to collect fluorescence. Finally, an image processing algorithm code was used by MATLAB to conduct data analysis. The mean intensities of sfGFP and CyOFP were acquired. RNA extraction and qRT-PCR analysis were conducted as described previously (Min et al., 2017).

2.4 | In situ spectral measurements of the c-type cytochromes

Shewanella strains were incubated overnight in 3 ml LB broth at 30°C with shaking at 200 rpm. The cultures were centrifuged at 7000*g* for 2 min. The resulting pellets were washed twice and resuspended with 3 ml phosphate buffered saline (PBS buffer). The UV/Vis spectroscopy equipped with a integrating sphere reflectance attachment was used to record spectra of the c-type cytochromes of the strains Δca/pYYDT, Δca/pbPAC, MR-1/pYYDT, and MR-1/pbPAC.

2.5 | Electrochemical tests

A dual-chamber microbial fuel cell (MFC) with a working volume of 90 ml was assembled as described previously (Min et al., 2017). Carbon felt (Beijing Sanye Carbon Co., China) with a specific surface area of 8 cm^2 was used as the anode and the cathode materials and a proton exchange membrane (GEFC-10N, GEFC Co., China) was used as the separator. The cathodic electrolyte was composed of 50 mM potassium ferricyanide in 50 mM phosphate buffer solution and the anodic electrolyte was made of 50 ml of *Shewanella* mineral medium with 20 mM lactate as electron donor. The composition of *Shewanella* mineral medium was referred to a previous study (Min et al., 2017). The bacteria were cultured for 16 hr in LB medium supplemented with 10 mM arabinose and 50 μg/ml kanamycin. Then, the cultures were centrifuged at 7000*g* for 2 min, washed twice and resuspended with PBS buffer. The bacterial suspension was subsequently added into the anode chamber at an initial optical density (OD_{600}) of 0.4. The voltage output of the MFCs inoculated with *Shewanella* strains was recorded every 10 min using a data acquisition system (USB2801, ATD Co., China). Moreover, 1 ml of sample was withdrawn from each anode chamber at the end of the electrochemical experiment and the fluorescence intensities of samples were recorded by fluorescence spectroscopy with an excitation wavelength of 440 nm and an emission wavelength of 525 nm. Linear sweep voltammetry (LSV), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) analysis were conducted as described previously (Min et al., 2017).

The conventional three-electrode microbial electrolysis cells (MECs) was used to investigate the outward EET abilities of *Shewanella* strains at a constant potential (Min et al., 2017). Δca/pYYDT and Δca/pbPAC were cultured in aerobic *Shewanella* mineral medium (with 20 mM lactate as the electron donor and 5% LB medium) until an OD_{600} of 0.5 was reached. Then, the cultures were directly transferred into MECs because Δca could not grow in *Shewanella* mineral medium with fumarate as a electron acceptor under anaerobic conditions. Different from the cultivation conditions for the strains Δca/pYYDT and Δca/pbPAC, after aerobic cultivation for 12 hr in *Shewanella* mineral medium with 20 mM lactate as the electron donor, 5% of MR-1/pYYDT and MR-1/pbPAC cultures were inoculated into anaerobic mineral salts medium (including 20 mM lactate, 50 mM sodium fumarate, and 0.05% casamino acids) until an OD_{600} of 0.5 was reached and then transferred into MECs. A CHI1030B electrochemical workstation (Chenhua Instrument Co., China) served as potentiostat and a constant potential of 0.2 V (vs Ag/AgCl) was applied to the carbon paper electrode (6 cm^2).

To investigate the inward EET abilities of *Shewanella* strains, their biofilm was formed in a three-electrode electrochemical cell (Yang et al., 2015). The working electrode (2.0 cm × 2.0 cm carbon felt) was poised at a potential of −0.56 V (vs. Ag/AgCl) and the electrolyte was continuously stirred by a multipoint magnetic stirrer. The inward current was measured by an electrochemical workstation (CHI1030B, Chenhua Co., China). After the steady state was reached for the baseline, fumarate was added into electrochemical cell with a final concentration of 50 mM. Cr(VI)-reducing biocathodes experiments were conducted as described previously (Xafenias et al., 2013).

2.6 | Cr(VI) bioreduction tests

Batch experiments characterizing the Cr(VI) reduction abilities of different *Shewanella* strains were conducted. Briefly, the strain

MR-1/pYYDT and MR-1/pbPAC were cultured overnight in LB medium at 30°C. The cultures were centrifuged at 7000*g* for 2 min, washed twice and resuspended with *Shewanella* mineral medium. Then, the bacterial suspension were injected into 100 ml serum vials containing 50 ml deoxygenated *Shewanella* mineral medium, 25 mg/L Cr(VI) and 20 mM lactate. The initial OD_{600} value of the *Shewanella* cells in the serum vials was 0.4. The Cr(VI) concentration was measured using the diphenylcarbazide (DPC) method (Urone, 1955).

3 | RESULTS AND DISCUSSION

3.1 | Functional expression of an exogenous adenylate cyclase encoding gene in *S. oneidensis* MR-1

Three adenylate cyclases encoding genes with respect to cAMP synthesis are reported to exist on the genome of *S. oneidensis* MR-1 (Charania et al., 2009). To explore the impact of the intracellular cAMP level on the physiological state and EET of MR-1, a mutant Δca lacking all endogenous adenylate cyclase encoding genes was constructed. Moreover, an exogenous adenylate cyclase encoding gene (*bPAC*) from the soil bacterium *Beggiatoa* sp. PS was introduced into Δca and MR-1, and the resulting strains are respectively referred as Δca/pbPAC and MR-1/pbPAC. The MR-1/pYYDT (the wild type containing the control plasmid) colonies showed brown-red after 12-hr cultivation in LB medium, while the Δca/pYYDT (Δca containing the control plasmid) showed pink (Figure 1a). The overexpression of *bPAC* in Δca led to a deeper colonies color than that of Δca. A similar phenomenon was also found in the MR-1/pbPAC (Figure 1a). A diffuse-transmission UV-visible spectroscopy was used to record the spectra of c-type cytochromes in the living *Shewanella* strains and characterize the c-type cytochromes levels of the different *Shewanella* strains (Luo et al., 2019). The optical spectrum of the oxidized c-type cytochromes had a Soret band centered at 408 nm, a visible region peak at 531 nm and a shoulder at 560 nm. Upon reduction, the Soret band λ_{max} shifted to 419 nm and α- and β-peaks at 525 and 552 nm. When sufficient electron donors were provided in culture medium, the c-type cytochromes in *Shewanella* were mainly in the reduced states (Luo et al., 2019). As shown in Figure 1b, the highest intensity of the specific peaks at 419 nm, 525 nm, and 552 nm was achieved by the strain MR-1/pbPAC. A higher intensity of the specific peaks was also observed in the strain Δca/pbPAC than that Δca/pYYDT (Figure 1b). All these results demonstrate that the exogenous adenylate cyclase encoding gene successfully expressed and the elevated intracellular cAMP enhanced the levels of the c-type cytochromes in the engineered strains.

Then, the relative levels of intracellular cAMP concentrations in the engineered and control strains were evaluated. To exclude any effects of endogenous adenylate cyclase, the relative levels of intracellular cAMP concentrations in the Δca/pYYDT and Δca/pbPAC were compared. A reporter module comprising two fluorescent proteins was constructed (Figure S1). Previous studies have shown

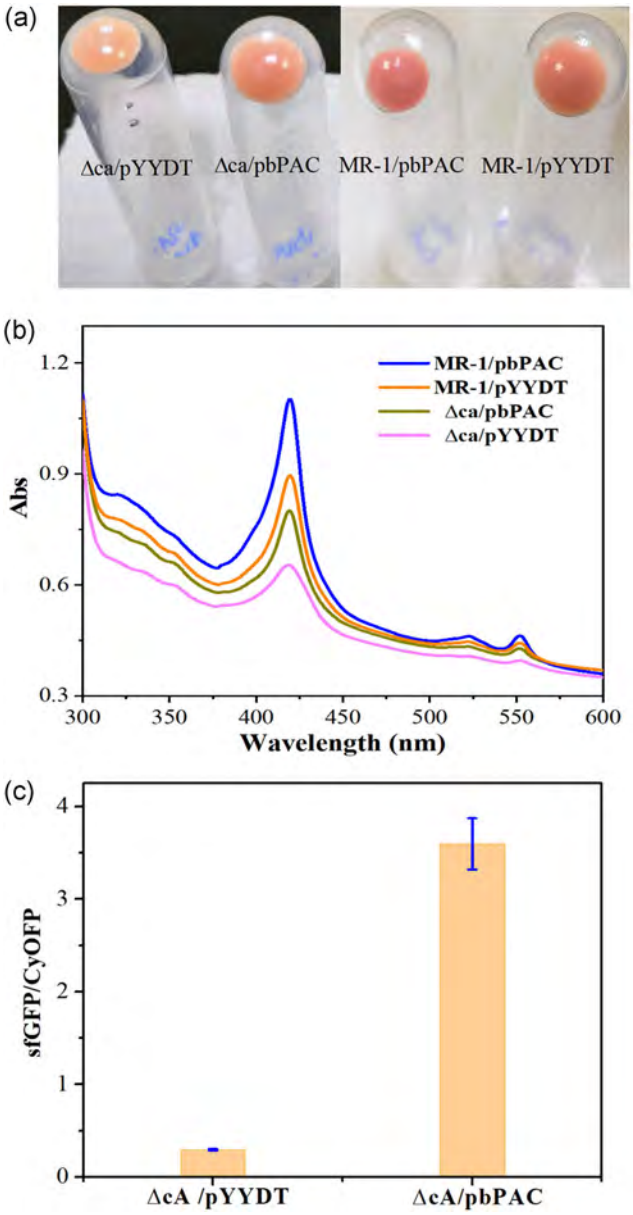

FIGURE 1 c-Type cytochromes levels and the intracellular cAMP concentrations of the different *Shewanella* strains. (a) The images of different *Shewanella* cells centrifuged after 12-hr cultivation in LB medium under aerobic conditions. (b) Spectral characterization of c-type cytochromes in the different *Shewanella* strains by diffuse-transmission UV-visible spectroscopy. (c) The fluorescent intensity ratios of sfGFP and CyOFP represent the relative levels of intracellular cAMP concentrations of the strains Δca/pYYDT and Δca/pbPAC. cAMP, cyclic adenosine 3′,5′-monophosphate; cyOFP, cyan-excitable orange-red fluorescent protein; LB, Luria-Broth; sfGFP, Superfolder green fluorescent protein

that CRP positively regulates the expression of the Mtr respiratory pathway genes by binding to the upstream regions of *omcA* promoter (Kasai et al., 2019). Thus, the promoter of *omcA* was used to drive the expression of a green fluorescent protein (sfGFP), while a constitutive promoter-J23102 was used to control the expression of a bright cyan-excitable orange fluorescent protein (CyOFP). In

this way, the relative levels of intracellular cAMP concentrations in the strains could be monitored by using the fluorescent intensity ratio of sfGFP to CyOFP (Figure 1c). As shown in Figure 1c, a higher intensity ratio was obtained by the strain Δca/pbPAC than by the Δca/pYYDT, and the relative level of cAMP concentration in the Δca/pbPAC was 11.2-fold higher than that in the Δca/pYYDT. Moreover, the growth of the engineered strains was also compared with the control. Consistent with a previous work (Yin et al., 2016), the aerobic growth of the Δca/pYYDT was only slightly impaired than that of the MR-1/pYYDT (Figure S2). Interestingly, the growth of the Δca/pbPAC was more severely impaired than that of the Δca/pYYDT. The growth of the MR-1/pbPAC was initially suppressed and ultimately reached the same level as that of the Δca/pYYDT. These results suggest that the engineered strains exhibited slight defects in aerobic growth than the control strain.

3.2 | Enhanced outward EET capacities in the strains Δca/pbPAC and MR-1/pbPAC

The current densities of the dual-chamber MFCs cultivated with the engineered strains and control strains were determined (Figure 2a). Most of the MFCs started up rapidly and achieved the maximum current density after about 20 hr. The MFCs with the strain Δca/pYYDT generated an extremely low maximum current density (107 mA/m^2), indicating that the cAMP–CRP regulatory system played an important role in the outward EET of the strain MR-1. The maximum current density of the MFC inoculated with the strain MR-1/pbPAC was 356 mA/m^2, which was 2.3 times higher than that of the Δca/pYYDT. The MFCs with the strain Δca/pbPAC and MR-1/pYYDT could generate a higher maximum current density (234 mA/m^2 and 237 mA/m^2, respectively) than that with the Δca/pYYDT, suggesting that the relative concentration of intracellular cAMP in the Δca/pbPAC reached the same level as that of the MR-1/pYYDT.

The polarization and power density curves indicate that the MFCs inoculated with the strains MR-1/pbPAC, MR-1/pYYDT, and Δca/pbPAC delivered higher power densities than the strain Δca/pYYDT (Figure 2b). The MFC inoculated with the strain MR-1/pbPAC achieved a maximum power density of 0.096 W/m^2, almost 7.0-fold higher than that with the strain Δca/pYYDT (0.012 W/m^2). The maximum power density for the MFCs inoculated with the strain MR-1/pYYDT, and Δca/pbPAC was 0.054 W/m^2 and 0.051 W/m^2, respectively, which was 3.5-fold and 3.25-fold higher than that for the strain Δca/pYYDT. Moreover, the lower slope in the V-j curves of the MFCs inoculated with the strains MR-1/pbPAC, MR-1/pYYDT, and Δca/pbPAC also implies a decreased internal ohmic resistance compared to that for the strain Δca/pYYDT. Since EIS could be used to

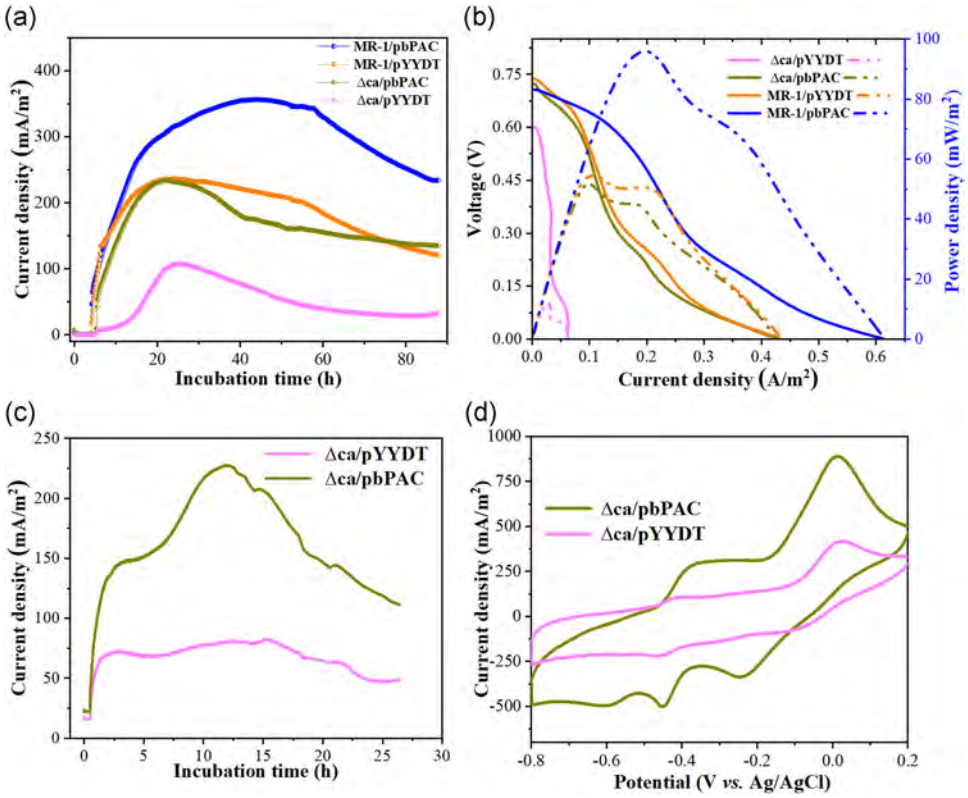

FIGURE 2 Outward EET performances of different *Shewanella* strains. Current output (a) and power density (b) of different *Shewanella* strains in MFCs. (c) Amperometric data from the MECs inoculated with the strains Δca/pYYDT and Δca/pbPAC. (d) CV characterization of the MECs with inoculations of the strains Δca/pYYDT and Δca/pbPAC under turnover condition, respectively. CV, cyclic voltammetry; EET, extracellular electron transfer; MFC, microbial fuel cell

determine the interfacial electron-transfer resistance between microbes and electrode (Min et al., 2017), the EIS analysis of the MFCs inoculated with the strains MR-1/pbPAC and Δca/pbPAC was performed (Figure S3). A smaller interfacial charge-transfer resistance (R_{ct}) represents a faster interfacial electron-transfer rate from microbes to electrode. The R_{ct} values of the MFCs inoculated with the strains MR-1/pbPAC (R_{ct} = 552), MR-1/pYYDT (R_{ct} = 879), and Δca/pbPAC (R_{ct} = 950) were remarkably lower than that for the strain Δca/pYYDT (R_{ct} = 3167), confirming that the decreased internal ohmic resistance contributed to the elevated electrochemical performances of the MFCs inoculated with the strains MR-1/pbPAC and Δca/pbPAC. All these results indicate that the improved synthesis of intracellular cAMP in the MR-1/pbPAC or Δca/pbPAC enhanced their outward EET capacities compared with their counterparts.

The feasibility of enhancing outward EET in other *Shewanella* strains such as *Shewanella decolorationis* S12 and *Shewanella putrefaciens* CN32 by the overexpression of the exogenous adenylate cyclase encoding gene was also examined (Figure S4). A higher peak current density value for the MFCs was achieved by the strain S12/pbPAC (0.37 A/m^2) than that of the strain S12/pYYDT (0.30 A/m^2). The polarization and power density curves indicate that the MFCs inoculated with the strain S12/pbPAC delivered a higher power density than the strain S12/pYYDT. Similarly, the overexpression of the exogenous adenylate cyclase encoding gene in *S. putrefaciens* CN32 also improved the electricity generation in the MFCs (Figure S4). Therefore, improving intracellular cAMP synthesis was an efficient approach to enhance the EET in *Shewanella* strains.

To further evaluate the impacts of the elevated intracellular cAMP level on the outward EET capacities in the strains MR-1/pbPAC and Δca/pbPAC, a MEC system poised at a constant potential (0.2 V vs. Ag/AgCl) was adopted (Figures 2c and S5). The current generation profiles show that the MECs inoculated with the strains MR-1/pYYDT and MR-1/pbPAC achieved start-up rapidly and took about 2.5 hr only to reach the maximum current density (Figure S5). A higher peak current density value was achieved by for the MEC with the strain MR-1/pbPAC (approximately 0.23 A/m^2) than that of the strain MR-1/pYYDT (about 0.14 A/m^2). Similarly, the maximum current density of the MECs inoculated with the strain Δca/pbPAC was much higher than that for the strain Δca/pYYDT (Figure 2c). Figure 2d shows the CV results of the MECs inoculated with the strains Δca/pYYDT and Δca/pbPAC. For the Δca/pYYDT, only a pair of anodic and cathodic peaks generating a flavin-mediated catalytic current (centered at −0.43 V vs. Ag/AgCl; Min et al., 2017) was identified in the CV curve under turnover conditions. The low expression levels of the c-type cytochromes in the Δca/pYYDT might lead to the lack of another pair of peaks corresponding to the outer membrane c-type cytochromes (centered at −0.25 V vs. Ag/AgCl) in the CV curve. For the Δca/pbPAC, two pairs of anodic and cathodic peaks respectively corresponding to the outer membrane c-type cytochromes and flavins (Min et al., 2017), were identified in the CV curve under turnover conditions. Moreover, the strain Δca/pbPAC showed a much higher peak intensity centered at −0.43 V versus Ag/AgCl in the CV curve than that of the strain Δca/pYYDT.

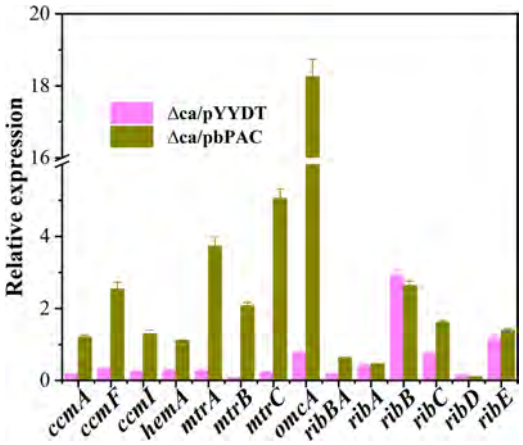

FIGURE 3 Comparison of the transcription levels of genes encoding the cytochrome *c* maturation system, the heme synthetic pathway, the Mtr respiratory pathway, and the flavin synthetic pathway in the strains Δca/pbPAC and Δca/pYYDT

Interestingly, flavin with a higher concentration existed in the anode supernatant of the MFC inoculated with the strain Δca/pbPAC than that for the strain Δca/pYYDT (Figure S6). These results imply that an enhanced outward EET rate in the strain Δca/pbPAC was ascribed to the synthesis of more outer membrane c-type cytochromes and flavins than the strain Δca/pYYDT.

To elucidate the mechanisms underlying the increased synthesis of outer membrane c-type cytochromes and flavins in the strain Δca/pbPAC, the transcription levels of genes encoding the cytochrome *c* maturation system, the heme synthetic pathway, the Mtr respiratory pathway, and the flavin synthetic pathway in the strains Δca/pbPAC and Δca/pYYDT were compared (Figure 3). In MR-1, the cytochrome *c* maturation system associated genes were contained in three operons, *ccmABCDE*, *ccmFGH*, and *ccmI* (Yin et al., 2016). The heme synthetic pathway in MR-1 entailed nine reactions and the first reaction catalyzed by HemA was the rating-limiting step in heme synthesis (Yin et al., 2016). The expression levels of *ccmA*, *ccmF*, *ccmI*, and *hemA* in the strain Δca/pbPAC were enhanced by about 5.8-, 6.6-, 4.1- and 2.8-fold, respectively, compared with the strain Δca/pYYDT. Previous studies demonstrate that the elevated intracellular cAMP concentrations in MR-1 increased the expression levels of the Mtr respiratory pathway associated genes (Kasai et al., 2019). Similarly, the levels of *mtrA*, *mtrB*, *mtrC*, and *omcA* in the strain Δca/pbPAC increased by about 12.9-, 24.9-, 22.9- and 22.8-fold, respectively, compared to the levels of the strain Δca/pYYDT. Furthermore, compared with the strain Δca/pYYDT, the expression levels of *ribBA*, *ribC*, and *ribE* in the strain Δca/pbPAC increased by about 250%-, 120%-, and 20%, respectively. However, the expression of *ribA*, *ribB*, and *ribD* in the strain Δca/pbPAC showed a similar level to the strain Δca/pYYDT. Although there were no CRP-binding sites located upstream of *ccmA*, *ccmF*, *ccmI*, *hemA*, *ribBA*, *ribC*, and *ribE*, these results demonstrate that the cAMP–CRP complex might indirectly regulate the expression of the genes encoding the cytochrome *c* maturation system, the heme synthetic pathway, and the flavin synthetic pathway in *Shewanella*.

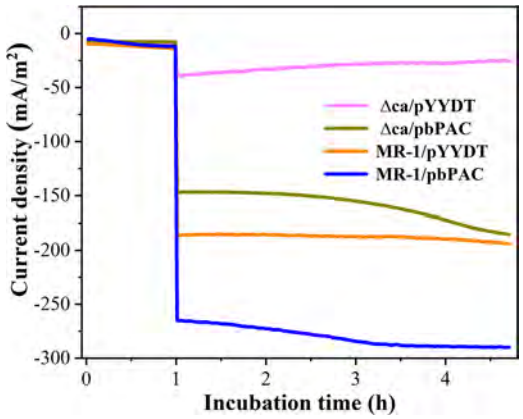

FIGURE 4 Inward EET performances of the different *Shewanella* strains. EET, extracellular electron transfer

3.3 | Enhanced inward EET capacities in the engineered strains

The inward EET capacities of the engineered and control strains were evaluated with the electricity consumption rate for the reduction of fumarate to succinate in the MEC system (Yang et al., 2015). Upon addition of 50 mM fumarate, prompt cathodic currents were recorded for all *Shewanella* strains (Figure 4). The strain Δca/pbPAC exhibited an obvious increase in the inward current density (185 mA/m^2), which was 3.7-fold higher than that of the strain Δca/pYYDT (39 mA/m^2). Similarly, the cathodic current density of the strain MR-1/pbPAC (290 mA/m^2) was almost 50% higher than that of the strain MR-1/pYYDT (194 mA/m^2). These results prove that the elevated intracellular cAMP concentrations in the strains Δca/pbPAC and MR-1/pbPAC also enhanced their inward EET capacities compared with their counterparts.

3.4 | Improved Cr(VI) reduction performance by the engineered strains

Our previous work has shown that coupling improved synthesis of flavins with metal-reducing conduits in MR-1 could improve its degradation rate of methyl orange (Min et al., 2017). Therefore, the upregulated expression levels of genes encoding the Mtr respiratory pathway and the flavin synthetic pathway in the strain MR-1/pbPAC might also enhance its capacity for the contaminant degradation. Cr (VI) reduction in MR-1 mainly occurred extracellularly (Huang

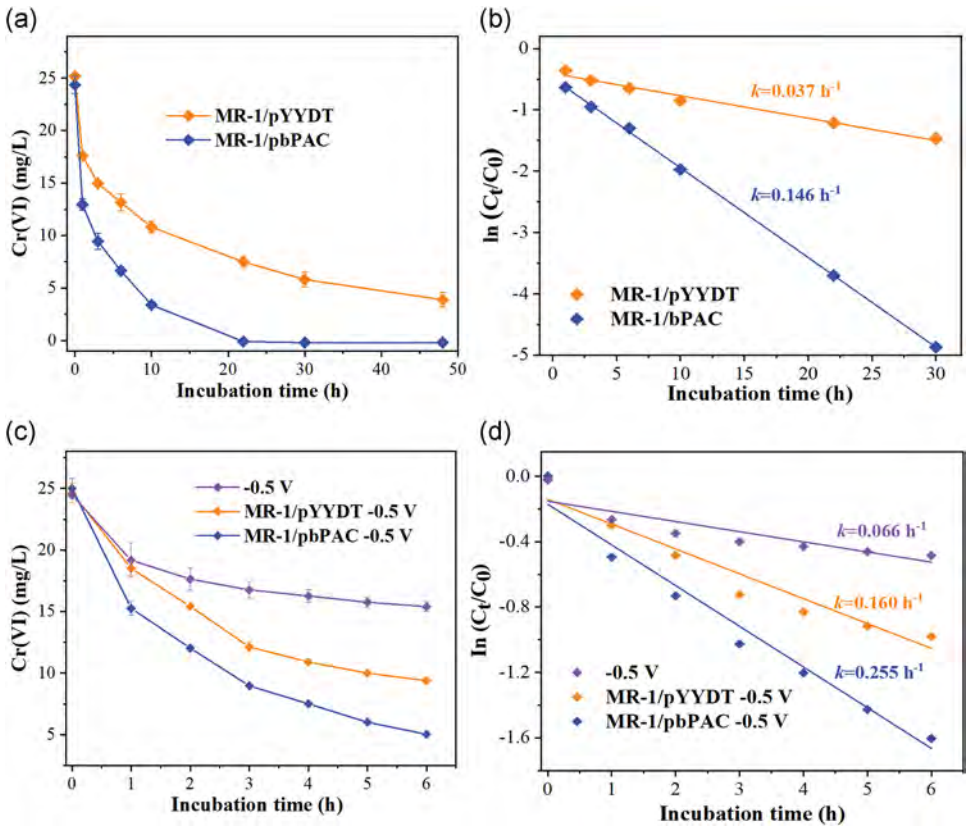

FIGURE 5 (a) Anaerobic reduction of Cr(VI) of 25 mg L^{-1} by the strain MR-1/pYYDT and MR-1/pbPAC in batch experiments. (b) Kinetic curves of Cr(VI) reduction by the strain MR-1/pYYDT and MR-1/pbPAC in the batch experiments. (c) The Cr(VI) concentration in the potentiostatically controlled cathodes. −0.5 V, MR-1/pYYDT-0.5V, and MR-1/pbPAC-0.5V represent the experiments with only mineral medium including 25 mg L^{-1} Cr(VI), mineral medium including 25 mg L^{-1} Cr(VI) and the strain MR-1/pYYDT, mineral medium including 25 mg L^{-1} Cr(VI) and the strain MR-1/pbPAC, respectively. (d) The Cr(VI) reduction kinetic curves in the biocathode experiments

et al., 2019; Myers, Carstens, Antholine, & Myers, 2000). The Mtr respiratory pathway plays an important role in Cr(VI) reduction and exogenous flavins could enhance the Cr(VI) reduction rate of the MR-1 (Belchik et al., 2011). It is reasonable to assume that the strain MR-1/pbPAC had a higher Cr(VI) reduction efficiency than the strain MR-1/pYYDT. In fact, Figure 5a shows that a more rapid Cr(VI) removal rate was achieved by the strain MR-1/pbPAC compared to the strain MR-1/pYYDT in the batch experiments. For the strain MR-1/pbPAC, Cr(VI) could be completely reduced within the initial 20 hr. However, the complete reduction of Cr(VI) to Cr(III) by the strain MR-1/pYYDT was accomplished after about 60-hr cultivation. Meanwhile, the first-order rate constant (k) was calculated to evaluate their Cr(VI) reduction rates (Figure 5b). Within the initial 30 hr, the k values increased by three times from 0.036 hr^{-1} for the strain MR-1/pYYDT to 0.146 hr^{-1} for the strain MR-1/pbPAC.

Moreover, Cr(VI) could be reduced and current was produced in the biocathodes in which *S. oneidensis* MR-1 as a biocatalyst and lactate as a carbon source co-existed (Xafenias et al., 2013). Thus, Cr(VI)-reducing biocathode experiments were also conducted to characterize the inward EET capacities of the strain MR-1/pYYDT and MR-1/pbPAC. Figure S7 shows that cathodic current produced by the potentiostatically controlled cells (−500 mV vs Ag/AgCl) inoculated with the strain MR-1/pbPAC increased when compared to that of the potentiostatically controlled cells inoculated with the strain MR-1/pYYDT or only mineral medium. Meanwhile, 1.1 mg Cr (VI) was reduced in the cathode chamber inoculated with the strain MR-1/pbPAC at the end of the 6 hr operation period, compared to the maximum Cr(VI) reduction of around 0.8 and 0.5 mg Cr(VI) by the cathode chamber inoculated with the strain MR-1/pYYDT and only mineral medium, respectively (Figure 5c). Moreover, the Cr(VI) reduction rate increased by 60% from 0.160 hr^{-1} for the strain MR-1/pYYDT to 0.255 hr^{-1} for the strain MR-1/pbPAC (Figure 5d). These results demonstrate that the elevated bidirectional EET capacity enhanced the Cr(VI) reduction performance of the strain MR-1/pbPAC compared with the control strain.

With the above results, we propose the underlying mechanisms for bidirectional EET regulated by the cAMP–CRP complex in *S. oneidensis* MR-1 (Figure 6). The exogenous adenylate cyclase catalyzes the cyclization of adenosine triphosphate (ATP) into cAMP and releases pyrophosphate (PPi). Accumulating cAMP could bind to CRP and then the cAMP-CAP complex activates the specific promoters. The cAMP-CAP complex could indirectly regulate the expression levels of the genes encoding the cytochrome *c* maturation system and the heme synthetic pathway, which leads to the increased synthesis of c-type cytochromes. It also directly regulates the expression levels of the genes encoding the Mtr respiratory pathway, which plays a central role in bidirectional EET of MR-1. Interestingly, cAMP-CAP complex also indirectly regulates the expression levels of the genes encoding the flavin synthetic pathway through a previously unrecognized mechanism. The coupling improved synthesis of both c-type cytochromes and flavins reduces the interfacial electron-transfer resistance between microbes and electrode and ultimately enhances the bidirectional EET of *S. oneidensis* MR-1.

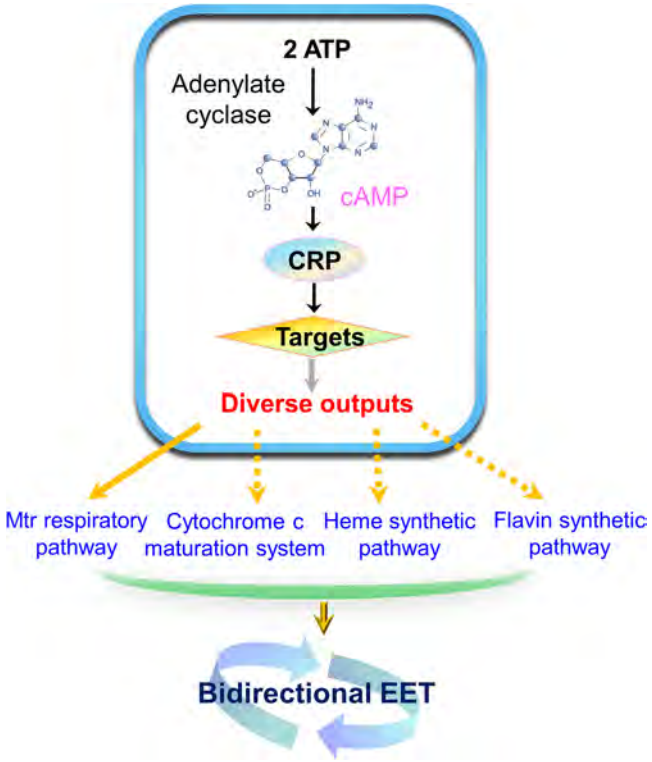

FIGURE 6 Proposed regulatory mechanisms of bidirectional EET mediated by the cAMP–CRP complex in *Shewanella* MR-1. cAMP, cyclic adenosine 3′,5′-monophosphate; CRP, cyclic adenosine 3′,5′-monophosphate receptor protein; EET, extracellular electron transfer

Compared with the other methods to improve the bidirectional EET in MR-1 (TerAvest & Ajo-Franklin, 2016), elevating intracellular cAMP levels offers a far more simple and efficient means. The same effect could be achieved through overexpression of only a single gene encoding adenylate cyclase or the co-expression of multiple genes encoding the flavin synthetic pathway and metal-reducing conduits in MR-1 (Min et al., 2017). Moreover, this method could also be applicable for the other *Shewanella* and DMRB strains. In addition to cAMP, heterologously overexpressing bis-(3′,5′)-cyclic diguanosine monophosphate (c-di-GMP) synthesis gene *ydeH* was reported to enhance biofilm formation and outward EET in MR-1 (T. Liu et al., 2015). Similarly, cyclic diadenosine monophosphate (c-di-AMP) was also identified to regulate the biofilm formation in diverse bacteria (Peng, Zhang, Bai, Zhou, & Wu, 2016). Recently, GacA was found to specifically regulate the intracellular cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP-AMP) level and was essential for Fe(III) particle-associated growth for *Geobacter sulfurreducensis* (Hallberg et al., 2019). These results imply that increasing the intracellular levels of other second messengers such as c-di-GMP, c-di-AMP, and cyclic AMP-GMP, should be the efficient approaches for constructing engineered *Shewanella* strains with the elevated bidirectional EET abilities. Furthermore, given the disparate regulatory targets by different second messengers (Hengge, Grundling, Jenal, Ryan, & Yildiz, 2016), the coupling improved synthesis of

multiple second messengers in *S. oneidensis* MR-1 would further enhance its bidirectional EET capacity.

4 | CONCLUSIONS

In this study, the introduction of an exogenous adenylate cyclase encoding gene significantly elevated the intracellular cAMP level in *S. oneidensis* MR-1. Accumulating cAMP–CRP complex positively regulated the expression levels of the genes encoding c-type cytochromes and flavins biogenesis and ultimately promoted the bidirectional EET of the genetically engineered strains. Moreover, improving the synthesis of the intracellular cAMP was also demonstrated to be efficient to enhance the bidirectional EET in other *Shewanella* strains. These engineered strains have potential applications in environmental remediation, wastewater treatment, and even electrochemical synthesis of high-value chemicals.

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