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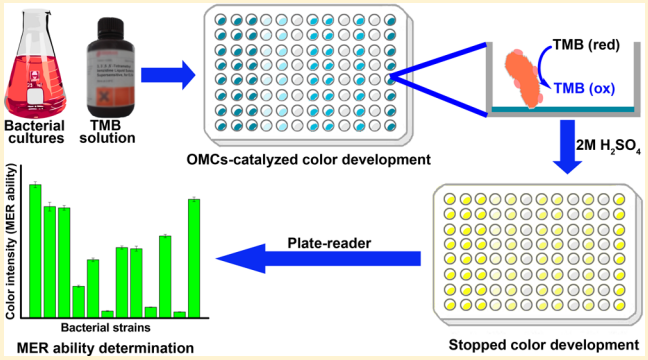
Rapid Measurement of Microbial Extracellular Respiration Ability Using a High-Throughput Colorimetric Assay

Shungui Zhou,* Junlin Wen, Junhua Chen, and Qin Lu

Guangdong Institute of Eco-environmental and Soil Sciences, Guangzhou 510650, P. R. China

Supporting Information

ABSTRACT: Microbial extracellular respiration (MER) involves the transfer of electrons to extracellular substrates and has significant environmental implications. Conventional methods for MER ability determination are reagent- and time-consuming, have a low throughput, or require noncommercial instruments. In this study, a plate-based colorimetric assay is proposed to measure MER ability. This method utilizes the peroxidase activity of the key components (multi-heme *c*-type cytochromes) of the extracellular electron-transfer network. The bacterial intrinsic peroxidase-catalyzed oxidation of chromogen (e.g., tetramethylbenzidine) resulted in a measurable color change correlated with the MER ability of the tested microorganisms. The results of the proposed colorimetric assay correspond well with those of traditional methods, such as the dissimilatory Fe(III) reduction method (Spearman's ρ of 0.946; $P < 0.01$) and the electricity generation method (Spearman's ρ of 0.893; $P < 0.01$). The proposed method allows researchers to identify extracellular respiring bacteria within several minutes and to measure their MER ability quantitatively by a plate-based assay.



INTRODUCTION

Extracellular respiring bacteria (ERB) make up a group of anaerobic or facultative anaerobic bacteria that can thrive under anaerobic conditions by coupling their cellular metabolism to respiratory reduction of various extracellular substrates, including solid phase metal minerals,[1] humic substances,[2] and soluble radionuclides.[3] This versatile microbial extracellular respiration (MER) plays an active and important role in the geochemical cycling of elements.[4−6] ERB have been used to remediate polluted environments,[7] synthesize nanoparticles,[8] and generate electricity.[9] However, their application potential can be further explored if more ERB species are identified, and developing an effective method for measuring the extracellular respiration ability of bacteria is critical to screening novel ERB from the natural environment.

Several methods have been used to measure MER ability. Traditional methods, including dissimilatory Fe(III) reduction[10] and humic substance reduction,[11] are typically reagent- and time-consuming. Electricity generation methods based on the principle of microbial fuel cells (MFCs) often require noncommercial equipment, such as U-tube MFCs,[12] nine-well pipet MFCs,[13] and microfabricated MFC arrays.[14] The electrochromic method[15] proposed by Yuan et al. in 2014 relies on the bioelectrochromic reaction of tungsten trioxide nanorods, which is rapid when used for qualitative assessments but is time-consuming and has a low throughput when utilized for quantitative measurements, requiring image capture and photograph analysis. Therefore, it is necessary to develop a rapid, low-cost, and high-throughput method for quantitatively determining MER ability.

As key components of the extracellular respiration network, outer membrane multi-heme *c*-type cytochromes shuttle electrons from cytoplasmic and inner membrane oxidizing enzymes toward the outside of the cell.[16,17] These outer membrane *c*-type cytochromes (OMCs) are iron porphyrin-containing enzymes[18] and can function catalytically via the chromogen/peroxide system. The peroxidase activity of iron porphyrin has been applied to visualize functional proteins that are involved in MER.[19] On the basis of the peroxidase activity of OMCs, a colorimetric assay has been developed in our laboratory for the rapid detection of *Shewanella oneidensis*.[20] In this study, the high efficiency of an OMC-catalyzed color-developing reaction based on peroxidase activity was combined with the high throughput of a microplate-based assay to offer a rapid and high-throughput method for qualitatively or quantitatively determining MER ability.

MATERIALS AND METHODS

Bacterial Culture and Preparation of Ferrihydrite. The details of the bacterial culture and ferrihydrite preparation are given in the Supporting Information.

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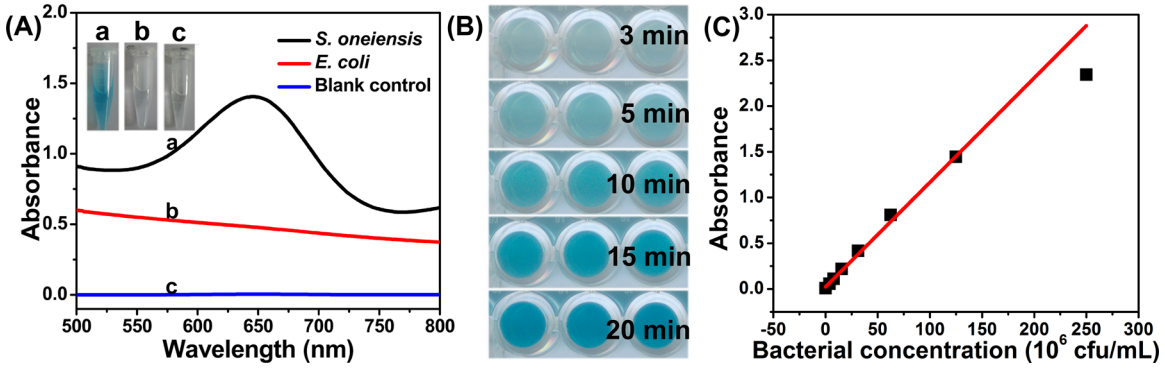

Figure 1. Evaluation of the proposed *c*-type cytochrome peroxidase activity-based colorimetric assay. (A) UV−vis spectra of reaction solutions containing (a) *S. oneidensis*, (b) *E. coli*, and (c) no bacteria. The inset shows photographs of the corresponding solutions. (B) Color development over time. (C) Correlation between cell density and color intensity (20 min for color development). $R^2 = 0.98$; $P < 0.001$ ($n = 5$). The error bars are not visible as they are smaller than the symbols.

UV−Vis Spectra of *c*-Type Cytochromes. UV−vis spectra of *c*-type cytochromes were measured in diffuse transmission (DT) mode to overcome the interference caused by light scattering by cell surfaces.[21] The washed cell cultures were centrifuged, and the resulting cell pellets were resuspended in 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer at pH 7.0. Cell suspensions with an optical density at 600 nm of 0.5 (approximately 2.5×10^8 cells/mL) were injected into quartz cuvettes (1 cm path length). The DT spectra of air-oxidized cells were obtained using a TU-1900 UV−vis spectrophotometer (Beijing, China).

Proposed Plate-Based Colorimetric Assay. Colorimetric assays were performed in a 96-well flat-bottom polystyrene microplate. One hundred microliters of washed cells suspended in pure water was mixed with an equal volume of a supersensitive 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (T4444, Sigma). The plate was incubated at room temperature for color development. After color development, the reaction was stopped with 2 M sulfuric acid (50 μL/well). OD_{450} was measured with a Bio-Rad (Tokyo, Japan) iMark microplate reader. In addition, to eliminate the possible background absorbance, all plates were measured in dual-wavelength reading mode by assigning the measurement (450 nm) and reference (630 nm) filters of the microplate reader.

RESULTS AND DISCUSSION

Method Evaluation and Optimization. *S. oneidensis* (a typical ERB) and *Escherichia coli* (not an ERB) were used to evaluate the feasibility of the proposed method. As shown in Figure 1A, a blue color developed in the tube with *S. oneidensis* after incubation for 20 min. In contrast, no blue color was observed in the tube containing *E. coli* or the tube without microbes (detection controls). When scanned from 500 to 800 nm, the *S. oneidensis* system produced a strong absorption peak at 650 nm (curve a), which is characteristic of the product (a charge-transfer complex of the TMB and the diimine oxidation product) from the peroxidase-catalyzed H_2O_2/TMB reaction.[22,23] However, the *E. coli* control (curve b) and the pure water control (curve c) did not produce similar peaks. To further validate the proposed colorimetric assay, *Geobacter sulfurreducens* PCA-1 (another well-known ERB) was also tested using this method. Compared to those of the controls (incubated with NBAF medium), color development was clearly observed for the tube containing *G. sulfurreducens* (Figure S1 of the Supporting Information). This result confirms that the developed colorimetric assay is able to identify ERB from those without MER ability.

Color developing time can influence color intensity. As shown in Figure 1B, a blue color was visible within 3 min, and then, the color intensity increased with time. After 20 min, a bright blue color was observed. In the following studies, 20 min was used as the working color developing time.

Cell density is another factor that affects color development. *S. oneidensis* with cell densities of 2.5×10^8, 1.25×10^8, 6.25×10^7, 3.13×10^7, 1.56×10^7, 7.81×10^6, and 3.91×10^6 cells/mL were tested. The results (Figure 1C) indicate that the color intensity is linearly correlated with cell number ($R^2 = 0.98$). A higher cell density results in a stronger color. In addition, in a typical dissimilatory Fe(III) reduction experiment, an inoculum density of approximately 10^7 cells/mL will increase to approximately 10^8 cfu/mL during the 14 day incubation period. To obtain adequate detection performance, a cell density of 2.5×10^8 cfu/mL was used in the following colorimetric assay.

Determination of MER Ability Using Conventional Methods. Panels A and B of Figure 2 show the results of the determination of MER ability using the dissimilatory Fe(III) reduction method (Supporting Information). Bacterial strains PCA-1, MR-1, SP200, S12, and SgZ-2 showed potent Fe(III) reduction, producing 3.11, 2.92, 2.51, 2.36, and 1.98 mM Fe(II), respectively, after an only 12 day incubation period. Weak Fe(III) reduction abilities were observed for strains MFC-3, CY01, SgZ-1, HS01, and PAH-1, with only 1.06, 1.68, 0.89, 0.66, and 0.32 mM Fe(II) produced, respectively, after a 15 day incubation. Strains of DMS10 and K12, similar to the abiotic control, exhibited nearly no Fe(III) reduction, reducing only 0.09 and 0.18 mM Fe(III), respectively, after a 30 day incubation.

The results of the MER ability determination using the electricity generation method (Supporting Information) are shown in Figure 2C. The MFCs inoculated with PCA-1, MR-1, SP200, S12, and SgZ-2 generated relatively high current densities of 148−451 mA/m^2. In contrast, low current densities of 31−47 mA/m^2 were observed for the MFCs containing MFC-3, CY01, SgZ-1, HS01, and PAH-1. The MFCs inoculated with DMS10 and K12 presented minimal electricity generation (7.56 and 4.42 mA/m^2, respectively).

Determination of MER Ability Using the Proposed Method. As shown in Figure 3A, the wells incubated with MR-1, S12, and SP200 (the first three columns from the left)

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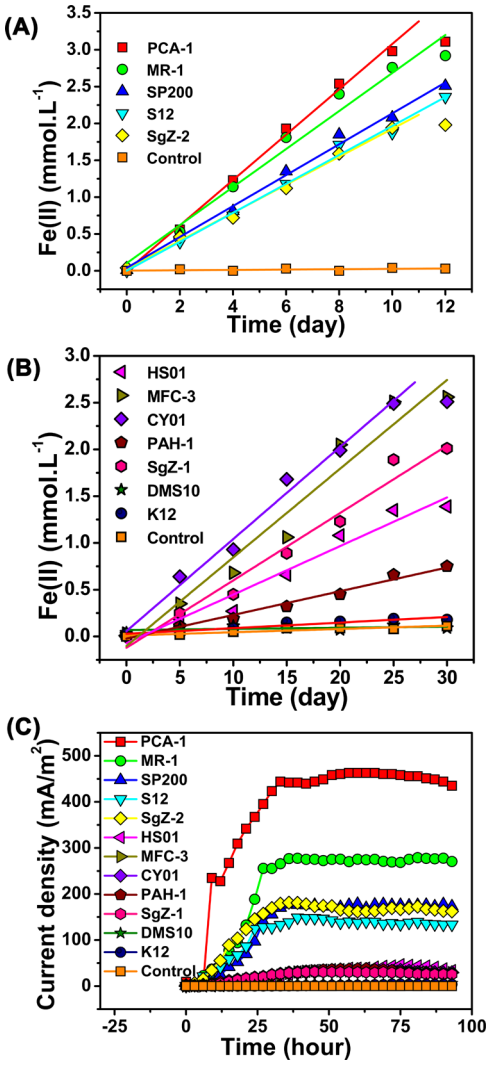

Figure 2. Determination of MER ability using conventional methods. (A and B) Dissimilatory Fe(III) reduction method. (C) MFC-based electricity generation method.

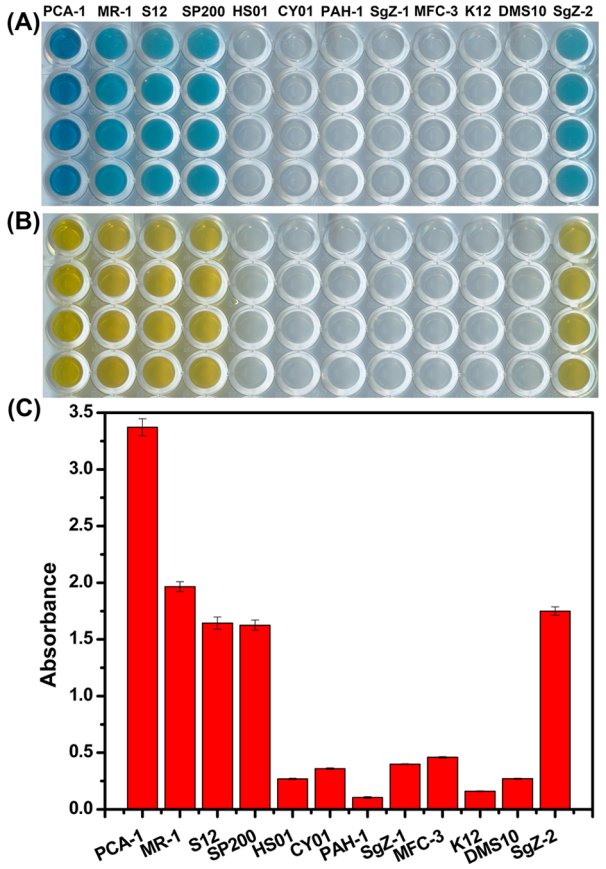

Figure 3. Determination of MER ability using the proposed plate-based colorimetric assay. (A) Before the reactions were stopped with 2 M sulfuric acid. (B) After the reactions were stopped with 2 M sulfuric acid. (C) Color intensities determined at 450 nm. The following bacteria were tested: *S. oneidensis* MR-1, *Shewanella decolorationis* S12, *Shewanella putrefaciens* SP200, *Aeromonas hydrophila* HS01, *Chrysoblephara guandongensis* CY01, *Pseudomonas aeruginosa* PAH-1, *Thauera humireducens* SgZ-1, *Pantoea agglomerans* MFC-3, *E. coli* K12, *Bacillus subtilis* DMS10, and *Fontibacter ferrireducens* SgZ-2 (from left to right, respectively). The cell concentration was 2.5×10^8 cells/mL, and the color development time was 20 min.

displayed a notable blue color, whereas the wells containing HS01, CY01, PAH-1, SgZ-1, MFC-3, K12, and DMS10 (columns 4−10 from the left) showed only slight color changes, if any. The wells in the first column from the right, which contained strain SgZ-2, a Fe(III)-reducing bacterium isolated from a microbial fuel cell, also exhibited a bright blue color. When a 2 M H_2SO_4 solution was added, the blue color (the charge-transfer complex) immediately turned yellow (the diimine oxidation product) (Figure 3B), which allowed for the rapid quantification of color intensities at 450 nm with a microplate reader, as shown in Figure 3C. The color intensities represent the MER ability levels of the tested bacterial strains. To facilitate a comparison of the proposed method with the conventional methods, the color intensities were further converted to the concentration of the related reaction product according to the Beer−Lambert law and then to the number of electrons (details in the Supporting Information).[23]

Comparison of the Proposed Method with the Conventional Methods. When MER ability was determined with the dissimilatory Fe(III) reduction method, MER ability can be represented by the observed rate constant (k_{obs}) of Fe(III) reduction.[24] As shown in Figure 4A, there was a significant (Spearman's ρ of 0.946; $P < 0.01$) correlation between the results obtained with the traditional Fe(III) reduction method and those obtained with the proposed method. When determined with the MFC-based electricity generation method, MER ability can be represented by the maximal current density.[25] MER ability determined with the proposed method was also significantly (Spearman's ρ of 0.893; $P < 0.01$) related to that determined with the electricity generation method (Figure 4B).

The significant correlation between the MER ability measured by the proposed method and that measured by conventional methods [dissimilatory Fe(III) reduction or MFC-based electricity generation] demonstrates that the proposed method can identify ERB and reliably determine the MER ability in a quantitative manner. In addition, when compared with the methods based on dissimilatory Fe(III) reduction, the proposed method is simple and inexpensive, requiring only 100 μL of bacterial suspension mixed with a color developing solution (containing TMB and H_2O_2). The developed approach, unlike the low-throughput electricity generation method, allows the measurement of 96 bacterial samples simultaneously with a plate-based assay. The color

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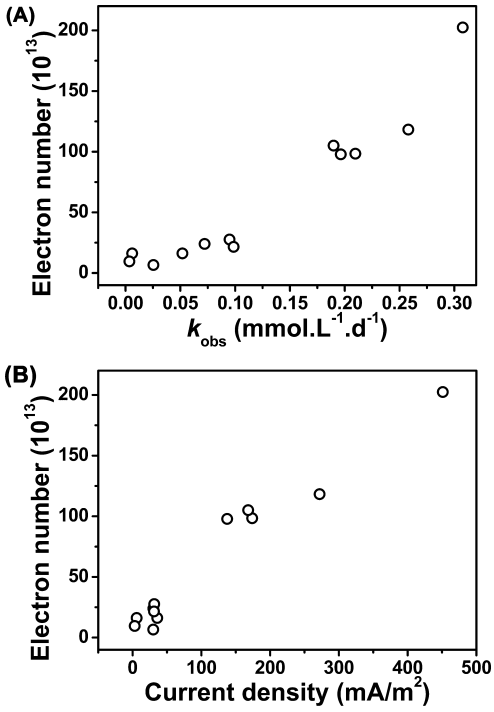

Figure 4. Correlations between the MER ability determined with the proposed method and with conventional methods. (A) Dissimilatory Fe(III) reduction method. The observed rate constant (k_{obs}) of Fe(III) reduction represents MER ability. Spearman's ρ was 0.946; $P < 0.01$. (B) MFC-based electricity generation method. The maximal current density of Figure 2C was used to represent MER ability. Spearman's ρ was 0.893; $P < 0.01$.

development can be conveniently monitored using a plate reader because of the solubility of chromogen (tetramethylbenzidine). In addition to the determination of MER ability, this method can be extended to screen OMC mutant strains, which is useful in exploring the electron-transfer mechanisms.

Determination of *c*-Type Cytochrome Content. UV–vis spectra of intact bacterial cells were recorded in diffuse transmission mode. As shown in Figure S2A of the Supporting Information, the spectra of all bacterial cells except S12 and DMS10 showed an intense absorption peak and a weak absorption peak at 410 and 530 nm, respectively. These peak positions are consistent with those of purified outer membrane decaheme *c*-type cytochromes in their oxidized forms.[26,27] Considering the critical role that *c*-type cytochromes play in extracellular electron transfer, the content of the *c*-type cytochromes may be correlated with the MER ability. To test this assumption, the content of the *c*-type cytochromes of a bacterium was estimated with the Kubelka–Munk (KM) function,[28] whose value at 410 nm is linearly related to the content of *c*-type cytochromes. Therefore, the content of *c*-type cytochromes can be represented by the KM function at 410 nm (Figure S2B of the Supporting Information).

Table S2 of the Supporting Information shows a significant Spearman correlation between the KM function and MER ability determined with the three methods. This correlation confirms the *c*-type cytochrome-dependent MER mechanism and supports the applicability of the proposed method in the determination of MER ability.

Bacteria that have MER ability can utilize several mechanisms to transfer electrons to extracellular electron acceptors, including those involving electron-shuttling molecules and conductive pili (containing *c*-type cytochromes[29]), in addition to directly contacting the electron acceptor via OMCs. Because the proposed colorimetric assay relies on the peroxidase activity of OMCs, this method may not be suitable for the ERB that transfer electrons via mediators, which may lead to false negative results in detecting mediator-dependent ERB. In addition, false positive results may also exist because of the release of cytochromes from the lysed cells. Despite its limitations, this study shows advantages in its ability to achieve high-throughput screening of novel ERB and rapid measurement of their extracellular reduction activity.

In summary, a plate-based colorimetric assay has been designed to measure MER ability utilizing microbial intrinsic *c*-type cytochrome peroxidase activity. This *c*-type cytochrome-based assay is rapid, has a high throughput, is inexpensive, and is suitable for both qualitative and quantitative assays. The developed colorimetric assay should offer an alternative approach for measuring MER ability in the fields of microbiology, biogeochemistry, and environment, among others.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional tables, figures, and text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone and fax: +86-20-87025872. E-mail: sgzhou@soil.gd.cn.

Notes

The authors declare no competing financial interest.

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