ANAEROBIC DEGRADATION OF ORGANIC POLLUTANTS DEPENDS ON THE ELECTRICITY PRODUCTION OF EXOELECTROGENIC BACTERIUM *SHEWANELLA ONEIDENSIS* MR-1

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Introduction

Shewanella, a typical metal-reducing bacterium, has a potential application on the remediation of various organic pollutants. Several *Shewanella* strains have been found to possess the ability to degrade some industrial dyes. However, the biological mechanism involved into the extracellular biodegradation was controversial. Previous studies have reported that peroxidases, laccase, cellobiose dehydrogenas, DCIP reductase, and azoreductase might be responsible for the reduction of the mixtures of different classes of textile dyes[1] and azo dyes[2]. Generally, the dye decolorization owing to the specific reductases was considered as the major mechanism. For *Shewanella*, the speculated azoreductase once was considered to be responsible for the reduction of azo dyes[3]. However, recent studies have found that the metal respiratory pathway (Mtr) played an important role in the degradation of azo dyes by *Shewanella*[4,5]. The extracellular *c*-type cytochrome MtrC was speculated to act as an azoreductase[6], but there was no clear evidence available to exclude the existence of specific azoreductases[7]. In our previous studies, Mtr respiratory pathway was also confirmed to be involved in the decolorization of non-azo metal-complex dye naphthol green B[8] and triphenylmethane dye aniline blue[9]. Thus, it is interesting that how *Shewanella* to perform when the coexistence of different types of dyes. If the degradation mechanism of dyes by *Shewanella* is due to the nonspecific electrons release from the cell surface through the Mtr pathway, then a competition should be expected to occur between two different types of dyes. However, reports about the decolorization process of dye mixture by *Shewanella* are rather scarce.

Therefore, in this study, focusing on the recent controversy concerning the decolorization mechanism by exoelectrogenic bacteria, mixtures of azo dye methyl orange (MO) and non-azo metal-complex dye naphthol green B (NGB) were employed to investigate whether nonspecific electron release resulted in the extracellular degradation of dyes or not, and to understand how the coexistence of more than one type of dyes influenced the degradation course of *Shewanella*. For this purpose, *Shewanella oneidensis* MR-1 was conducted to investigate the decolorization performance to the mixtures of MO and NGB under anaerobic conditions. The Mtr pathway mutants and excess electron shuttles were used to evaluate the influence of electricity generation on the decolorization ability of *Shewanella*. This work may facilitate a better understanding about the anaerobic bleaching mechanisms of dye mixture by *Shewanella* and other electricity-generating microorganisms, and be beneficial to their application in bioremediation.

Materials and methods

The wild-type *S. oneidensis* MR-1 (ATCC@ number is 700550™) and strains deleted of the genes *cymA* (Δ*cymA*), *mtrA* (Δ*mtrA*), *mtrB* (Δ*mtrB*), or both *mtrC* and *omcA* (Δ*mtrC/omcA*), were kindly provided by Professor K.H. Nealson from the University of Southern California. All strains were routinely cultivated in LB medium at 30 ^{o}C until the late stationary phase.

The dye mixture medium used for the anaerobic decolorization experiments contained 18 mM lactate as the electron donor and the sole carbon source[10]. Each of 0.1 mM MO and NGB were added as the electron acceptors. The medium was buffered with 50 mM 4-(2-hydroxyerhyl) piperazine-1-erhanesulfonic acid (HEPES) to pH 7.0. Each serum vial containing 80 ml dye mixture medium was bubbled with N_2 for 10 min, then sealed with butyl bubber stoppers to ensure anaerobic condition. The cell suspension was inoculated into the serum vial with a initial cell density of 4~6 $\times 10^7$ cells ml^{-1}. All the experiments in this study were repeated in triplicate.

To elucidate the degradation mechanism in dye mixture decolorization, wild-type of *S. oneidensis* MR-1 and Mtr mutants were used to investigate whether the electricity generation capacity of this strain is related with

its decolorization. In addition, the influence of riboflavin on the dye mixture degradation by *S. oneidensis* MR-1 was also investigated by adding riboflavin of different concentrations (0, 1, 3, and 5 μM).

Results and discussion

Dynamic changes of the absorption spectra during the decolorization process were observed by UV–visible spectroscopic analysis. As shown in Fig. 1, there existed two absorption peaks at 465 and 714 nm, corresponding to characteristic absorption peaks of MO and NGB, respectively. The absorption peak at 465 nm decreased gradually in the first 1.5 h while that at 714 nm remained almost unchanged. The disappearance of the peak at 465 nm reflected the cleavage of the conjugated azo-bond in the MO[3,5]. The absorption peak at 714 nm began to decrease after MO was decolorized completely after 1.5 h incubation, indicating the occurring of NGB reduction. The color of dye mixture changed from dark brown to green in the first 1.5 h and then gradually became colorless after 1.5 h. These results clearly show that *S. oneidensis* MR-1 had the ability to decolorize the dye mixture. Reduction of NGB was not observed until MO was exhausted, suggesting that MO was easier to be degraded by *S. oneidensis* MR-1 than NGB. In the heat-killed control, no significant color change was observed. This indicates that decolorization of dye mixture by *S. oneidensis* MR-1 was mainly attributed to biodegradation, rather than biosorption.

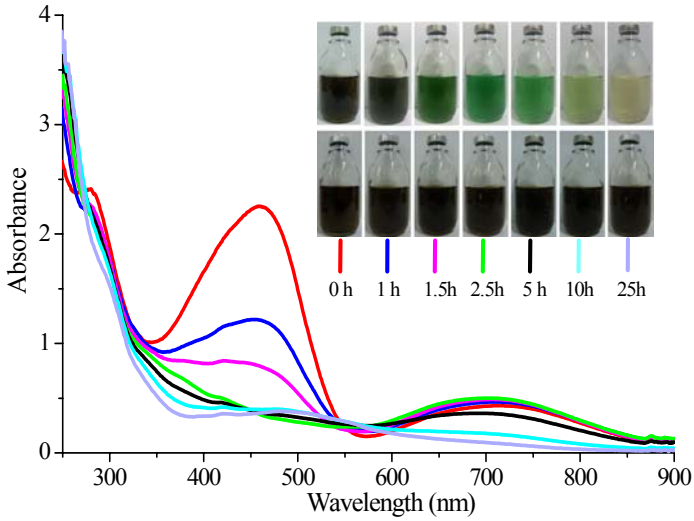

Fig. 1 Evolution of UV–vis spectra of decolorization of MO and NGB mixture at different times by *S. oneidensis* MR-1 cells (above) and heat-killed control (below). The concentration of MO and NGB mixture was both 0.1 mM.

The decolorization mechanism of dye mixture was investigated by the wild type of *S. oneidensis* MR-1 and its Mtr pathway mutants. As shown in Fig. 2A and 2B, block of the Mtr respiratory pathway resulted in an obvious decrease in the decolorization rate. For the degradation of MO, the mutants without MtrA, MtrB, or OmcA/MtrC showed a decolorization rate of less than 13.5%, 13.7% or 41.4% after 2.5 h, respectively, which was comparable to the wide type. For wild type, the decolorization of NGB was not observed until the MO was exhausted. Likewise, *mtrA*, *mtrB*, or *mtrC/omcA* mutants exhibited similar performance as the wild type. For these three strains, NGB decolorization began at 15, 40 or 40 h, respectively, when MO had been depleted. Due to the significant loss of the decolorization ability, the *cymA* mutant did not decolorize MO completely during 60 h, and no degradation of NGB was observed. These results indicate that Mtr respiratory pathway played an important role in the decolorization of dye mixture. Considering that both dyes could not enter the cell and only be decolorized through the extracellular degradation, MO was easier to get the electrons from cell surface than NGB and thus was preferentially degraded.

Recent studies have proved that *Shewanella* could decolorize a variety of dyes through the Mtr respiratory pathway[5,8,9,11]. However, it is still considered that some speculated special reductases, rather than the unspecific electron released from the cell surface, may contribute to the biodecolorization of dyes[3,7]. In the present study,

Mtr respiratory pathway was found to be the main pathway for the reduction of MO and NGB by *S. oneidensis* MR-1. Especially, as electron acceptors, there was a significant competition between MO and NGB. Considering that MO and NGB were both degraded outside the cells, it is reasonable to speculate that nonspecific electron released from cell surface through the Mtr pathway, rather than the specific terminal reductases, resulted in the dye degradation.

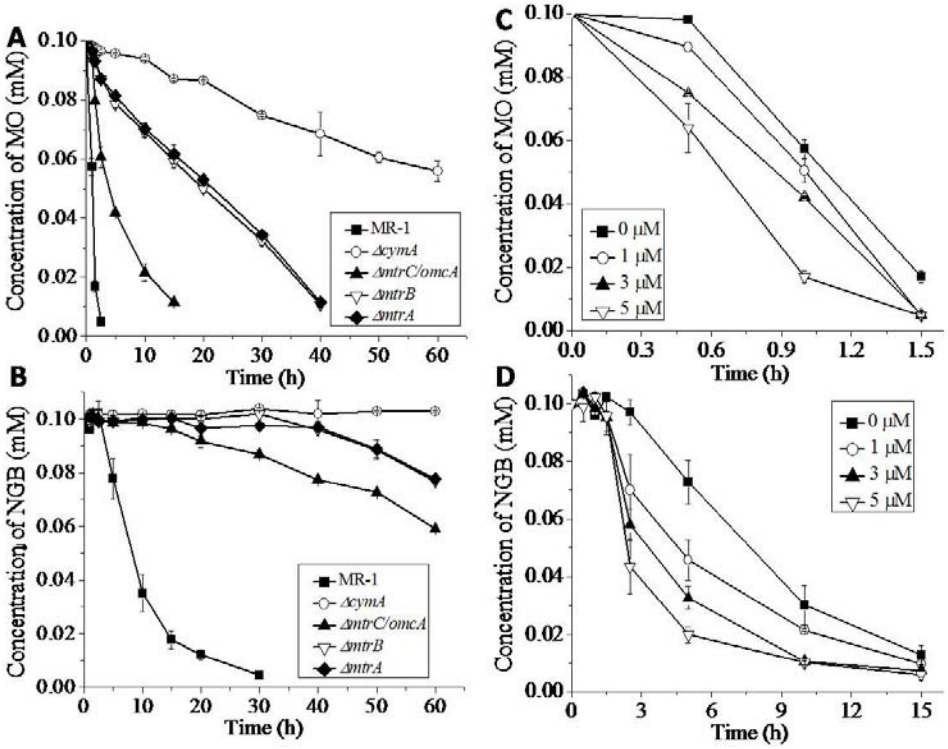

Fig 2 Effect of Mtr respiratory pathway (A and B) and extracellular electron shuttle (C and D) on the dye mixture reduction by *S. oneidensis* MR-1. All values represent means ± SD.

As shown in Fig. 2C and 2D, addition of riboflavin significantly accelerated the biodegradation of dye mixture. The decolorization rate was improved with the increase of riboflavin concentration. When 5 μM riboflavin was added, the removal efficiency of MO and dye mixture increased to 1.9-fold after 1 h incubation and 2.9-fold after 5 h incubation, respectively, compared with the control without riboflavin addition. Since riboflavin is mainly reduced outside the outer membrane through Mtr pathway[12] and acts as electron shuttles between cell surface and extracellular electron acceptors, the reduction of dye mixture should be attributed to the electron released from the cell surface, rather than the special enzyme. Thus, dye mixture could be reduced directly by the outer membrane cytochromes MtrC/OmcA or indirectly via flavins as the electron shuttles. Interestingly, addition of excess electron shuttles only accelerated the total rate of decolorization, but not the preference of MO. In any addition concentration of riboflavin, decolorization of NGB did not occur until MO was exhausted.

In summary, the decolorization ability of *S. oneidensis* MR-1 to dye mixture under anaerobic conditions was investigated for the first time. Block of the Mtr respiratory pathway reduced the decolorization rate. Addition of extracellular electron shuttles significantly accelerated the decolorization. There was obvious competition for electrons between MO and NGB. Those results reveal that the decolorization capacity is closely associated with the electricity production, rather than the hypothetical terminal reductase. This study might contribute to understanding the biodecolorization mechanisms of exoelectrogens in wastewater treatment.

Acknowledgements

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