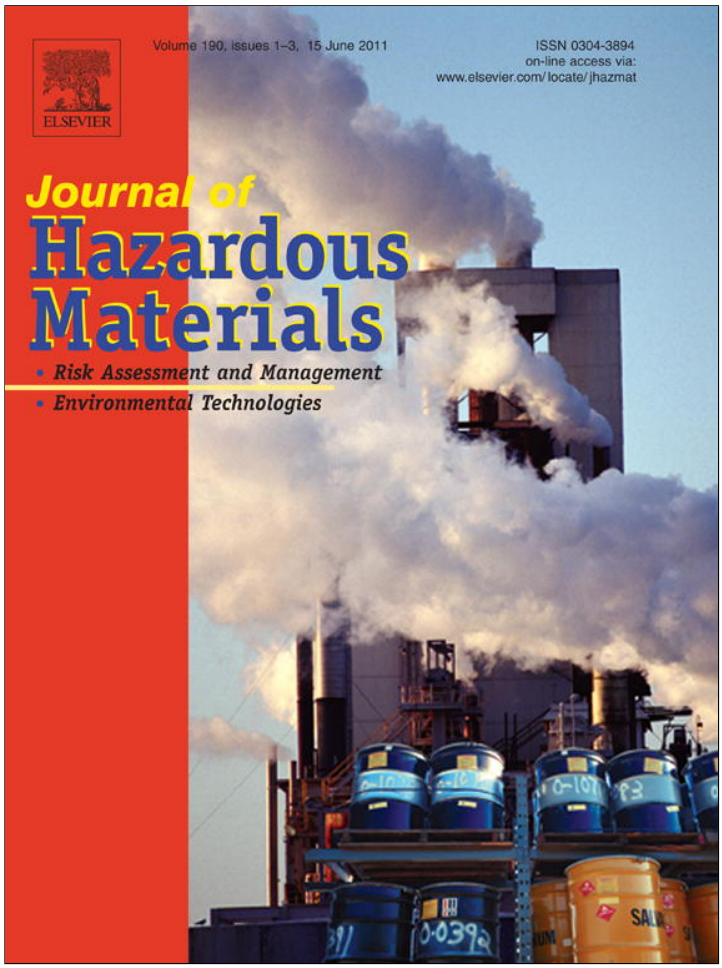

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Short communication

Enhancement of cadmium bioremediation by endophytic bacterium *Bacillus* sp. L14 using industrially used metabolic inhibitors (DCC or DNP)

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ABSTRACT

Bioremediations of cadmium by endophytic bacterium (EB) L14 (*Bacillus* sp.) in the presence of industrially used metabolic inhibitors (DCC or DNP) were investigated. In the presence of DCC or DNP, the biomass population of EB L14 was greatly inhibited. However, the cadmium removal of EB L14 increased from 73.6% (in the absence of DCC or DNP) to 93.7% and 80.8%, respectively. The analysis of total and intracellular cadmium concentrations during 24 h of incubation indicated that this enhanced cadmium removal was the inhibition effect of DCC or DNP on the cations export resistance system of EB L14. This unique property strongly indicated the superiority of this endophyte for practical application in cadmium bioremediation in the presence of industrially used metabolic inhibitors.

1. Introduction

Cadmium which was listed as *Known to be Human Carcinogens* in the 11th Report on Carcinogens (http://ntp.niehs.nih.gov/index.cfm?objectid=32BA9724-F1F6-975E-7FCE50709CB4C932) receives increasing attention as one of the most toxic heavy metals [1,2]. Most physicochemical strategies for cadmium removal appear to be expensive, inefficient and labor-intensive [2]. Bioremediation, which involves the use of living microbes to remove heavy metals, has been considered to be a safe and economic alternative to physicochemical strategies due to their ability of self-replenishment, continuous metabolic uptake of metals after physical adsorption, and the potential for optimization through development of resistant species and cell surface modification [3–5].

Metabolic inhibitors, such as DCC (N,N′-dicyclohexylcarbodiimide, specific ATPase inhibitor) and DNP (2,4-dinitrophenol, uncoupler of oxidative phosphorylation), could greatly inhibit the growth of the microbes and may reduce the continuous metabolic metals uptake of the living cells which may be used for bioremediation. Recently their detrimental effects on bioremediation of heavy metals by living microbes, such as algae [6], bacteria [7], fungi [8], and yeast [9], have been documented. Unfortunately, DCC has been widely used in chemical and pharmaceutical industry since the early 1950s as a stabilizing agent, coupling agent, and condensing agent (http://ntp.niehs.nih.gov/?objectid=03DAE7A9-C42A-08AA-016B1AAA9F1B57B3), while DNP is an organic compound which has been used in manufacture dyes, wood preservatives and insecticides (http://ntp.niehs.nih.gov/index.cfm?objectid=BD5C9584-123F-7908-7B140212F871BF77). As the results, the more metabolic inhibitors applied in industry, the higher concentration they will be in the industrial effluent. Eventually the existences of metabolic inhibitors in the industrial effluent will be a crucial limitation to application of living microbes for the *in situ* heavy metals bioremediation. Hence, it is important and urgent to obtain potential microbes with promising bioremediation efficiency in the presence of industrially used metabolic inhibitors for practical heavy metal bioremediation.

Our previous study indicated that EB L14 (bacterial endophyte of cadmium hyperaccumulator) possessed unique bioremediation behavior to divalent heavy metal and its efficiency might be greatly promoted through inhibiting the activities of ATPase [4]. In this study, the cadmium bioremediation behaviors of EB L14 in the presence of industrially used metabolic inhibitors (DCC or DNP) were determined and the bioremediation efficiencies were also assessed.

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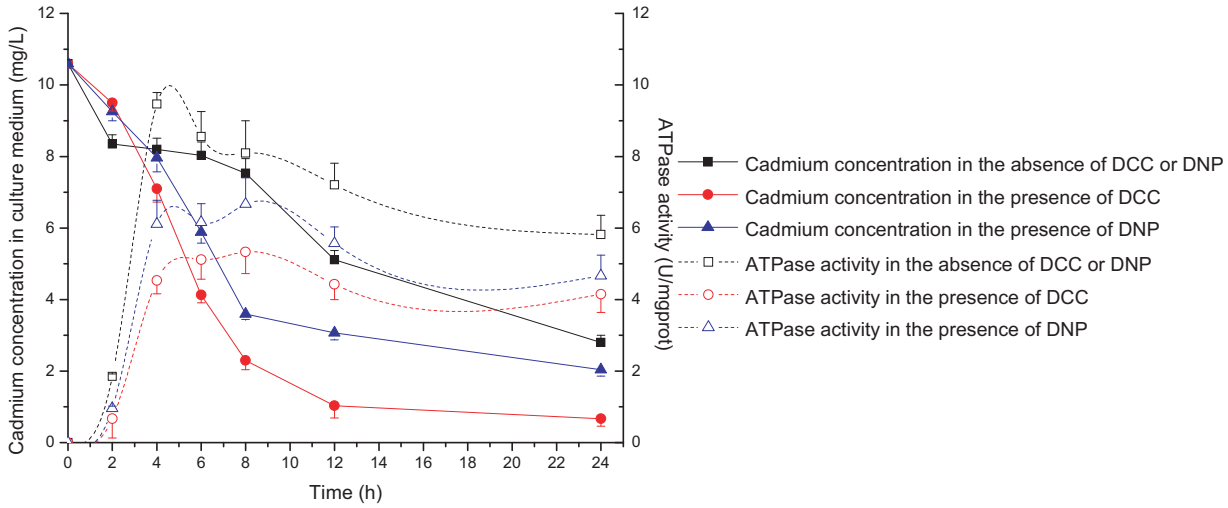

Fig. 1. ATPase activity and cadmium ion removal by EB L14 with or without the presence of DCC or DNP (solid lines cadmium concentrations, dash lines ATPase activities).

2. Experimental

2.1. Experimental microbe, growth and culture conditions

EB L14 (*Bacillus* sp.) was previously isolated from cadmium hyperaccumulator *Solanum nigrum* L. and was found to uptake cadmium with high efficiency [4]. It was maintained and cultured in LB (pH 7.2–7.4) medium at 30 °C and agitated at 150 rpm. All the experiments were conducted in triplicate at same culture conditions.

2.2. Analytical technique

The optical densities of the cultures were determined by using UV–vis Spectrophotometer (Cary 300, Varian, USA).

The Cd (II) concentrations were determined by the flame atomic absorption spectrometry (FAAS) using Z2000 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Japan), The hollow cathode lamp was operated at 5 mA and the analytical wavelength was set at 228.8 nm.

2.3. Bioremediation of cadmium by growing EB L14 with/without metabolic inhibitors

To assess the effect of industrially used metabolic inhibitors on cadmium bioremediation efficiency of EB L14, the cadmium removal in concentrations from 0.01 to 2 mM of N,N′-dicyclohexylcarbodiimide (DCC) or 2,4-dinitrophenol (DNP) were monitored in previous experiments. The results showed that DCC and DNP had the best effects in the concentrations of 0.5 and 1 mM, respectively.

0.2 mL(about 1×10^8 CFU/mL) of cells was withdrawn from exponentially growing homogenous culture of EB L14 and inoculated into 200 mL freshly prepared LB medium in 500 mL conical flasks containing 10 mg/L Cd (II), 10 mg/L Cd (II) + DCC (0.5 mM) and 10 mg/L Cd (II) + DNP (1 mM), respectively. Samples (10 mL) were taken from the culture flasks at predefined time intervals (0, 2, 4, 6, 8, 12 and 24 h) harvested by centrifugation (2576 × *g*, 25 min, 4 °C). The Cd (II) concentration of supernatant was analyzed to determine the cadmium removal by EB L14.

The sediment was applied to analyze the total and intracellular Cd (II) uptake by EB L14. According to the methods modified from Harish [6], the sediment was suspended in deionized-distilled water and then averagely divided into two parallel samples. One parallel sample was washed three times (10 min each time) with 10 mL of EDTA (0.02 M) solution and gently shaken. Afterwards, cells were centrifuged and washed with deionized-distilled water. The intracellular Cd (II) uptake was measured in this sample. Another sample was only washed by deionized-distilled water under the same condition as control and the total cadmium taken by cells was measured in this sample.

The two parallel samples were dried at 80 °C overnight and the dried biomass were weighted. Subsequently, dried biomass were respectively digested with double acid [HNO_3:$HClO_4$ mixture (10:1, v/v)] in boiling water bath for 2 h. After cooling, the samples were diluted to 10 mL with deionized-distilled water and analyzed for Cd (II) concentrations.

2.4. ATPase activity of EB L14 with/without metabolic inhibitors

The ATPase activity of EB L14 was determined as described by Guo et al. [4].

All experiment was repeated three times and the results reported in this article are average values.

3. Results and discussion

3.1. Effects of DCC and DNP on growth of EB L14

The growths of EB L14 with or without presence of DCC or DNP at initial Cd (II) concentration of 10 mg/L were presented in supplementary Fig. 1 (dash lines). It indicated that in the absence of DCC and DNP the endophyte exhibited a lag phase of 4–5 h and a maximum specific growth rate (μ_{max}) of 0.52 h^{-1}. Although EB L14 showed a similar lag phase in the presence of DCC or DNP, the μ_{max} of the strain did decrease to 0.37 and 0.43 h^{-1}, respectively. Furthermore, the cell biomass level (dry weight) after 24 h was inhibited as compared to the control (without DCC or DNP), reducing from 2.84 g/L to 1.96 and 2.16 g/L, respectively.

Generally, hazardous heavy metals, such as cadmium, chromium, copper and zinc, always have detrimental effects on microorganisms. Hassen [10] investigated the effect of heavy metals on *Pseudomonas aeruginosa* and *Bacillus thuringiensis*. Results showed that the inhibition was variable according to the metal and its concentrations. Li [9] demonstrated that μ_{max} of *Rhodotorula* sp. Y11 were decreased as the increase of Cd (II)

concentrations. Slow growth of the *Trichoderma viride* strain was observed at different concentrations of copper, and the lag growth phase was very extended [8]. However, our previous study has already indicated that EB L14 seems preferred to inhabiting in such hazardous divalent heavy metals at relatively low concentration (10 mg/L) rather than in metal free conditions. This phenomenon was called hormesis and probably might be the side effect of abnormal ATPase activities increases which was planned to provide energy to help the endophyte reduce the toxicity of heavy metals by exporting the cations [4]. As shown in Fig. 1, the ATPase activities of EB L14 was greatly inhibited in the presence of DCC (ATPase inhibitor), which testified the above explanation of hormesis phenomenon and was consisted with the report of Leedjärv [11]. In addition, DNP (uncoupler of oxidative phosphorylation), another metabolic inhibitor, had a relatively weaker inhibition effect.

3.2. Effects of DCC and DNP on cadmium bioremediation efficiency

The Cd (II) concentrations in culture medium during 24 h incubation with or without DCC or DNP were also shown in Fig. 1 (solid lines), respectively. It was noticed that the Cd (II) concentration in medium of EB L14 could be divided into three stages. At early lag phase (0–2 h), the Cd (II) concentration decreased rapidly from 10.6 mg/L to 8.35 mg/L. Subsequently, as more and more Cd (II) ions accumulated in the interior of the cells, the ATPase activities of EB L14 was turned up to protect the strain from the toxic effect of cadmium by exporting the cations [4]. This is why the Cd (II) concentration in medium balanced at about 8 mg/L despite the biomass of EB L14 was in logarithmical growth (2–8 h). Finally, this balance was broken and the concentration reduced to 2.8 mg/L amounting to about 73.6% cadmium removal. However, this balance was disappeared in the presence of DCC or DNP (Fig. 1). The Cd (II) concentrations firstly decreased rapidly as the increase of biomass till log phase (0–8 h). Then the decreasing rates were slow down as far as the end of incubations resulting in 93.7% and 80.8% cadmium removal, respectively.

As shown in Fig. 1, the ATPase activities of EB L14 with DCC or DNP in presence were greatly inhibited during 24 h incubation, especially in the cadmium concentration balance period (2–8 h). In addition, DCC had a stronger inhibition effect on the ATPase activities of EB L14 than DNP (Fig. 1, dash lines).

Metabolic inhibitors, such as DCC and DNP, could greatly inhibit the growth of the microbes and may reduce the continuous metabolic metals uptake of the living cells which may be used for heavy metals treatment. As the widely application of metabolic inhibitors (e.g. DCC and DNP) in industry, there is no doubt that they will eventually discharge into the industrial effluent and thus become a crucial limitation to applying living microbes for practical heavy metals bioremediation. To the best of our knowledge, EB L14 is the first reported microbe, whose metal bioremediation efficiency did not decrease like any other reported strains [6–9] but increase in the presence of metabolic inhibitors. Our previous studies have showed that endophytes of hyperaccumulator may be potential resources of highly efficient candidates for heavy metal bioremediation due to their unique characteristics which were obtained in their original special inhibition niches [2,4]. Hence, this excellent property of EB L14 may also originate from its unique inhibition niches. The excellent remediation efficiencies in the presence of industrially used metabolic inhibitors strongly suggested the superiority of EB L14 for practical application in cadmium bioremediation.

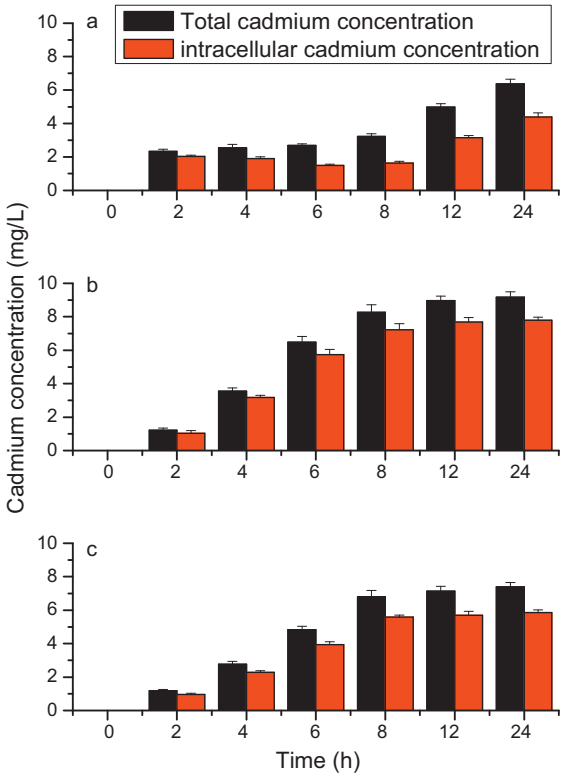

Fig. 2. The total and intracellular cadmium concentrations of EB L14 during 24 h incubation with or without the presence of DCC or DNP. (a) In the absence of DCC or DNP, (b) in the presence of DCC, (c) in the presence of DNP.

3.3. Effects of DCC and DNP on total and intracellular cadmium uptake

To determine if the enhancement of cadmium bioremediation efficiency was accomplished by inhibiting the export mechanism of EB L14, the total and intracellular Cd (II) concentrations were both detected with and without DCC or DNP during 24 h incubation.

Similar with Cd (II) concentration curve in culture medium, the total and intracellular Cd (II) concentrations of EB L14 in absences of DCC and DNP could also be divided into three stages (Fig. 2a). At first 2 h of incubation, the total and intracellular Cd (II) concentrations both increased rapidly from 0 to 2.33 and 2.03 mg/L, respectively. Subsequently (2–8 h), the intracellular Cd (II) concentrations decreased from 2.03 mg/L to 1.49 mg/L (2–6 h), and then increased slightly to 1.63 mg/L (6–8 h) despite the increasing biomass of EB L14. However, the extracellular uptake of cadmium is strongly depended on biomass population [4] and thus it should increase with the increase of the biomass population during the log phase. As the total cadmium uptake was the summation of extracellular (biosorption) and intracellular (bioaccumulation) cadmium uptake, the decreased intracellular cadmium uptake of EB L14 and increased extracellular uptake resulted in the balance of total Cd (II) concentration in this stage. Finally, the total and intracellular Cd (II) concentrations both increased to 6.37 and 4.4 mg/L after 24 h incubation, respectively. Situations were different in the presence of DCC or DNP. It was noticed that the intracellular Cd (II) concentrations were never reduced and the total Cd (II) concentration balance were also disappeared (Fig. 2b and c). The total and intracellular Cd (II) concentrations of EB L14 were all gradually increased as the increase of biomass. After 24 h of incubation, the total Cd (II) concentrations were 9.17 and 7.39 mg/L in presence of DCC and DNP, respectively. These indicated that the metabolic inhibitors, especially ATPase inhibitor DCC, could inhibit the Cd (II) ions export

Table 1
The mass balance of supernatant and total cadmium concentrations in EB L14 with or without in presence of DCC and DNP.

	Supernatant cadmium concentrations	Total cadmium uptake concentrations	Mass balance (summation of supernatant and total cadmium concentration)
Control			
Time (h)			
0	10.6 ± 0.10	0	10.6 ± 0.10
2	8.35 ± 0.26	2.33 ± 0.14	10.68 ± 0.40
4	8.2 ± 0.31	2.54 ± 0.21	10.74 ± 0.52
6	8.03 ± 0.38	2.69 ± 0.09	10.72 ± 0.47
8	7.53 ± 0.41	3.23 ± 0.16	10.76 ± 0.57
12	5.51 ± 0.26	4.97 ± 0.21	10.08 ± 0.47
24	2.8 ± 0.20	6.37 ± 0.27	9.17 ± 0.47
DCC			
Time (h)			
0	10.6 ± 0.11	0	10.6 ± 0.11
2	9.60 ± 0.23	1.19 ± 0.13	10.79 ± 0.36
4	7.10 ± 0.37	3.55 ± 0.20	10.65 ± 0.57
6	4.13 ± 0.22	6.49 ± 0.34	10.62 ± 0.56
8	2.30 ± 0.26	8.27 ± 0.45	10.57 ± 0.71
12	1.03 ± 0.34	8.97 ± 0.27	10.00 ± 0.61
24	0.67 ± 0.21	9.17 ± 0.33	9.84 ± 0.54
DNP			
Time (h)			
0	10.6 ± 0.12	0	10.6 ± 0.12
2	9.26 ± 026	1.39 ± 0.08	10.65 ± 0.34
4	7.97 ± 0.4	2.77 ± 0.16	10.74 ± 0.56
6	5.89 ± 0.31	4.83 ± 0.21	10.72 ± 0.52
8	3.60 ± 0.16	6.81 ± 0.37	10.41 ± 0.53
12	3.07 ± 0.20	7.13 ± 0.30	10.20 ± 0.50
24	2.40 ± 0.18	7.39 ± 0.26	9.43 ± 0.44

resistance system of EB L14 and thus resulted in the promotion of cadmium uptake.

It was also noticed that the extracellular Cd (II) uptakes (biosorption uptake) of EB L14 gradually increased with the increase of biomass with or without DCC or DNP (Fig. 2). These suggested that the cadmium binding site of EB L14 may not be affected by the presence of metabolic inhibitors.

3.4. Cadmium recovery

As can be seen in Table 1, the mass balances were perfectly kept at around 10.6 mg/L in the first 8 h of incubation with or without DCC and DNP. However, these balances were broken from then on, which suggested that beside the extracellular and intracellular cadmium uptake, the abiotic precipitate also played a role in the overall cadmium bioremoval.

4. Conclusions

The cadmium bioremediation efficiency of EB L14 in the presence of DCC or DNP did not decrease like other reported strains but increase from 73.6% to 93.7% and 80.8%, respectively. This unique property indicated that EB L14 was the suitable candidate for cadmium removal in practical industrial effluent in which the concentrations of metabolic inhibitors have been going up day by day.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.04.023.

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