Reductive degradation of chloramphenicol by Geobacter metallireducens

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Geobacter metallireducens is known to be capable of removing nitroaromatic compounds via an oxidation mode. However, little attention has been paid to investigate the reductive removal of chlorinated nitroaromatic compounds by G. metallireducens. In this study, G. metallireducens was used to reduce chloramphenicol (CAP), a typical chlorinated nitroaromatic antibiotic. Cyclic voltammograms and chronoamperometry highlighted a higher peak current for CAP reduction by G. metallireducens compared to the control without bacteria. G. metallireducens efficiently reduced CAP (20 mg/L) with acetate as the sole electron donor, and the removal efficiency reached (97.6±4.9)% within 6 d. Aromatic amine (AMCl2), AMCl (dechlorinated AMCl2) and AM (dechlorinated AM) were identified as reduction products by liquid chromatography-mass spectrometry. However, the removal efficiency declined to (25.0±3.6)% when the CAP dosage increased to 80 mg/L. Transcriptomic analysis indicated the significant upregulation of genes related to electron transfer, such as pilus assembly protein gene (2.8 folds), NADH-quinone oxidoreductase subunit K2 gene (4.5 folds) and many c-type cytochrome genes such as cytochrome c biogenesis protein ResB (Gmet 2901, 4.6 folds), cytochrome c (Gmet 0335, 4.4 folds) and cytochrome c7 (Gmet 2902, 3.4 folds). Furthermore, a gene related to chlorinated contaminant removal (Gmet 1046, 5.4 folds) was also upregulated, possibly resulting in enhanced CAP reduction. This work deepened our knowledge of the bioremediation ability of G. metallireducens with respect to environmental contaminants and provided a potential strategy to treat antibiotics with electrochemically active bacteria.

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1 Introduction

Antibiotics, as effective and cheap drugs, have been used to cure disease and save lives for over 70 years [1]. Among these antibiotics, chloramphenicol (CAP) is extensively used for the control of numerous bacterial diseases in humans and animals [2,3]. Although CAP usage has been severely restricted in food-producing animals by the U.S. Food and Drug Administration owing to its hematotoxicity, embryotoxicity and potential genotoxicity, it is still used in developing nations because of its wide availability and low production cost [4]. More recently, CAP has been frequently...
detected in wastewater effluents, aquatic environments and soils, which may pose an adverse threat to human health and environmental ecosystems [5]. Furthermore, the existence of CAP in the environment induces the formation of antibiotic resistant genes (ARGs) that are regarded as emerging pollutants [6]. Therefore, it is of great significance to establish an effective method to remove CAP from wastewater and aquatic environments.

Many physicochemical methods such as adsorption, metal-based catalytic reduction and advanced oxidation processes are efficient for chloramphenicol removal but suffer from high chemical costs and short service lives [7–9]. Alternatively, biodegradation offering cost-effectiveness, sustainability and eco-friendliness has emerged as a promising CAP removal strategy [10,11]. Bioelectrochemical systems (BES) employing electrode-respiring microbes in cathodes could effectively reductively remove CAP [12]. Furthermore, Liang et al. [13] found that the antibacterial activity of CAP was quickly eliminated during the CAP bioelectroreduction process in BES. However, reduction of CAP on the biocathode significantly relied on the microbes with extracellular electron transfer ability [14]. Electrochemically active bacteria (EAB) that possess extracellular electron transfer capability have not only been used for electricity production but also explored for bioremediation of contaminants in wastewater. Currently, increased attention is focused on the removal of CAP using EAB, because CAP can be effectively converted to aromatic amines with less toxicity and greater mineralization capacity than the former [5,15].

*Geobacter* are well-known EAB species that extensively exist in sedimentary and soil environments for driving the cycling of Fe, Mn and other associated trace metals; thus, *Geobacter* is also used to study the extracellular electron transfer mechanisms among minerals and solid electrodes [16,17]. Moreover, recent studies have suggested that *Geobacter* species are capable of degrading various contaminants via reduction or oxidation under anaerobic conditions [18,19]. For example, Zhang et al. [18] found that *Geobacter metallireducens* can oxidize benzene to carbon dioxide with the production of phenol and benzoate as accumulated intermediates. Liu et al. [20] demonstrated that *Geobacter sulfurreducens* PCA efficiently reduced methyl orange into amines. Furthermore, *G. sulfurreducens* PCA showed a higher decolorization efficiency compared with the currently known methyl orange reducing bacteria. In our previous study, *Geobacter* species were also detected to be the dominant bacteria for removal of pharmaceutically active compounds in a BES reactor [21]. Therefore, *Geobacter*-mediated bioremediation of contaminants may be an effective and simple treatment technology. However, the potential for bioremediation of chlorinated contaminants in pure culture using *Geobacter* species has only been studied with a few contaminants. Few studies have been reported on the feasibility of using *Geobacter* species for the degradation of CAP.

This study aims to investigate bioreduction of CAP by *G. metallireducens* and unveil the underlying mechanism. The electrochemical behavior of *G. metallireducens* was characterized by cyclic voltammetry (CV), differential pulse voltammetry (DPV) and chronoamperometry (CA). The reduction capacity of *G. metallireducens* at different initial CAP dosages was also explored. In addition, the reduction products were identified, and the kinetic parameters were calculated. Finally, transcriptomic analysis was conducted to reveal the variation of gene expression of *G. metallireducens* during CAP reduction. These findings may advance the application of EAB for the bioremediation of antibiotic contaminants.

### 2 Materials and methods

#### 2.1 Bacterial strains and growth conditions

*G. metallireducens* GS-15 (ATCC 53774) was cultured anaerobically at 30°C in freshwater-acetate medium (FWA) with 30 mM acetate as the electron donor and 20 mM nitrate as the electron acceptor, as previously described [22,23]. The strain was cultured for more than 4 days to reach a stationary phase. The cell growth in FWA-nitrate medium was monitored at an optical density of 540 nm.

#### 2.2 Removal of CAP by *G. metallireducens*

CAP removal experiments were conducted in 100 mL serum bottles with a working volume of 40 mL. Serum bottles were added with CAP to a final concentration controlled at 20 mg/L after the strain reached the stationary phase. The acetate remaining in the cultures (22.7 mM) further served as the electron donor for *G. metallireducens* metabolism. The medium without *G. metallireducens* was compared as the control. Meanwhile, the removal of CAP by a heat-inactivated strain (high-pressure steam sterilization pot at 121°C for 20 min) was also investigated as the control according to the above described methods, for the purpose of knowing the function of live cells in CAP removal. The removal capacity tests were conducted by ranging CAP concentrations from 20 to 80 mg/L. During the CAP removal experiments, samples were withdrawn in an anaerobic glovebox that was sparged with N₂:H₂ (95:5) (v:v) mixed gas. All of the experiments were conducted in triplicates.

#### 2.3 Characterization of *G. metallireducens* by electrochemical methods

The electrochemical activity of *G. metallireducens* for CAP
reduction was explored through the methods of CV, CA and DPV in a conventional three-electrode electrochemical cell using a CHI660 electrochemical workstation (Chenhua, China). A glassy carbon electrode with a diameter of 3 mm served as the working electrode, whereas a Pt sheet electrode and an Ag/AgCl electrode were used as the counter electrode and reference electrode, respectively. Before use, the glassy carbon electrode was first abraded with emery paper, and then polished with 0.3 and 0.05 μm alumina slurries for 3 min, followed by rinsing with ultrapure water and sonicing in ultrapure water and ethanol for 3 min, respectively, and dried under an infrared lamp environment [24]. CV measurement was conducted in the phosphate buffered solution (PBS) containing 20 mg/L CAP in the absence of G. metallireducens (OD540=0.2) at a scanning rate of 5 mV/s between –1 V and 1 V. DPV was performed at a scanning rate of 5 mV/s between –1 V and 1 V. The G. metallireducens was rinsed with PBS three times to remove acetate, and then resuspended in PBS prior to CV testing. For the CA test, a constant potential of –0.6 V was applied in the PBS with or without G. metallireducens. CAP was successively injected into the PBS when the basic line was stable. All of the experiments were carried out under a nitrogen environment.

2.4 Analytical methods

Aqueous samples were withdrawn at different time intervals and filtered over 0.22 μm filters. The concentration of CAP was determined using a high-performance liquid chromatography (HPLC, 1260 Infinity, Agilent, USA) with a PDA detector. The separation was performed using a C18 column. The mobile phase consisted of methanol and water (65:35) (v/v) at a flow rate of 1 mL/min and the detection wavelength was 275 nm. The concentration of acetate was determined using HPLC with a refractive index detector. The separation was performed with a Hi-Plex HPLC column, and the mobile phase consisted of water containing 5 mM H2SO4 at a flow rate of 0.6 mL/min. During detection, the column temperature was controlled at (60±2)°C. The reduction products were first extracted from the medium by solid-phase extraction using Waters HLB Oasis® SPE cartridges and then identified by liquid chromatography-mass spectrometry (LC-MS, LCQ Fleet ion trap mass spectrometer, Thermo Fisher, USA). The LC-MS was equipped with an electrospray ionization source and operated in positive/negative polarity mode. The total biomass of G. metallireducens was quantified in terms of protein, which was determined using the bicinechonic acid method through a BCA protein assay kit (Solarbio, Beijing).

2.5 Transcriptomic analysis

The samples of G. metallireducens in the FWA medium containing nitrate at the end of the CAP removal experiment were collected for transcriptomic analysis. The G. metallireducens in the FWA medium containing nitrate without the addition of CAP was collected as a control. Both conditions were run in parallel in separate vials. All of the RNA samples were extracted using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer’s instructions. The quality and quantity of RAN were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) and Nanodrop 2000 spectrophotometer (Nanodrop Technology, Wilmington, USA), respectively. To prepare a library for transcriptome sequencing, a total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA), following the manufacturer’s recommendations, and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an illumina hiseq platform, and paired-end reads were generated. The data analysis was performed according to the method of Zhu et al. [6].

3. Results and discussion

3.1 Electrochemical analysis of G. metallireducens

Electrochemical analyses including CV, DPV and CA were conducted to explore the catalytic activity of G. metallireducens towards CAP reduction. As shown in Figure 1(a), a reduction peak was observed at around –0.6 V (vs. Ag/AgCl) for G. metallireducens, which was ascribed to the reduction of CAP [12]. A similar reduction peak was also observed for the PBS control containing only CAP, further indicating that the reduction peak appeared around –0.6 V belonged to CAP reduction, because there were no other redox mediators besides CAP existing in the PBS. These results suggested that G. metallireducens could directly reduce CAP with electrodes as an electron donor. The potential for reductive degradation of chlorinated contaminants by Geobacter species that employing electrodes as electron donors has been documented [25]. In addition, the peak current density of G. metallireducens (0.67 mA/cm²) was 1.9-fold higher than that of control (0.36 mA/cm²), further indicating that G. metallireducens catalyzed the reduction of CAP. Similar results were also reported by Liang et al. [12]. They found that the reduction peak current of CAP with EAB was much higher than that without EAB in CV curves.

The catalytic activity of G. metallireducens for CAP re-
production was further investigated through measuring CA plots when intermediately spiked by CAP at a fixed potential of –0.6 V (Figure 1(b)). The current increased immediately for the *G. metallireducens* after the addition of CAP, while that of the control increased slightly. Although an obvious current signal was observed for the control over time, it was retarded by more than 380 s when compared to *G. metallireducens*. The maximum reduction current for the *G. metallireducens* reached 0.14 mA/cm², which was larger than control (0.10 mA/cm²). In addition, the reduction percentage of CAP by *G. metallireducens* (75.8%) was also higher than the control (46.3%). In addition, the DPV signal response of *G. metallireducens* was also more intense than that of the control, further indicating that CAP could be effectively reduced by *G. metallireducens* (Figure 1(c)). All these results revealed that *G. metallireducens* was capable of directly reducing CAP.

### 3.2 Confirmation of CAP reduction by *G. metallireducens*

As a chlorinated nitroaromatic antibiotic, the reductive removal of chlorine or nitro substituents from CAP would lead to the change in CAP concentration with concurrent reducing its biotoxicity and removing its antibacterial activity [26]. As shown in Figure 2, 20 mg/L CAP was nearly completely removed by *G. metallireducens* within 6 d with a removal percentage of (97.6±4.9)% when acetate was supplied as electron donor. Conversely, the CAP concentration of the control with heat-inactivated bacteria was negligibly declined, indicating that adsorption and chemical process for CAP removal can be rule out. Meanwhile, no change of CAP concentration was observed in the control of FWA medium containing only CAP. Therefore, the removal of CAP in this system was solely attributed to microbially-mediated processes.

The deprotonated molecular ions of *m/z* ratios of 321 and 323 were identified as CAP, as shown in Figure S1. The intermediates of CAP transformation were also identified, and a low amount of aromatic amine (AMCl2) at the *m/z* of 293 was observed [27]. As reported in previous study, the nitro group reduction process has been proposed to involve several steps including nitroso compounds and hydroxylamino compounds as intermediates [28]. In this study, LC-MS results confirmed the existence of nitroso intermediate from CAP reduction. However, no hydroxylamino compounds were detected, possibly due to low inoculum concentration. As the toxicity of AMCl2 was 500-fold less than its corresponding nitroaromatic, suggested that *G. metallireducens* could at least detoxify CAP even if not completely degraded it. Subsequently, the AMCl2 was dechlorinated to inactive antibacterial products AMCl (at the *m/z* of 281) and AM (at the *m/z* of 247), which may have potential significance in reducing the selection pressure on the generation of antibiotic resistance bacteria and ARGs in eco-environments. Interestingly, Liang et al. [13] found that the relative abundances of overall ARGs and specific CAP resistance related major facilitator superfamily transporter genes showed a negative relationship with reduction efficiency of CAP to AMCl, and these may help to reduce the ecological risk of ARGs evolution. It was previously suggested that AMCl2 dechlorination to AM may be possible, but it requires strict environments such as utilizing a biotic cathode with a negative enough potential or employing specific dechlorinating bacterial consortium [12]. *G. metallireducens* with excellent extracellular electron transfer ability might induce complete CAP dechlorination. Similar result was also reported by McCormick and Adriaens [29].
They found that carbon tetrachloride was fully dechlorinated by *G. metallireducens*, and followed by production of low molecular carbon compounds such as CO and CH₄. In addition, previous studies demonstrated that *Geobacter* species could reduce nitrate and chlorinated contaminants simultaneously, suggesting no direct competition for electron donors during the CAP dechlorination and nitrate ammonification processes [30].

### 3.3 Reduction ability of *G. metallireducens* at different CAP dosages

It is known that CAP dosage played a key role in the degradation process when using pure strains because CAP itself exhibits the effect of bacteriostasis to some extent [31]. Therefore, the effect of the initial dosage of CAP on removal performance by *G. metallireducens* was investigated. It was observed that the removal percentages of CAP at initial dosages of 10 and 20 mg/L were (94.6±3.7)% and (97.6±4.9)%, respectively, while these values declined gradually from (62.2±2.9)% to (25.0±3.6)% when the CAP dosage was increased from 40 to 80 mg/L (Figure 3). The CAP reduction was fitted to first-order kinetics according to the following equation:

\[
\ln\left(\frac{C}{C_0}\right) = -k_{\text{app}}t, \tag{1}
\]

where \(C\) and \(C_0\) are the real-time concentration and the initial concentration, respectively, \(k_{\text{app}}\) is the apparent rate constant, and \(t\) is the reaction time.

The apparent removal rate constants of CAP dosages at 10 and 20 mg/L were 0.69 d⁻¹ and 0.66 d⁻¹, respectively, which were much higher than those of CAP dosages in the range of 40~80 mg/L (0.15~0.04 d⁻¹). Additionally, the protein contents decreased from 0.013 μg/mL to 0.004 μg/mL when the CAP dosages increased from 10 mg/L to 80 mg/L on the 6th day (Figure S2). These results suggested that high CAP dosage has an inhibitory effect on the growth of *G. metallireducens*, which may result in the reduced CAP removal performance. However, the protein content increased slightly for 10 mg/L CAP on the 6th day because low amount of antibiotics could selectively stimulate the growth of *G. metallireducens* [32].

In addition, the demand of acetate for CAP reduction was also examined. Theoretically, complete reduction of 1 mole of CAP into AM consumes 10 moles of electrons; meanwhile, oxidation of one mole of acetate produces 8 moles of electrons via microbial metabolism. In other words, the reduction of 1 mole of CAP will consume 1.25 moles acetate. The concentration of residual acetate was approximately 22.7 mM before the addition of CAP into the FWA medium, suggesting that the acetate amount was sufficient for CAP reduction even though the initial concentration of CAP was 80 mg/L (Figure S3). These results indicated that acetate amount may not affect the removal efficiency of CAP.

### 3.4 Transcriptomic analysis of *G. metallireducens*

To identify the mechanism of CAP reduction by *G. metallireducens*, gene transcript profiles were compared as the strain was grown in the FWA medium containing 0 and 20 mg/L CAP (Figure 4). The expression of alcohol dehydrogenase gene (Gmet 1046) was highly upregulated (5.4 folds). As alcohol dehydrogenase can degrade chloroalkane and chloroalkene contaminants, this may partially explain the effective dechlorination performance of *G. metallireducens* [33]. The expressions of NADH-quinone oxidoreductase subunit K2 (Gmet 3345, 4.5 folds), NADH dehydrogenase subunit G (Gmet 3349, 4.5 folds), porin (Gmet 0532, 2.5 folds), outer membrane protein assembly factor BamD (Gmet 3014, 2.2 folds) and pilus assembly protein (Gmet 1399, 2.8 folds) genes were also upregulated. These five genes encode the enzymes related to electron transfer in *G. metallireducens*. Especially, pilus assembly protein gene encoded type IV pili, which has been demonstrated to be electrically conductive and capable of mediating...
a long-distance electron transfer [34]. These genes endowed G. metallireducens with a remarkable extracellular electron transfer ability which played a vital role in CAP reduction.

In addition, the expressions of 17 genes encoding c-type cytochromes such as cytochrome c biogenesis protein ResB (Gmet 2901, 4.6 folds), cytochrome c (Gmet 0335, 4.4 folds) and cytochrome c7 (Gmet 2902, 3.4 folds) were upregulated (Figure S4). Previous studies demonstrated that c-type cytochromes localized onto the outer membrane or in the periplasm and were involved in the electron transfer chain in direct contact with extracellular electron acceptors in Geobacter species [35,36]. However, 13 genes related to c-type cytochromes were downregulated, suggesting that not all the c-type cytochromes participated in transferring electrons for CAP reduction.

On the basis of the above results, a possible mechanism of CAP reduction by G. metallireducens was proposed (Figure 5). Acetate was oxidized by G. metallireducens and transformed in the tricarboxylic acid (TCA) cycle, where the reducing force was formed. Then, the electrons were transferred via NAD/NADH redox couple to the cytochrome and pili, which further transferred electrons to CAP and induced CAP reduction to AMCl2, AMCl and AM.

4 Conclusion

In this study, G. metallireducens was demonstrated to reduce CAP under a pure culture condition. CAP was reduced to a complete dechlorination product (AM). Meanwhile, other reduction products such as AMCl2 and AMCl were also observed. Cyclic voltammograms and chronoamperometry results revealed that G. metallireducens had a remarkable catalytic activity for CAP reduction. The removal efficiency of CAP exhibited a negative correlation with the initial CAP dosages. Some genes related to extracellular electron transfer and chlorinated contaminant removal were upregulated, which may contribute to the CAP removal by G. metallireducens. This study may broaden the application of Geobacter to the bioremediation of environments polluted by antibiotics.

Supporting Information

The supporting information is available online at tech.scichina.com and link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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