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Enhanced abiotic and biotic contributions to dechlorination of pentachlorophenol during Fe(III) reduction by an iron-reducing bacterium *Clostridium beijerinckii* Z

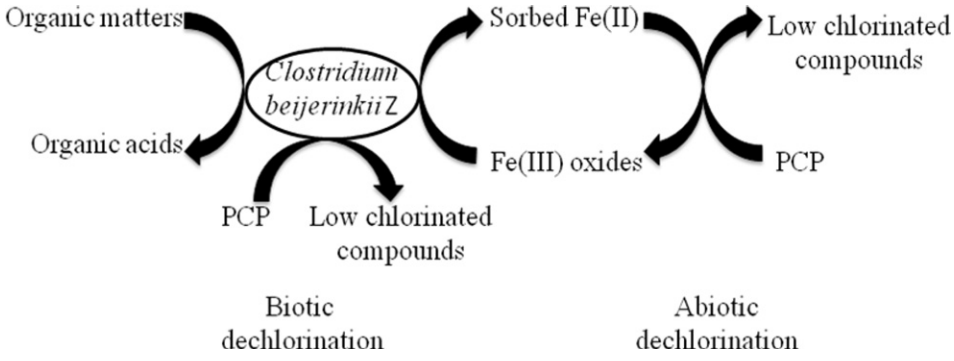
Yan Xu [a], Yan He [a,*], Xiaoli Feng [a], Luyi Liang [b], Jianming Xu [a,*], Philip C. Brookes [a], Jianjun Wu [a]

[a] College of Environmental and Natural Resource Sciences, Zhejiang Provincial Key Laboratory of Subtropical Soil and Plant Nutrition, Zhejiang University, Hangzhou 310058, China
[b] Experiment Teaching Center for Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China

HIGHLIGHTS

- A novel Fe(III) reducing bacterium *Clostridium beijerinckii* Z was isolated and could dechlorinate pentachlorophenol.
- Anaerobic transformation of PCP by *C. beijerinckii* Z could be accelerated by simultaneous reduction of Fe(III).
- Biochemical electron transfer coupling between Fe redox cycling and reductive dechlorination was the mechanism involved.
- The finding increases our knowledge of *Clostridium* sp. regarding their multiple functions for dechlorinating pollutants.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel Fe(III) reducing bacterium, *Clostridium beijerinckii* Z, was isolated from glucose amended paddy slurries, and shown to dechlorinate pentachlorophenol (PCP). Fifty percent of added PCP was removed by *C. beijerinckii* Z alone, which increased to 83% in the presence of both *C. beijerinckii* Z and ferrihydrite after 11 days of incubation. Without *C. beijerinckii* Z, the surface-bound Fe(II) also abiotically dechlorinated more than 40% of the added PCP. This indicated that the biotic dechlorination by *C. beijerinckii* Z is a dominant process causing PCP transformation through anaerobic dechlorination, and that the dechlorination rates can be accelerated by simultaneous reduction of Fe(III). A biochemical electron transfer coupling process between sorbed Fe(II) produced by *C. beijerinckii* Z and reductive dehalogenation is a possible mechanism. This finding increases our knowledge of the role of Fe(III) reducing genera of *Clostridium* in dechlorinating halogenated organic pollutants, such as PCP, in anaerobic paddy soils.

1. Introduction

Pentachlorophenol (PCP) is one of the most significant chlorinated organic pollutants. It was introduced in the 1930s as a wood preservative and is now widely used in both industry and agriculture as a fungicide, insecticide, and herbicide (Crosby et al., 1981). Its bioaccumulation has led to the extensive contamination of soil, surface and ground water, where it can be toxic to microorganisms, plants, animals, and humans (Xiao et al., 2010). Previous studies of the bioremediation of chlorinated organic pollutants such as PCP have verified that microbially reductive dechlorination is an eco-friendly and cost-competitive alternative to

* Corresponding authors. Tel./fax: +86 571 8898 2069.
E-mail addresses: yhe2006@zju.edu.cn (Y. He), jmxu@zju.edu.cn (J. Xu).

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purify the soil environment for the transformation of these chlorinated compounds under contaminated anoxic conditions (Zhang et al., 2012). This has initiated much research to isolate specific microorganisms with the ability for direct anaerobic dechlorination. Other work has focused on understanding the environmental factors required for the efficient biodegradation of chlorinated organic pollutants by these microorganisms (Bouchard et al., 1996; Yoshida et al., 2007; Lu et al., 2010). To date, some anaerobic dehalogenating bacteria, isolated from pure cultures, have demonstrated the strong coupling of PCP dechlorination to bacterial growth. For example, the first anaerobic dehalogenating bacterium, *Desulfomonile tiedjei* strain DCB-1, to be isolated was obtained from a methanogenic consortium capable to reductively dechlorinate PCP at the *meta* position (Shelton and Tiedje, 1984). *Desulfitobacterium dehalogenans* JW/IU-DC1, a Gram-positive anaerobic bacterium, has the ability to dehalogenate a wide range of chlorophenols, including PCP at the *ortho* position (Bouchard et al., 1996). Similarly, a spore-forming anaerobic bacterium, *Desulfitobacterium frappieri* strain PCP-1, which dechlorinates PCP to 3-chlorophenol, can also be induced to dechlorinate at the *ortho* position (Utkin et al., 1994).

Essentially, the anaerobic transformation of chlorinated organic pollutants in anaerobic environments is a microbial dechlorinated respiration induced electron transfer metabolic process, during which the chlorinated organic pollutants serve as electron acceptors (Payne et al., 2011). This process is largely regulated by the coordination and competition of the available electron donors and acceptors (Nies and Vogel, 1990; Zhang et al., 2010). Some organic acids with low molecular weights, such as lactate and glucose, usually act as electron donors in the form of readily metabolizable carbon sources (Nies and Vogel, 1990), while various ionic species, such as NO_3^-, Fe(III), Mn(IV) and SO_4^{2-}, usually act as competitive electron acceptors by microorganisms during dechlorinated respiration (Hofstetter et al., 1999; Kotik et al., 2013).

Fe(III) is usually abundant in soil, and dissimilatory Fe(III) reducers are widely distributed in various environments and among different phylogenetic groups. Therefore the relationship between the redox cycling of Fe and Fe(III) reducing bacteria and the anaerobic transformation of chlorinated organic pollutants has attracted increasing attention (Li et al., 2009b, 2010). For example, *Desulfuromonas chlorethenica* can use tetrachloroethylene and trichloroethylene as electron acceptors for growth, in addition to Fe(III) (Krumholz et al., 1996). The growth of *Anaeromyxobacter dehalogenans* strain 2CP-C is coupled with both dissimilatory Fe(III) reduction and reductive dechlorination, so the dehalogenation of *ortho*-substituted halophenols can occur rapidly (He and Sanford, 2003). The typical dominant bacteria in Fe enriched soil, *Clostridium* sp., is also abundant in sites contaminated with chlorinated compounds (Li et al., 2008, 2009b). The anaerobic PCP-degrading consortium in an anaerobic sludge bed (UASB) reactor contained gene sequences of the genus *Clostridium*, which unexpectedly increased over 140 days (Tartakovsky et al., 2001). The significantly accelerated Fe(III) reduction and organochlorine pesticide DDT transformation in the soils after addition of lactate and glucose may be due to the increased population of *Clostridium* (Chen et al., 2013). The above studies suggest that Fe(III) reducing bacteria might have multiple functions for transforming chlorinated organic pollutants, probably not through the direct dechlorination mechanism as used by dechlorinating bacteria, but through the indirect catalytic dechlorination mechanism that is coupled with the biochemical electron transfer processes between Fe redox cycling and reductive dechlorination. To testify this possibility, new studies on the ability of commonly occurring Fe(III) reducing bacteria to perform the anaerobic transformation of chlorinated organic pollutants are warranted.

Our aims were to (1) isolate Fe(III) reducing bacterial species from paddy soil, and then identify their ability to reduce Fe(III) in different Fe(III) compounds and (2) test the ability of the isolated Fe(III) reducing bacteria to transform PCP, and then determine the mechanisms involved. Our hypothesis is that previously identified Fe(III) reducing species have the ability to dechlorinate PCP by coupling the biochemical electron transfer processes between the Fe redox cycling and reductive dechlorination. Thus they may also simultaneously be the microbial population involved in PCP dechlorination in anaerobic environments.

2. Materials and methods

2.1. Paddy slurry incubation with different carbon sources

The paddy soil used was collected from the 0 to 20 cm depth of a rice field at Jiaxing in the Zhejiang province of China. The soil pH, measured in a 1:2.5 soil/water (w:v) suspension, was 6.0. Soil organic carbon was 19.41 g kg^{-1} and the active Fe content was 6.14 g kg^{-1}. It contained 15.9% sand, 54.9% silt, and 29.2% clay. The soil was air-dried and sieved <1 mm before use.

A method of paddy slurry incubation was used to estimate the Fe(III) reduction efficiency of soil bacterial communities supplied with different carbon substrates. A batch of serum bottles was autoclaved prior to adding 3 g air-dried soil and 1 mL ferrihydrite suspension (Fe concentration 16.69 g L^{-1}) to each. They were then mixed with one of the following carbon sources (1 mL), glucose (0.25 mol L^{-1}), pyruvate (0.5 mol L^{-1}), lactate (0.5 mol L^{-1}), or acetate (0.75 mol L^{-1}), to each provide 18.02 mg C g^{-1} soil, and 1 mL water to provide a final soil: water ratio of 1:1. Ferrihydrite was prepared according to Lovley and Phillips (1986) and characterized by X-ray diffraction (XRD). The carbon sources were sterilized by passing each through a 0.22 μm filter membrane and ferrihydrite was sterilized with ultraviolet before adding to the soil samples. Soil samples for the control treatments were autoclaved three times to ensure sterilization prior to use. Each serum bottle was purged with N_2 for 10 min and then sealed with a butyl rubber stopper covered with aluminum foil. All vials were incubated at 32 °C in an anaerobic chamber under a N_2 stream (Electrotex, AW200SG, England) and the Fe(II) concentrations determined at regular intervals.

2.2. Enrichment and isolation of Fe reducing bacteria

The bacteria were isolated from the slurry at the end of the glucose incubation. The basal medium, modified from Lovley and Phillips (1988), contained: NH_4Cl, 1.0; $CaCl_2 \cdot H_2O$, 0.02; KCl, 0.9; $NaH_2PO_4 \cdot H_2O$, 0.6; and yeast extract, 0.05 g L^{-1}. Then 10 mL each of a trace mineral solution and a vitamin solution were added (Lovley et al., 1993). In addition, glucose (5 g L^{-1}) and Fe(III) citrate (6.44 g L^{-1}) or ferrihydrite (6.49 g L^{-1}) was added as carbon substrates and electron acceptors. The media were sterilized by autoclaving for 20 min and cooled to room temperature under a constant stream of 80% N_2 and 20% CO_2. Slurry (1 mL), as mentioned above, was transferred into sterilized serum bottles containing 50 mL glucose–Fe(III) medium, capped with butyl rubber stoppers, then incubated at 32 °C in the dark. During incubation, when the color of the medium in the serum bottles became lighter, the mixture was transferred into new media at a volume of 10% as inoculum (v/v). After subculturing seven to ten times, a stable microbial culture with the potential to reduce Fe(III) was obtained. Standard anaerobic techniques were used throughout and the samples incubated in an anaerobic chamber with a N_2 stream.

To isolate the Fe(III) reducing bacteria, the incubation solution was diluted serially and plated on agar plates containing glucose and Fe(III) compounds (either Fe(III) citrate or ferrihydrite). Selected well developed colonies were streaked three times onto new agar slants before further study.

For identification of the strain, genomic DNA was extracted from the cells grown on agar plates with standard extraction procedures using a FastDNA Spin Kit (Takara). The amplification of 16S rRNA genes was performed in a total volume of 25 μL containing the universal primers 11F and 1387R. The PCR amplification product was purified with the GENECLEAN Kit (Sango) and the recovered fragments were cloned