Journal of Bioscience and Bioengineering
VOL. 119 No. 2, 188–194, 2015

www.elsevier.com/locate/jbiosc

Humin as an electron donor for enhancement of multiple microbial reduction reactions with different redox potentials in a consortium

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Received 17 May 2014; accepted 24 July 2014
Available online 28 August 2014

A solid-phase humin, acting as an electron donor, was able to enhance multiple reductive biotransformations, including dechlorination of pentachlorophenol (PCP), dissimilatory reduction of amorphous Fe (III) oxide (FeOOH), and reduction of nitrate, in a consortium. Humin that was chemically reduced by $NaBH_4$ served as an electron donor for these microbial reducing reactions, with electron donating capacities of 0.013 mmol e^-/g for PCP dechlorination, 0.15 mmol e^-/g for iron reduction, and 0.30 mmol e^-/g for nitrate reduction. Two pairs of oxidation and reduction peaks within the humin were detected by cyclic voltammetry analysis. 16S rRNA gene sequencing-based microbial community analysis of the consortium incubated with different terminal electron acceptors, suggested that *Dehalobacter* sp., *Bacteroides* sp., and *Sulfurospirillum* sp. were involved in the PCP dechlorination, dissimilatory iron reduction, and nitrate reduction, respectively. These findings suggested that humin functioned as a versatile redox mediator, donating electrons for multiple respiration reactions with different redox potentials.

[**Key words:** Solid-phase electron donor; Humic substance; Humin; Microbial respiration; Electron acceptors]

Redox mediators, which are reversibly oxidized and reduced, can accelerate reactions by lowering the activation energy (1), and thereby enhance microbial transformation. Over the past two decades, different redox mediators have been used to promote anaerobic biotransformation processes in numerous studies (2,3). Naturally occurring humic substances (HSs; humic and fulvic acids) and their quinoid analog anthraquinone-2,6-disulfonate (AQDS) have been widely used as redox mediators to facilitate the reduction of several substrates, including chlorinated aliphatic compounds (4,5), nitrate (6), azo dyes (4,7), and oxidized metal ions such as Fe (III) (8,9), Cr(VI) (10), and U(VI) (10). However, the redox-mediating functions of solid-phase HSs were not investigated until 2010 when the enhancement of microbial iron reduction by solid-phase HSs was first reported (11). When solid-phase HSs are used, there are no losses during *in situ* remediation, and the required mediator is effectively retained within the system. Use of solid-phase HSs could eliminate the need for continuous dosing with dissolved redox mediators in environmental applications (4,12). Therefore, a solid-phase redox mediator would be convenient for *in situ* bioremediation of oxidized pollutants.

The redox-mediating potential of naturally derived humin, the insoluble fraction of HSs at any pH, had not been reported until we showed for the first time that various humins obtained from soils and sediments functioned as solid-phase redox mediators in the microbial reductive dehalogenation of pentachlorophenol (PCP) and tetrabromobisphenol A (13,14). In our recent report, we showed that humin could be electrochemically reduced by a negatively poised electrode and could enhance PCP dechlorination by serving as a redox mediator (15). In all of these studies, *Dehalobacter* was thought to be involved in the dehalogenation. It is noteworthy that humin could maintain microbial PCP dechlorination activity as redox mediator, whereas dissolved humic acid could not. The humin was very stable, and was able to serve as a redox mediator even after various chemical and heat treatments (13). Given the ubiquity and stability of humin, this naturally occurring material might have great potential as a solid-phase redox mediator for reducing oxidized pollutants. Moreover, many researchers have observed the need for soils or sediments to maintain anaerobic dehalogenating activity (16–18). Therefore, it is important to study solid-phase humin as a potential redox mediator for anaerobic bioremediation of contaminated soil and groundwater.

However, little is known about the capacity of humin as an electron donor for multiple microbial reduction reactions. Therefore, this study aimed to determine whether humin can serve as an electron donor of multiple microbial respiration reactions with different terminal electron acceptors, namely microbial PCP dechlorination, dissimilatory iron reduction, and nitrate reduction to ammonium. Their standard redox potentials are +399 mV for $Eo'_{(PCP/2,3,4,5\text{-tetrachlorophenol})}$, +340 mV for $Eo'_{(FeOOH/Ferrous)}$, and +360 mV for $Eo'_{(nitrate/ammonium)}$ (19–21). Structural analyses of the microbial community based on 16S rRNA gene sequencing of the microbial consortium incubated with different terminal electron acceptors was used to identify the microorganisms that were responsible for these redox reactions.

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.07.010

MATERIALS AND METHODS

Bacterial culture and growth conditions An anaerobic humin-dependent PCP dechlorinating culture was used as the source culture for the humin evaluation experiments. This culture has been maintained in our laboratory for at least 2 years, and the culture maintenance procedures have been described previously (13). The anaerobic humin medium was prepared in 60-mL serum bottles with an N_2 headspace, and it contained 20 mL of mineral medium, 0.3 g of freeze-dried humin, filter-sterilized vitamin solution (22), and 10 mM formate (30 mM when nitrate was used as an electron acceptor), which served as both the carbon source and the electron donor. The bottles were sealed with Teflon-faced butyl rubber stoppers and an aluminum crimp cap. The mineral medium contained (per L) 0.05 g of NH_4Cl, 0.05 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.4 g of K_2HPO_4, 1 mL of trace element SL-10 solution, 1 mL of Se/W solution, and 15 mM MOPS buffer (pH 7.2) (23). Humin was obtained from Kamajima paddy soil as described previously (13) and was provided as a freeze-dried form. After the appropriate incubation periods, active cultures were transferred (5%, v/v) to fresh medium (described above) containing 20 μM PCP, 4 mM amorphous Fe (III) oxide (FeOOH) (24), or 5 mM nitrate as the electron acceptor to enrich the PCP-dechlorinating, iron-reducing, or nitrate-reducing consortia, respectively. After three transfers, the three cultures were analyzed to assess the microbial activities of the reduction reactions and the structures of microbial communities. These PCP-dechlorinating, iron-reducing, and nitrate-reducing cultures are referred to hereafter as the PCP-HM-culture, Fe-HM-culture, and NO_3^--HM-culture, respectively. For all conditions, triplicate cultures were prepared in addition to an autoclaved negative control (abiotic control) and a non-humin control (biotic control).

Chemically reduced humin preparation The humin was chemically reduced by mixing 5 g of humin and 100 mL of 0.1 M $NaBH_4$ in *N*,*N*-dimethylacetamide with continuous magnetic stirring at room temperature for 30 h (13). After reduction, acetic acid was added to bring the pH to 7 and to eliminate excess $NaBH_4$, and the humin was then rinsed with distilled water. Prior to the reduction procedure, the solution was thoroughly flushed with nitrogen. The reduction and drying were done under anaerobic conditions in a glove chamber (7450000; COY, Grass Lake, MI, USA). The chemically reduced humin (red-humin) was designated as red-humin. Active cultures (PCP-HM-culture, Fe-HM-culture, or NO_3^--HM-culture) were transferred (5%, v/v) into fresh medium containing 0.3 g of intact humin or red-humin and the electron acceptor 20 μM PCP, 4 mM FeOOH, or 2 mM nitrate, respectively, without formate. All of the experiments were performed in triplicate.

Chemical and electrochemical analyses In the cultures, PCP and its metabolites were analyzed by using a gas chromatography-mass spectrometry system (Shimadzu 2010; Shimadzu, Kyoto, Japan) equipped with a DB-5MS column (J&W Scientific, Folsom, CA, USA) (17). Ferrous ion concentration was measured spectrophotometrically using the ferrozine method (11). Nitrate and nitrite were determined by compact ion chromatography with a Metrohm 761 (Herisau, Switzerland) equipped with a SI-90 4E column and a conductivity detector (25). Ammonium was determined by the phenol-hypochlorite method (26). Cyclic voltammetry (CV) measurements were obtained under anaerobic conditions with a potentiostat (HSV-110; Hokuto Denko Inc., Osaka, Japan), consisting of a graphite working electrode (5 mm × 15 cm) (Tokai Carbon, Tokyo, Japan), an Ag/AgCl reference electrode (HX-R8; Hokuto Denko Inc.), and a twisted platinum counter electrode (0.8 mm × 1 m; Nilaco, Tokyo, Japan). The scan rate was 10 mV s^{-1}, with a potential range of −0.6–0.8 V (vs. Ag/AgCl). After being electrically reduced at an applied potential of −500 mV (vs. a standard hydrogen electrode; SHE) under continuous magnetic stirring for 2.5 h, the humin sample was provided at a concentration of 7.5 g L^{-1} in an anaerobic mineral medium as the electrolyte. As a control, CV measurements were also obtained after the anaerobic mineral medium was reduced at the same time as the same negative potential cathode in the absence of humin.

DNA extraction and denaturing gradient gel electrophoresis After three transfers, the PCP-HM-culture, Fe-HM-culture, NO_3^--HM-culture, and non-humin controls were subjected to DNA analyses. Genomic DNA was extracted from 1 mL of each culture with the Isoplant DNA Extraction Kit (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's protocol. The bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) with universal primers (357f with a GC clamp and 517r) (27). DNA extraction and denaturing gradient gel electrophoresis (DGGE) analysis of the PCR products was performed as described by Yoshida et al. (17). The DNA bands were excised from the DGGE gel and sequenced as described previously (15). BLAST was used to search GenBank for similar sequences.

Analysis of the 16S rRNA gene clone library The 16S rRNA genes in the bacterial communities were amplified by PCR for clone analysis with the high-fidelity KOD FX Neo enzyme (Toyobo, Osaka, Japan) and primers 27f and 1492r (28). The PCR products were cloned using the pCR8/GW/TOPOTA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequences of the 16S rRNA inserts from the recombinant clones were determined by the Dragon Genomics Center of Takara Bio (Yokkaichi, Mie, Japan) using primer 341f. Sequences were analyzed using the Ribosomal Database Project classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp), and similarity searches of the GenBank database were performed by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The tree was constructed by using the neighbor-joining method with CLUSTAL X and MEGA 4.0 software.

Real-time quantitative PCR For real-time quantitative PCR (qPCR), the specific primer sets used were Eub341F and Eub534R for total bacteria (27), Dhb477F and Dhb647R for *Dehalobacter* (29), Sulfuro114F and Sulfuro421R for *Sulfurospirillum* (30), and Bac1023F (5′-actgcctacgaggtgctgcatg-3′) and Bac1202R (5′-gtaagggccgtgctgatttgacg-3′) for *Bacteroides*. Bac1023F and Bac1202R were designed specifically for this study. qPCRs were performed using a LightCycler system (Roche Diagnostics, Mannheim, Germany) and the LightCycler Fast-start DNA Master SYBR green I Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) as previously described (14). Statistical analysis was conducted with the IBM SPSS Statistics software package (version 21.0.0) using one-way analysis of variance (ANOVA) with post-hoc tests (Tukey's honestly significant difference test or Dunnett's T3 test) for the qPCR results as described previously (14). Differences were considered significant when the *P* values were less than 0.05.

Nucleotide sequence accession numbers The nucleotide sequence data obtained in this paper have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers from AB936296 to AB936313.

RESULTS

Anaerobic microbial reduction of various electron acceptors via humin Fig. 1 shows time courses of PCP dechlorination (Fig. 1A), iron reduction (Fig. 1B), and nitrate reduction (Fig. 1C) in cultures with or without humin after three transfers. None of the abiotic controls showed activity regardless of whether PCP, FeOOH, or nitrate was used as the terminal electron acceptor (data not shown), suggesting that these reduction reactions were attributed to microbial activity. After 10 days, all of the PCP (20 μM) in the PCP-HM-culture was dechlorinated to monochlorophenol (MCP) or phenol, whereas no dechlorination activity was observed in the biotic control in the absence of humin. This confirmed that humin was essential for microbial PCP dechlorination. Almost all of the PCP in the PCP-HM-culture was dechlorinated to phenol after 13 days.

In the Fe-HM-culture, FeOOH was reduced to ferrous ion, and 1.47 mM ferrous ion was formed after 6 days. In contrast, in the biotic control without humin, only 0.13 mM ferrous ion was produced after 8 days, which was substantially lower than that measured in the presence of humin. This suggested that microbial iron reduction was enhanced in the presence of humin.

Under nitrate-reducing conditions, 5 mM nitrate in the NO_3^--HM-culture was reduced to ammonium within 3 days. Conversely, in cultures without humin (biotic control), nitrate reduction was concomitant with nitrite accumulation, and only a very small amount of nitrite was reduced to ammonium. Even after 20 days, 3.3 mM nitrite remained in the culture, suggesting that humin enhanced nitrate reduction.

These results provide evidence that microbial dechlorination of PCP, dissimilatory iron reduction, and nitrate reduction were enhanced by humin which was acting as an electron donor, as described below.

Reduced humin as an electron donor for microbial reactions A previous study showed that PCP-dechlorinating activity was stably maintained when $NaBH_4$-reduced humin was used as an electron donor without formate, and that humin functioned as a solid-phase electron donor for the microorganisms that dechlorinate PCP (13). To determine if red-humin can serve as electron donor for microbial iron reduction and nitrate reduction and its electron donating capacity for PCP dechlorination, dissimilatory iron reduction, and nitrate reduction, organisms were transferred into fresh medium without formate, but with 0.3 g of red-humin. When red-humin was used as an electron donor, all of the PCP was dechlorinated to MCP (1.3 μM) and phenol (17.5 μM) within 7 days, whereas negligible levels of PCP dechlorination products were formed in the presence of intact humin. Likewise, much more FeOOH and nitrate were reduced to