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ORIGINAL ARTICLE

Fe(III) oxide reduction and carbon tetrachloride dechlorination by a newly isolated *Klebsiella pneumoniae* strain L17

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anthraquinone-2,6-disulfonate, carbon tetrachloride, dechlorination, Fe(III) oxide, *Klebsiella pneumoniae*, reduction.

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Abstract

Aims: To isolate an iron-reducing bacterium and examine its ability of Fe(III) oxide reduction and dechlorination.
Methods and Results: A fermentative facultative anaerobe, strain L17 isolated from subterranean sediment, can reduce Fe(III) oxides and carbon tetrachloride (CT). It was identified as *Klebsiella pneumoniae* by 16S rRNA sequence analysis. Strain L17 can metabolize fermentable substrates such as citrate, glycerol, glucose and sucrose coupled with the reduction of hydrous ferric oxide, goethite, lepidocrocite and hematite. Fe(III) reduction was influenced by crystal structure of Fe(III) oxide, type of fermentable substrate, metabolic status of the strain, and significantly enhanced by addition of anthraquinone-2,6-disulfonate (AQDS). Strain L17 could dechlorinate CT to chloroform, and the rate was accelerated in the presence of Fe(III) oxide and AQDS. Biotic dechlorination by strain L17 and abiotic dechlorination by sorbed Fe(II) were proposed as the two main mechanisms. AQDS might accelerate the dechlorination by transferring electrons from strain L17 to Fe(III) oxide and CT.
Conclusions: *K. pneumoniae* L17 can reduce Fe(III) oxides and CT. The two reductions can occur simultaneously, and be significantly promoted by AQDS.
Significance and Impact of the Study: This is the first report of a strain of *K. pneumoniae* capable of reducing Fe(III) oxides and CT. As a strain of environmental origin, strain L17 may have the potential for bioremediation of chlorinated compound-contaminated sites.

Introduction

Dissimilatory iron-reducing bacteria (DIRB), which can oxidize organic matter with the reduction of Fe(III) to Fe(II) under anoxic conditions, are very common in groundwaters, soils and sediments, and can influence the aqueous geochemistry, surface chemistry and mineralogy of these environments (Fredrickson and Gorby 1996; Chapelle 2001). They are phylogenetically diverse, including both facultative and obligate anaerobes, classified as fermentative, photosynthetic, organic acid-oxidizing and hydrogen-oxidizing (Scala *et al.* 2006). For example, fermentative bacteria *Clostridium butyricum* (Park *et al.* 2001) can reduce Fe(III) as an electron sink during glucose metabolism. *Rhodobacter capsulatus* (Dobbin *et al.* 1996) was able to reduce Fe(III) under anaerobic photoheterotrophic growth conditions in the photosynthetic metabolism. *Geobacteraceae* and *Shewanella* (Lovley *et al.* 2004) are representative species of the organic-acid-oxidizing family and have been extensively investigated with respect to their ability of Fe(III) reduction. Many DIRB including some *Geobacter* and *Shewanella* species can oxidize hydrogen coupled with Fe(III) reduction (Lovley *et al.* 1989; Caccavo *et al.* 1994).

It is well-known that the contamination of organic chlorinated compounds is a widespread environmental concern because of their mutagenic, carcinogenic and toxic effects (Vogel *et al.* 1987). Some DIRB have been found to be capable of using chlorinated compounds as electron acceptor. For example, *Desulfuromonas michiganensis* (Sung *et al.* 2003) could reductively dechlorinate tetrachloroethylene using acetate as electron donor. *Shewanella putrefaciens* (Picardal *et al.* 1993) is also capable of dechlorinating carbon tetrachloride (CT) to chloroform (CF) and other unidentified products. As many studies have reported that chlorinated compounds can be biodegraded to less toxic products, microbial reductive dechlorination has been valued as an important approach for remediation of chlorinated compounds (Holliger *et al.* 1999; Janssen *et al.* 2001). Meanwhile, the Fe(II) species produced in subsurface environments by DIRB may reduce chlorinated contaminants abiotically (Kim and Picardal 1999; Mccormick and Adriaens 2004).

The capability of dechlorination by DIRB may be more prevalent than currently recognized as the evaluation of such ability was not always a part of the characterization of many DIRB (Lovley *et al.* 2004). The research investigating the effect of microbial Fe(III) reduction on the fate of chlorinated contaminants was mainly conducted with the two genera, *Shewanella* and *Geobacter* (Petrovskis *et al.* 1994; Picardal *et al.* 1995; Kim and Picardal 1999; Mccormick and Adriaens 2004). In this study, a new DIRB named strain L17 was isolated and identified as a fermentative facultative anaerobe, and characterized as a strain of the genus *Klebsiella*. Previous studies have demonstrated that some strains of *Klebsiella* sp. can reduce Fe(III) citrate (Baldi *et al.* 2001) and biodegrade some chlorinated compounds, such as chlorpyrifos (Ghanem *et al.* 2007), 4-chloroaniline (Vangnai and Petchkroh 2007), chlorinated *s*-triazines (Ernst and Rehm 1995), endosulfan and its toxic metabolite, endosulfan sulfate (Kwon *et al.* 2005). However, very little research has been conducted regarding its ability of both Fe(III) oxide reduction and dechlorination. Therefore, the objectives of this study were to (i) characterize strain L17 and identify available organic matter for Fe(III) reduction by strain L17, (ii) determine the rate and extent of Fe(III) oxide reduction by strain L17, (iii) investigate the transformation of CT by strain L17 in the presence and absence of Fe(III) oxide. In addition, quinones-containing humic substances, such as anthraquinone-2,6-disulfonate (AQDS), can function as an electron shuttle for microbial reduction of Fe(III) oxides (Lovley *et al.* 1998). In this study, we also evaluate the effect of AQDS on the microbial Fe(III) oxide reduction and CT dechlorination by strain L17.

Materials and methods

Enrichment and isolation of the strain

Standard anaerobic techniques were used for all experiments in this study. All anaerobic media were boiled and cooled under a constant stream of 80% N_2–20% CO_2, dispensed into aluminum-sealed culture bottles under the same gas phase, capped with butyl rubber stoppers, and sterilized by autoclaving (121°C, 20 min). Besides the sterile media, inoculation and sampling were conducted by using sterile syringes and needles. All vials were incubated in a Bactron Anaerobic/Environmental Chamber II (Shellab, Sheldon Manufacturing Inc., Cornelius, OR) at 30°C in dark.

A sample of subterranean forest sediment (Zhaoqing, China) was extruded into glass tubes, stoppered, and immediately transported back to the laboratory and stored in the anaerobic chamber, serving as an inoculum for enrichment. The basal medium, modified from ATCC 1768 medium, contained the following components (in grams per litre of deionized H_2O): $NaHCO_3$, 2·5; NH_4Cl, 0·25; $NaH_2PO_4{\cdot}2H_2O$, 0·68; KCl, 0·10. In addition, the trace mineral solution and vitamin solution were added at 1% (v/v) (Lovley and Phillips 1988). For enrichment, glucose (5 mmol l^{-1}) and soluble ferric citrate (50 mmol l^{-1}) were added as organic matter and electron acceptor, respectively. The enrichment was initiated by adding 5·0 g (wet weight) anaerobic sediment to sterile serum bottles containing the glucose-Fe(III) basal medium (20 ml) in the anaerobic chamber. The headspaces (5·2 ml) of serum bottles were evacuated and replaced with 80% N_2–20% CO_2. Medium without inoculum was incubated under the same conditions as the controls. By regular subculturing (10–15 days intervals), 10% (v/v) inoculum was transferred to a fresh glucose-Fe(III) basal medium three times. To obtain pure-culture isolates, the enriched populations were serially diluted and plated onto glucose-Fe(III) basal medium agar plates for single-colony isolation. Distinct colonies were picked and streaked three times on glucose-Fe(III) basal medium agar slants before further characterization.

Identification of the strain

The obtained bacterial strain was characterized by using standard physiological–biochemical procedures, including cell size and morphology, gram reaction, tests for cytochrome oxidase, indole, methyl-red, acetyl methyl carbinol (AMC) and citrate. Additional phenotypic characteristics were determined by the Biolog microbial identification system. Extraction of genomic DNA and amplification of 16S rRNA gene were conducted as

described by Li *et al.* (2007b). A preliminary sequence similarity search was performed against known sequences available in the GenBank using BLAST (Altschul *et al.* 1997). Multiple alignments with corresponding nucleotide sequences of representatives of the genus *Klebsiella* retrieved from GenBank were carried out using CLUSTAL x program (Thompson *et al.* 1997). The neighbour-joining phylogenetic tree was performed by using the software package MEGA version 4 (Kumar *et al.* 2004), and it was evaluated using the bootstrap values (Felsenstein 1985) based on 1000 replicates.

Fe(III) oxides preparation and characterization

Hydrous ferric oxide (HFO) was prepared by slowly neutralizing 0·4 mol l^{-1} solution of $FeCl_3{\cdot}6H_2O$ with 1 mol l^{-1} NaOH in a high-density polyethylene container (Mccormick and Adriaens 2004). As the circumneutral pH was approached, thick red slurry of HFO formed. At pH 7·0, the slurry was allowed to ripen for 2–6 h, with slight decrease in pH (usually < 1 pH unit). The slurry was adjusted back to pH 7·0 and centrifuged at 3600 ***g*** for 20 min at 4°C. After decanting the supernatant, the solid was resuspended and washed with Milli-Q water and centrifuged again. This procedure was repeated seven times in order to reduce the chloride content to less than 1 mmol l^{-1}. Goethite (α-FeOOH) was prepared according to the procedures of Schwertmann and Cornell (1991). 0·05 mol of hydrated ferric nitrate ($Fe(NO_3)_3{\cdot}9H_2O$) was dissolved in 0·45 mol potassium hydroxide, and then diluted to 1000 ml. The solution was refluxed at 70°C for 60 h. Then the hydrosol was washed until neutral with Milli-Q water, dried at 65°C for 48 h and ground to pass through 100-mesh sieve. Lepidocrocite (γ-FeOOH) was synthesized by mixing $FeCl_2{\cdot}4H_2O$, $(CH_2)_6N_4$ and $NaNO_2$ in Milli-Q water, and hematite (α-Fe_2O_3) was formed by sintering lepidocrocite powder at 420°C for 2 h at 2°C min^{-1} temperature increase rate (Li *et al.* 2007a). Goethite, lepidocrocite and hematite were well crystallized by the X-ray diffraction (XRD). The displayed characteristic *d*-values were consistent with those of the literature values. According to the Brunauer–Emmett–Teller (BET) examination, the medium surface area of goethite was 120·9 m^2 g^{-1}, while that of lepidocrocite and hematite was 115·4 m^2 g^{-1} and 29·4 m^2 g^{-1}, respectively.

Fe(III) reduction and CT dechlorination experiments

Harvested cells of the obtained strain were used for the Fe(III) reduction and CT dechlorination experiments. Cells were grown in nutrient broth under aerobic conditions on a rotary shaker at 180 rev min^{-1} at 30°C, and harvested by centrifugation at 6900 ***g*** for 10 min at 4°C when it approached the exponential phase. The pellets were washed thrice and resuspended in sterile fresh basal medium to an optical density of 1·8 to 2·2 (λ = 600 nm). In all Fe(III) reduction and dechlorination experiments, 1 ml of the suspension was added to 25·2-ml serum bottles containing 19 ml of basal medium. Based on the correlation between culture optical density and viable cell counts determined by serial dilution and plating, the density of 2·2 corresponded to approximately 3×10^9 cells ml^{-1}.

To study the effect of different organic matters on Fe(III) oxide reduction, 10 mmol l^{-1} of formate, acetate, propionic acid, lactate, citrate, ethanol, glycerol, glucose and sucrose were respectively added into the inoculated basal medium with 50 mmol l^{-1} of HFO. To investigate the rate and extent of Fe(III) oxide reduction by strain L17, harvested cells were inoculated in the glycerol (10 mmol l^{-1}) basal medium containing 50 mmol l^{-1} of HFO, α-FeOOH, γ-FeOOH and α-Fe_2O_3, respectively. AQDS (97·0%; Fluka, Tokyo, Japan) was added at a final concentration of 100 μmol l^{-1} to estimate its effect on the microbial Fe(III) reduction. In addition, growth of cells was examined in the basal medium containing citrate (10 mmol l^{-1}), harvested cells, and HFO (50 mmol l^{-1}) or AQDS (100 μmol l^{-1}) as electron acceptor. Cell suspension for growth test was harvested at an optical density of <0·5 (λ = 600 nm).

Dechlorination experiments were conducted in 25·2-ml serum bottles containing 20 ml of basal medium using carbon tetrachloride (99·9%; Sigma-Aldrich) as the target compound. The bottles were purged with 80% N_2–20% CO_2 and sealed with Teflon-coated butyl rubber stoppers and crimp seals before CT was added. A methanol-based stock solution of CT (10 μl) was injected into the serum bottles to provide an aqueous concentration of 8·0 μmol l^{-1}, with a syringe needle long enough to guarantee the addition below the surface of the medium. To achieve equilibration between headspace and aqueous phase, the first sample was collected after 6 h. α-FeOOH (25 mmol l^{-1}) and AQDS (100 μmol l^{-1}) were added to study their effect on microbial dechlorination. Controls using sterile water were conducted to evaluate any loss of CT over the dechlorination experiment. Both Fe(III) reduction and dechlorination experiments were conducted in replicate, and the standard anaerobic techniques and anaerobic incubation conditions were the same as enrichment and isolation procedures.

Analytical methods

The total concentration of Fe(II), including dissolved and sorbed Fe(II), was determined by extracting Fe(II) from the samples using 0·5 mol l^{-1} HCl for 1·5 h (Fredrickson

and Gorby 1996) and assaying the extract using 1,10-phenanthroline colorimetric assay (Picardal *et al.* 1995). Dissolved Fe(II) was determined by removing the mineral and sorbed Fe(II) from the aqueous phase using a 0·22-μm syringe filter and then assaying the filtrate by 1,10-phenanthroline (Roden and Zachara 1996). Sorbed Fe(II) was calculated as the difference between the total and dissolved Fe(II).

The analysis of CT and its products in culture liquid was performed according to EPA method 5030C (Purge-and-trap for aqueous samples Revision 3, May 2003), using a purge and trap sample concentrator (Teledyne Tekmar velocity XPT, Mason, OH) equipped with a GC–MS system (Shimadzu QP2010 Plus) fitted with an EC-5 column (30 m × 0·25 mm × 0·25 μm). External standards were prepared from methanol stocks in gas-tight serum vials with the same gas-to-liquid volume ratio as the reaction vials. A volume of 5 ml of standards or samples was injected into the purging chamber immediately using syringes and needles. After an 11-min purge with He gas (99·999%) at 40 ml min^{-1} flow rate at ambient temperature, the purge and trap system was placed in the desorb mode and the trap was rapidly heated to 250°C for 1·5 min without a flow of carrier gas passing through the trap. Three replicate injections were used to determine the mean mass of CT and products in each bottle at each sampling point. Variation in sterile-water controls over the whole dechlorination period was <5%.

Results

Identification of the strain

The strain designated as L17 was a fermentative facultative anaerobe, which formed white rounded opaque colonies on aerobic LB agar plates and yellow rounded opaque colonies on anaerobic glucose–Fe(III) basal medium agar plates. Cells were straight rods, of 0·6∼0·8 μm in width and 1·0∼1·7 μm in length. Strain L17 was gram-negative, nonmotile, cytochrome oxidase negative, indole negative, methyl red negative, acetyl methyl carbinol (AMC) positive and citrate positive. Optimum temperature and pH for anaerobic growth were 30°C and 6·6, respectively. The 16S rRNA gene sequence of strain L17 was submitted to the GenBank database with the accession number EU419756. Based on 16S rRNA gene sequences analysis, strain L17 showed a close relationship with members of the genus *Klebsiella* in the phylogenetic tree, having the highest similarity of 99·50% with *K. pneumoniae* ATCC 13883^T. Its position in the 16S rRNA phylogenetic tree is shown in Fig. 1. The strain isolated in this study was consequently identified as *K. pneumoniae* strain L17, and has been deposited in China Center for Type Culture Collection (CCTCC AB 208106).

Effect of organic matter on HFO reduction

Data in Fig. 2 showed that when formate, acetate, propionic acid, lactate or ethanol was added, no more Fe(II) was produced in the inoculated vials than the control vials, suggesting that strain L17 could not reduce HFO in the presence of these organic compounds under anoxic conditions. The concentrations of Fe(II) in the inoculated vials with citrate, glycerol, glucose and sucrose were twofold or even greater than those in the controls. The concentrations of Fe(II) in these inoculated vials increased with time, and in the order: glycerol < citrate < glucose < sucrose after 10 days. For a given organic matter, additional tests showed that Fe(II) increased when more organic matter was provided (data not shown). Therefore, it can be concluded that strain L17 can reduce HFO when glycerol, citrate, glucose or sucrose is available, and the reduction rate is influenced by the type and concentration of the organic matter.

Fe(III) oxide reduction

During 25-day anaerobic Fe(III) oxide reduction, visual colour change was observed in the inoculated vials,

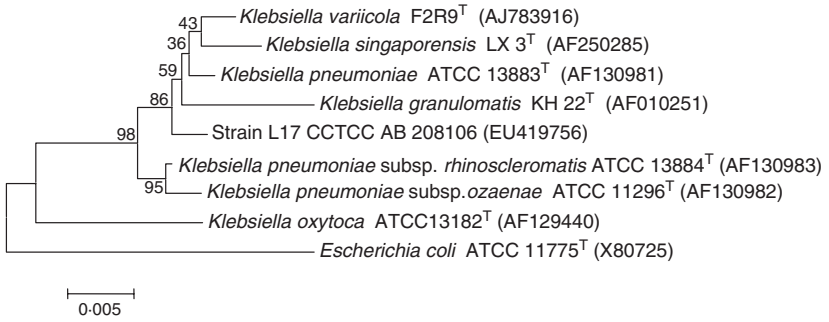

Figure 1 Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences, showing the position of strain L17 among phylogenetic neighbours. Bootstrap values (expressed as a percentage of 1000 replications) are given at the nodes. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bar, 0·005 substitutions per nucleotide position.

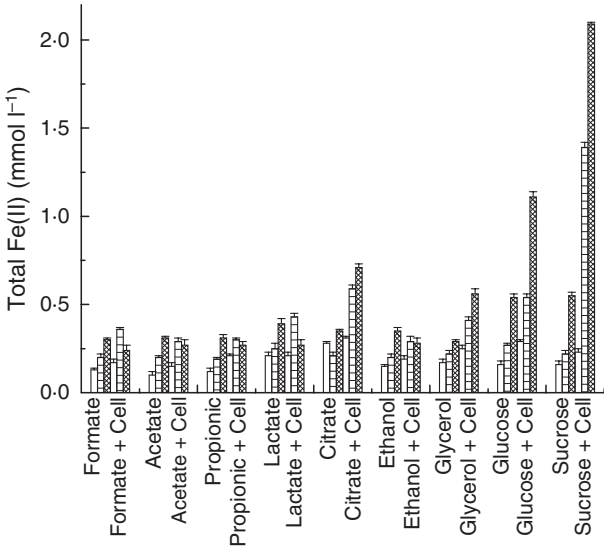

Figure 2 Effect of different organic matter on total Fe(II) production in HFO reduction by strain L17. (□) 0 days, (▤) 5 days, (▩) 10 days. Data are mean ± SD (n = 2). Some error bars are not visible because of their small size.

especially in the inoculated vials with AQDS addition. They were dark-red to brown for HFO, yellow to brown for α-FeOOH, orange to dark-brown for γ-FeOOH, and red to dark-brown for α-Fe_2O_3, relative to the original colour remained in the sterile control vials throughout the experiment. Figure 3 showed that more Fe(II) was produced in the inoculated vials than in the uninoculated controls, and the Fe(II) production increased as time elapsed. After 25 days, 0·23 mmol l^{-1}, 0·50 mmol l^{-1}, 0·30 mmol l^{-1} and 0·07 mmol l^{-1} of Fe(II) was produced in the inoculated vials with HFO, α-FeOOH, γ-FeOOH and α-Fe_2O_3, respectively. The zero-order kinetic constants (k) of Fe(II) production over the first 15-day reduction were listed in Table 1, in an order of α-FeOOH > γ-FeOOH > HFO > α-Fe_2O_3. From the results, Fe(III) oxide reduction only occurred in the inoculated vials, indicating that the reduction of Fe(III) to Fe(II) is a biological transformation rather than a chemical reaction. Furthermore, when AQDS was added, the Fe(II) production increased significantly in all inoculated vials. The Fe(II) concentrations increased to 4·59 mmol l^{-1}, 4·80 mmol l^{-1}, 2·38 mmol l^{-1} and 0·45 mmol l^{-1} after 25 days for HFO, α-FeOOH, γ-FeOOH and α-Fe_2O_3,

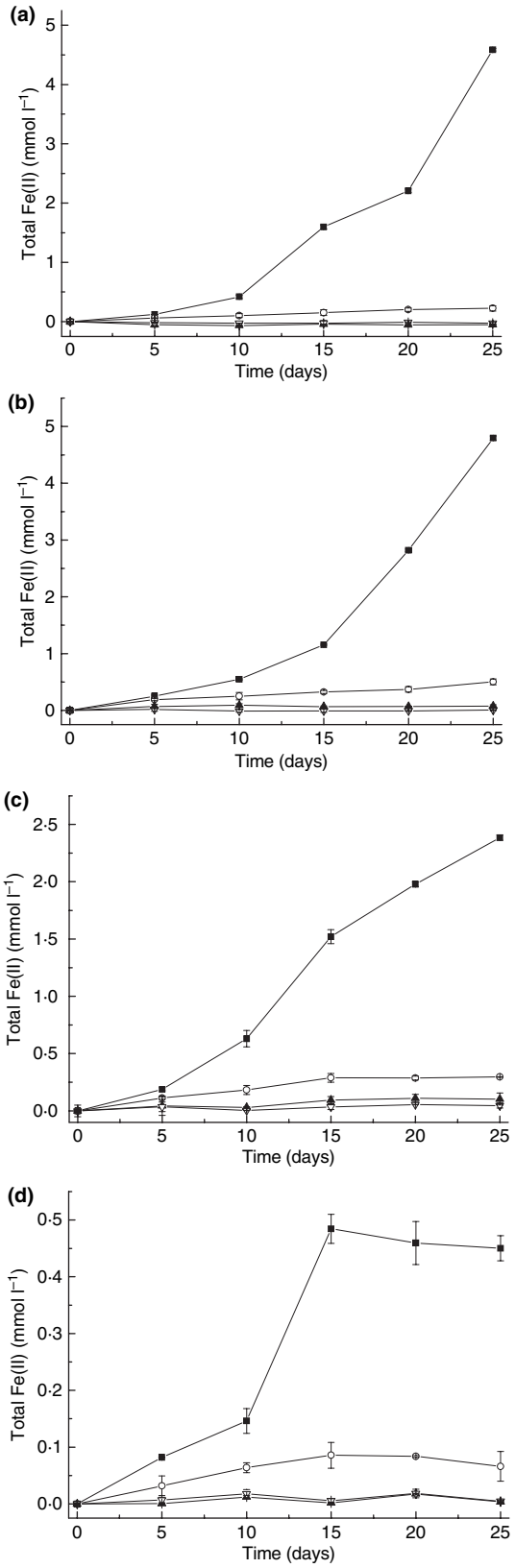

Figure 3 Total Fe(II) production in Fe(III) oxide reduction by strain L17 with glycerol. (a) HFO, (b) α-FeOOH, (c) γ-FeOOH and (d) α-Fe_2O_3. (■) Fe(III) oxide/strain L17/AQDS, (○) Fe(III) oxide/strain L17, (▲) Fe(III) oxide/AQDS, (▽) Fe(III) oxide alone. Data are mean ± SD (n = 2). Some error bars are not visible because of their small size.

Table 1 The zero-order kinetic constants*(k) of Fe(II) formation by strain L17 over the first 15-day reduction of various Fe(III) oxides

	Iron oxides		Iron oxides + AQDS	
	k (mmol l^{-1} d^{-1})	R	k (mmol l^{-1} d^{-1})	R
HFO	0·0104	0·9949	0·0822	0·8737
α-FeOOH	0·0238	0·9443	0·0687	0·9699
γ-FeOOH	0·0192	0·9959	0·0859	0·9386
α-Fe_2O_3	0·0060	0·9958	0·0261	0·9067

*Values were calculated from data in Fig. 3.

respectively. The zero-order kinetic constants (k), as shown in Table 1, were approximately eightfold, threefold, fivefold and fourfold greater than that in the absence of AQDS. Thus, strain L17 can reduce various Fe(III) oxides to Fe(II), and the reduction rate can be significantly accelerated by the presence of AQDS.

In the 3-day anaerobic growth test, no detectable increase in cell numbers was observed when HFO was added. However, when AQDS was added, the amount of viable cells increased from $6{\cdot}03 \times 10^3$ to $2{\cdot}80 \times 10^8$, and the colourless AQDS was reduced to the yellow AH_2DS. Thus, Fe(III) oxide reduction did not stimulate noticeable anaerobic growth of strain L17, but AQDS reduction did.

Carbon tetrachloride reduction

Figure 4a shows the CT transformation by strain L17 in the presence and absence of α-FeOOH and AQDS. Data showed that 75·3% of CT was transformed by strain L17 after 21 days, indicating that strain L17 was capable of dechlorinating CT under anaerobic conditions. At the same time, 82·7% of CT was transformed when α-FeOOH was added, and the percentage of transformation was increased to 96·6% when both α-FeOOH and AQDS were added, indicating that the CT transformation can be enhanced by the presence of α-FeOOH and AQDS. In the control vials, without strain L17, α-FeOOH or AQDS alone was not capable of CT transformation (data not shown). The results of GC–MS spectra showed that chloroform was the only identified dechlorination product in those inoculated vials. At the beginning of incubation, CF was produced at the very similar level in different experiments (Fig. 4b). The trends of CF formation began to diverge after 4 days, following an order of strain L17 < strain L17/α-FeOOH < strain L17/α-FeOOH/AQDS. Approximately <30% of the transformed CT was detected as CF in strain L17/α-FeOOH/AQDS experiment after 21 days. Figure 5 shows the concentrations of total (i) and sorbed Fe(II) (ii) produced in the CT transformation experiments by strain L17 with the addition of α-FeOOH/AQDS. Relative to no Fe(II) formation in the controls, 0·38 mmol l^{-1} and 0·35 mmol l^{-1} of total and sorbed Fe(II) were produced after 8 days in the strain L17/α-FeOOH experiment. Within the same time, in the strain L17/α-FeOOH/AQDS experiment, the concentrations of total and sorbed Fe(II) were 6·83 mmol l^{-1} and 6·31 mmol l^{-1}, respectively.

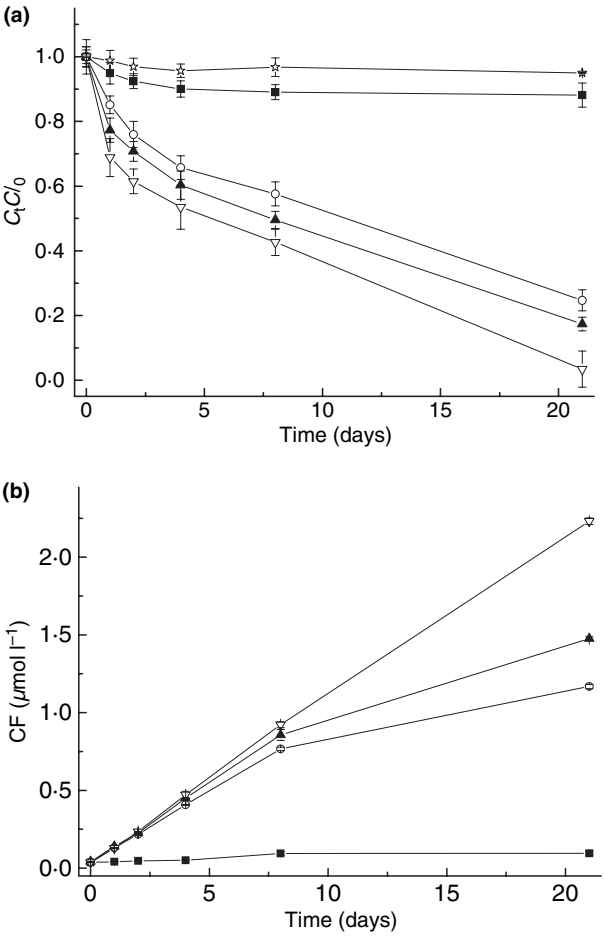

Figure 4 CT Transformation (a) and CF formation (b) by strain L17 in different experiments with glucose. The initial concentration of CT was 8 μmol l^{-1}. (☆) vials in sterile water only, (■) uninoculated vials, (○) strain L17, (▲) strain L17/α-FeOOH, (▽) strain L17/α-FeOOH/AQDS. Data are mean ± SD (n = 3). Some error bars are not visible because of their small size.

Discussion

In this study, we isolated a fermentative facultative anaerobe *K. pneumoniae* strain L17, which can reduce not only various crystalline Fe(III) oxides but also CT. To the best of our knowledge, this is the first report of such two types of reductive capability of *K. pneumoniae* under

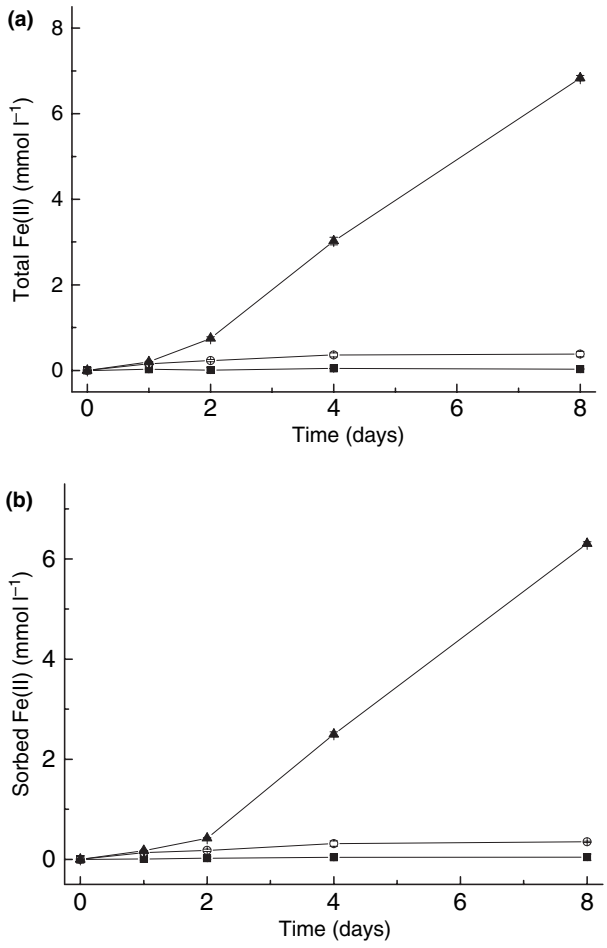

Figure 5 Total Fe(II) (a) and sorbed Fe(II) (b) production in CT transformation by strain L17 in different experiments with glucose. (■) α-FeOOH, (○) strain L17/α-FeOOH, (▲) strain L17/α-FeOOH/AQDS. Data are mean ± SD (n = 3). Some error bars are not visible because of their small size.

anaerobic conditions. These findings extend the diversity of DIRB associated with dechlorination.

Fe(III) oxide reduction

The understanding of the factors influencing microbial Fe(III) reduction is important to relate the physiological characteristics of DIRB to their potential environmental role (Lovley *et al.* 2004). HFO, α-FeOOH, γ-FeOOH and α-Fe_2O_3, representing different kind of iron oxides, are abundant in the soils (Straub *et al.* 2001). The properties of Fe(III) oxide, such as crystallinity, surface area, solubility and phase identity, have been demonstrated as major factors controlling microbial reduction of Fe(III) oxide (Liu *et al.* 2001; Roden 2003; Bonneville *et al.* 2004). Roden and Zachara (1996) found that the rate and extent of microbial Fe(III) oxide reduction increased linearly with surface area, regardless of the degree of Fe(III) oxide crystallinity or structural form. In this study, microbial Fe(III) reduction of α-Fe_2O_3 was the lowest because of its high crystallinity and low BET surface area (29·4 m^2 g^{-1}). The microbial reduction of α-FeOOH yielded a higher rate compared with that of γ-FeOOH with similar surface areas (115∼120 m^2 g^{-1}). It is possible that, for different types of Fe(III) oxides, significant difference of crystal structural properties may influence the rate of microbial Fe(III) reduction.

On the other hand, microbial Fe(III) oxide reduction may strongly depend on the characteristic of DIRB, such as growth stage, source of electron donors and metabolic status. It is well-known that DIRB can be separated into two major groups, one can support growth by conserving energy from electron transfer to Fe(III) and the other cannot (Lovley *et al.* 2004). In this study, Fe(III) serving as the sole electron acceptor did not stimulate noticeable anaerobic growth of strain L17. As one of the fermentative DIRB, strain L17 reduced <2% of added Fe(III) in its fermentative metabolism. Lovley (1987) demonstrated that <5% of electron equivalents can be transferred to Fe(III) in the fermentative metabolism, while most of the electron equivalents are recovered in organic fermentation products and hydrogen. Moreover, the minor transfer of electron equivalents to Fe(III) during fermentation has not been demonstrated to cause any increase in cell yield (Lovley and Phillips 1989). Therefore, Fe(III) reduction may be only a minor pathway for electron flow when strain L17 ferments organic matter, and such fermentative metabolic status may limit the extent and rate of Fe(III) oxide reduction by strain L17.

The addition of AQDS was expected to accelerate the limited rate of Fe(III) oxide reduction by strain L17 in this study, as AQDS can serve to shuttle electrons from DIRB to Fe(III) oxides in anaerobic soils and sediments, and this electron shuttling permits DIRB to indirectly reduce Fe(III) oxides faster than Fe(III) is reduced in the absence of humic substances (Lovley *et al.* 1998). Furthermore, anaerobic respiration with humic substances as the terminal electron acceptor can yield energy to support cell growth by some bacteria (Lovley *et al.* 1996). We observed production of AH_2DS and an increase in cell numbers when AQDS was added as electron acceptor, and the addition of AQDS significantly accelerated the microbial reduction of crystalline Fe(III) oxides. The enhancement may be because of growth in cells and abiotic Fe(III) reduction by AH_2DS, which was formed from reduction of AQDS by strain L17. This suggests that Fe(III) oxide reduction by strain L17 may be favoured in natural environment where humic substances are present.

Carbon tetrachloride dechlorination

The new DIRB, *K. pneumoniae* strain L17, appeared to have the ability to dechlorinate CT to CF under anaerobic conditions. This result was similar to that for the fermentative *Escherichia coli* K-12 and *Enterobacter cloacae* DG6, and it was suggested that the capability of the *Enterobacteriaceae* family for dechlorination might be related to some existing capabilities of the cell (Criddle *et al.* 1990). Nonstoichiometric CF formation in our microbial experiments may be a consequence of reactions between the trichloromethyl radical and nonspecified biochemicals in the bacterial cells, which was explained in the previous research on CT dechlorination by strains of *S. putrefaciens* (Picardal *et al.* 1993; Petrovskis *et al.* 1994).

Carbon tetrachloride transformation by strain L17 was improved by the presence of α-FeOOH, and reductive dechlorination and Fe(III) oxide reduction could occur simultaneously. Kim and Picardal (1999) proposed two main hypotheses to explain the increased CT transformation rate by the addition of Fe(III) oxide. First, as some DIRB can couple growth with the Fe(III) reduction, the addition of Fe(III) oxide may increase cell numbers. Secondly, Fe(II) produced from microbial Fe(III) oxide reduction can dechlorinate CT directly. It has been reported that the Fe(II) species, such as sorbed Fe(II) (Kim and Picardal 1999) and nanoscale magnetite/-maghemite particles (Mccormick and Adriaens 2004) formed by DIRB in subsurface environments may abiotically donate electrons to react with chlorinated contaminants. In this study, no noticeable increases in the cell numbers were observed because strain L17 cannot gain energy for growth when Fe(III) oxide served as electron acceptor. However, more than 93% and 86% of the HCl-extractable Fe(II) was determined as sorbed Fe(II) in the CT transformation tests in the absence and presence of AQDS, respectively. Therefore, the enhancement of CT transformation was more likely a consequence of abiotic reduction by the sorbed Fe(II) formed from microbial Fe(III) reduction by strain L17.

The mechanism of CT transformation by *K. pneumoniae* L17 in the presence of Fe(III) oxide and AQDS may involve three processes. First, strain L17 can directly dechlorinate CT in anaerobic glucose metabolism. Secondly, strain L17 can reduce Fe(III), and form sorbed Fe(II), which can dechlorinate CT abiotically. Thirdly, AH_2DS, which is formed from AQDS reduction by strain L17, can transfer electron to CT indirectly by abiotic Fe(III) reduction. Iron is cycled between Fe(III) and Fe(II) redox states as a result of being reduced by strain L17 and oxidized by CT. On the other hand, AQDS is cycled with AH_2DS as a result of being reduced by strain L17 and oxidized by Fe(III). Both cycles indicate that Fe(III) oxides and AQDS can serve as a redox mediator for the reduction of CT or other degradable pollutants under anaerobic environments.

In conclusion, a natural sediment isolate, *K. pneumoniae* strain L17 is able to reduce various crystalline Fe(III) oxides and CT. These two reduction processes can occur simultaneously, and be significantly promoted by the presence of AQDS. *K. pneumoniae*, as a member in the *Enterobacteriaceae* family, is a naturally-occurring population of fresh water and soils. Although strain L17 can transfer only a minor portion of the electron equivalents in the fermentable substrates to Fe(III), the fermentation products, such as fatty acids and alcohols (Zeng *et al.* 1996), may be subsequently metabolized by other Fe(III) reducers with Fe(III) reduction in natural subsurface environments (Lovley and Phillips 1989). Moreover, the sorbed Fe(II) from microbial Fe(III) reduction can enhance CT transformation abiotically. As a strain of environmental origin, which can survive under both aerobic and anaerobic conditions, strain L17 may have the potential to be used for bioremediation of chlorinated compound-contaminated environments containing Fe(III) oxides and humic substances. Further research will be conducted to confirm the electron transfer in the CT dechlorination and the interactive mechanism among strain L17, Fe(III) oxide, AQDS and CT.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl Acids Res* **25**, 3389–3402.

Baldi, F., Minacci, A., Pepi, M. and Scozzafava, A. (2001) Gel sequestration of heavy metals by *Klebsiella oxytoca* isolated from iron mat. *FEMS Microbiol Ecol* **36**, 169–174.

Bonneville, S., Cappellen, P.V. and Behrends, T. (2004) Microbial reduction of iron(III) oxyhydroxides-effects of mineral solubility and availability. *Chem Geol* **212**, 255–268.

Caccavo, F., Lonergan, D.J., Lovley, D.R., Davis, M., Stolz, J.F. and McInerney, M.J. (1994) *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. *Appl Environ Microbiol* **60**, 3752–3759.

Chapelle, F.H. (2001) *Ground-Water Microbiology and Geochemistry*. New York: J Wiley.

Criddle, C.S., Dewitt, J.T. and McCarty, P.L. (1990) Reductive dehalogenation of carbon tetrachloride by Escherichia coli K-12. *Appl Environ Microbiol* **56**, 3247–3254.

Dobbin, P.S., Warren, L.H., Cook, N.J., McEwan, A.G., Powell, A.K. and Richardson, D.J. (1996) Dissimilatory iron(III) reduction by *Rhodobacter capsulatus*. *Microbiology* **142**, 765–774.

Ernst, C. and Rehm, H.-J. (1995) Utilization of chlorinated *s*-triazines by a new strain of *Klebsiella pneumoniae*. *Appl Microbiol Biotechnol* **42**, 763–768.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–789.

Fredrickson, J.K. and Gorby, Y.A. (1996) Environmental processes mediated by iron-reducing bacteria. *Curr Opin Biotechnol* **7**, 287–294.

Ghanem, I., Orfi, M. and Shamma, M. (2007) Biodegradation of Chlorpyrifos by *Klebsiella* sp. isolated from an activated sludge sample of waste water treatment plant in Damascus. *Folia Microbiol* **52**, 423–427.

Holliger, C., Wohlfarth, G. and Diekert, G. (1999) Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiol Rev* **22**, 383–398.

Janssen, D.B., Oppentocht, J.E. and Poelarends, G.J. (2001) Microbial dehalogenation. *Curr Opin Biotechnol* **12**, 254–258.

Kim, S. and Picardal, F.W. (1999) Enhanced anaerobic biotransformation of carbon tetrachloride in the presence of reduced iron oxides. *Environ Toxicol Chem* **18**, 2142–2150.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Kwon, G.-S., Sohn, H.-Y., Shin, K.-S., Kim, E. and Seo, B.-I. (2005) Biodegradation of the organochlorine insecticide, endosulfan, and the toxic metabolite, endosulfan sulfate, by *Klebsiella oxytoca* KE-8. *Appl Microbiol Biotechnol* **67**, 845–850.

Li, F.B., Li, X.Z., Li, X.M., Liu, T.X. and Dong, J. (2007a) Heterogeneous photodegradation of bisphenol A with iron oxides and oxalate in aqueous solution. *J Colloid Interface Sci* **311**, 481–490.

Li, W.J., Xu, P., Schumann, P., Zhang, Y.Q., Pukall, R., Xu, L.H., Stackebrandt, E. and Jiang, C.L. (2007b) *Georgenia ruanii* sp. nov., a novel actinobacterium isolated from forest soil in Yunnan (China) and emended description of the genus *Georgenia*. *Int J Syst Evol Microbiol* **57**, 1424–1428.

Liu, C., Kota, S., Zachara, J.M., Fredrickson, J.K. and Brinkman, C.K. (2001) Kinetic analysis of the bacterial reduction of goethite. *Environ Sci Technol* **35**, 2482–249.

Lovley, D.R. (1987) Organic matter mineralization with the reduction of ferric iron: a review. *Geomicrobiol J* **5**, 375–399.

Lovley, D.R. and Phillips, E.J.P. (1989) Requirement for a microbial consortium to completely oxidize glucosein Fe(III)-reducing sediments. *Appl Environ Microbiol* **55**, 3234–3236.

Lovley, D.R., Phillips, E.J.P. and Lonergan, D.J. (1989) Hydrogen and formate oxidation coupled to dissimilatory reduction of iron or manganese by *Alterornonas putrefaciens*. *Appl Environ Microbiol* **55**, 700–706.

Lovley, D.R., Coates, J.D., Blunt-Harris, E.L., Phillips, E.J.P. and Woodward, J.C. (1996) Humic substances as electron acceptors for microbial respiration. *Nature* **382**, 445–448.

Lovley, D.R., Fraga, J.L., Blunt-Harris, E.L., Hayes, L.A., Phillips, E.J.P. and Coates, J.D. (1998) Humic substances as a mediator for microbially catalyzed metal reduction. *Acta Hydrochim Hydrobiol* **26**, 152–157.

Lovley, D.R., Holmes, D.E. and Nevin, K.P. (2004) Dissimilatory Fe(III) and Mn(IV) reduction. *Adv Microb Physiol* **49**, 219–286.

Lovley, D.R. and Phillips, E.J.P. (1988) Novel mode of microbial energy metabolism organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl Environ Microbiol* **54**, 1472–1480.

Mccormick, M.L. and Adriaens, P. (2004) Carbon tetrachloride transformation on the surface of nanoscale biogenic magnetite particles. *Environ Sci Technol* **38**, 1045–1053.

Park, H.S., Kim, B.H., Kim, H.S., Kim, H.S., Kim, G.T., Kim, M., Chang, I.S., Park, Y.K. *et al.* (2001) A novel electrochemically active and Fe(III)-reducing bacterium phylogenetically related to *Clostridium butyricum* isolated from a microbial fuel cell. *Anaerobe* **7**, 297–306.

Petrovskis, E.A., Vogel, T.M. and Adriaens, P. (1994) Effects of electron acceptors and donors on transformation of tetrachloromethane by *Shewanella putrefaciens* MR-1. *FEMS Microbiol Lett* **121**, 357–364.

Picardal, F.W., Arnold, R.G., Couch, H., Little, A.M. and Smith, M.E. (1993) Involvement of cytochromes in the anaerobic biotransformation of tetrachloromethane by *Shewanella putrefaciens* 200. *Appl Environ Microbiol* **59**, 3763–3770.

Picardal, F.W., Arnold, R.G. and Huey, B.B. (1995) Effects of electron donor and acceptor conditions on reductive dehalogenation of tetrachloromethane by *Shewanella putrefaciens* 200. *Appl Environ Microbiol* **61**, 8–12.

Roden, E.E. (2003) Fe(III) oxide reactivity toward biological versus chemical reduction. *Environ Sci Technol* **37**, 1319–1324.

Roden, E.E. and Zachara, J.M. (1996) Microbial reduction of crystalline iron(III) oxides influence of oxide surface area and potential for cell growth. *Environ Sci Technol* **30**, 1618–1628.

Scala, D.J., Hacherl, E.L., Cowan, R., Young, L.Y. and Kosson, D.S. (2006) Characterization of Fe(III)-reducing enrichment cultures and isolation of Fe(III)-reducing bacteria. *Res Microbiol* **157**, 772–783.

Schwertmann, U. and Cornell, R.M. (1991) *Iron oxides in the laboratory: preparation and characterization*. New York: VCH.

Straub, K.L., Benz, M. and Schink, B. (2001) Iron metabolism in anoxic environments at near neutral pH. *FEMS Microbiol Ecol* **34**, 181–186.

Sung, Y., Ritalahti, K.M., Sanford, R.A., Urbance, J.W., Flynn, S.J., Tiedje, J.M. and Loffier, F.E. (2003) Characterization of two tetrachloroethene-reducing, acetate-oxidizing bacteria and their description as *Desulfuromonas michiganensis* sp. nov. *Appl Environ Microbiol* **69**, 2964–2974.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Vangnai, A.S. and Petchkroh, W. (2007) Biodegradation of 4-chloroaniline by bacteria enriched from soil. *FEMS Microbiol Lett* **268**, 209–216.

Vogel, T.M., Criddle, C.S. and McCarty, P.L. (1987) Transformation of halogenated aliphatic compounds. *Environ Sci Technol* **21**, 722–736.

Zeng, A.-P., Menzel, K. and Deckwer, W.-D. (1996) Kinetic, dynamic, and pathway studies of glycerol metabolism by *Klebsiella pneumoniae* in anaerobic continuous culture: II. analysis of metabolic rates and pathways under oscillation and steady-state conditions. *Biotechnol Bioeng* **52**, 561–571.

