Nitrogen fixation by reductively dechlorinating bacteria

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Summary
Significant advances in the ecology, physiology and genetics of reductively dechlorinating bacteria have revealed their important environmental roles in bioremediation and in the global chlorine cycle. N₂ fixation has been widely observed in symbiotic, associative and free-living bacteria. Here we show physiological and molecular evidence that reductively dechlorinating bacteria are capable of fixing atmospheric nitrogen. Furthermore, N₂ fixation in some of these dechlorinating bacteria stimulated reductive dechlorination, which should help predict and regulate the environmental function of dechlorinating bacteria in situ bioremediation of chlorinated pollutants. These results imply that N₂ fixation in dechlorinating bacteria interacts with other biogeochemical cycles to control the nitrogen status of the anaerobic ecosystem.

Introduction
A wide range of man-made and naturally produced chlorinated hydrocarbons has been released into the environment. These compounds include many of the most toxic and environmentally persistent pollutants. Their wide distribution causes a significant risk to public health and the environment. Fortunately, reductively dechlorinating bacteria are capable of transforming chlorinated hydrocarbons through reductive dechlorination in anaerobic environments such as anoxic soils, groundwaters and sediments (Mohn and Tiedje, 1992; Fetzner, 1998), in an energy-generating process termed halorespiration. Bacterial culture, carbon isotope fractionation and molecular biology studies have shown that halorespiring anaerobes are widely distributed in nature, implying their important environmental roles in bioremediation and in the global chlorine cycle (Drenzek et al., 2001; Smidt and de Vos, 2004; Watts et al., 2005). Some reductively dechlorinating bacteria also conduct alternative physiological activities such as nitrate and sulfate reduction, depending on environmental conditions. In addition, reductive dechlorination may be compatible with other important physiological features such as nitrogen fixation through long-term evolutionary adaptation.

N₂ fixation is an energy-consuming process and has been widely observed in symbiotic, associative and free-living bacteria. This bacterial physiological feature plays a tremendous role in ecological sustenance, world agriculture and global nitrogen cycle. Nitrogen availability can limit microbial growth and affect ecosystem activity (Dixon and Kahn, 2004). Although N₂ fixation is widely distributed among Bacteria and Archaea (Raymond et al., 2004), this significant physiological feature has not been revealed in reductively dechlorinating bacteria. Results from sequencing of three whole genomes of the halorespiring bacteria Desulfobacterium hafniense and Dehalococcoides indicated that nitrogen fixation-related genes are found in D. hafniense strain Y51 (Nonaka et al., 2006) and Dehalococcoides strain 195 (Seshadri et al., 2005), but are missing in Dehalococcoides strain CBDB1 (Kube et al., 2005). In this research we provide experimental evidence that reductively dechlorinating bacteria are capable of fixing atmospheric nitrogen.

Results and discussion

nifH cloning and phylogenetic analysis

We detected N₂ fixation capability in halorespiring anaerobes Desulfomonile tiedjei (Shelton and Tiedje, 1984), Sulphurospirillum multivorans (Scholz-Muramatsu et al., 1995), Desulfovibrio dechloracetivorans (Sun et al., 2000) and Desulfitobacterium dehalogenans (Utkin et al., 1994), which were isolated from distinct habitats and represent Gram-negative and Gram-positive bacteria. Partial nifH fragments (approximate 390 bp) were amplified using degenerate primers (Mehta et al., 2003) corresponding to the conserved regions of nifH. All these dechlorinating
bacteria possessed nifH homologues. In *D. dechloracetivorans*, two divergent nifH homologues (*nifH-1* and *nifH-2*) were found. *nifH-2* grouped within cluster I of the NifH phylogenetic tree (Fig. 1), which includes α-, β- and γ-proteobacterial nitrogenases; *nifH-1* fell into cluster III, which includes diverse anaerobic bacteria like sulfate reducers and reductive dechlorinators.

Single *nifH* homologues were found in three other bacteria and grouped into different clusters: *S. multivorans* into cluster I, *D. tiedjei* into cluster III and *D. dehalogenans* into cluster IV respectively. The distribution of *nifH* homologues in these dechlorinating bacteria coincides with conventional opinion that *nifH* was phylogenetically diverse in nature (Ueda *et al*., 1995; Zehr *et al*., 1998;
suggesting that lateral transfer of \textit{nifH} genes may have occurred in diverse environments. Previous studies have shown that some symbiotic and free-living bacteria harbour multiple \textit{nifH} homologues (Lilburn \textit{et al}., 2001; Choo \textit{et al}., 2003), but how these \textit{nifH} homologues respond to environmental cues and are expressed remains unknown. The divergence of \textit{nifH} genes in these bacteria may proffer them ecological advantages in natural environments.

\textbf{N}_2-dependent growth and acetylene reduction activity

The nitrogenase activity in \textit{D. dechloracetivorans} and \textit{S. multivorans} was demonstrated by their \textit{N}_2-dependent growth and their \textit{NH}_4^+-repressible acetylene reduction activity (Fig. 2). Dechlorination products such as phenol and benzoate were analysed by high-performance liquid chromatography (Agilent 1100 Series) with reverse C\textsubscript{18} column (Phenomenex). Dichloroethene (DCE) was monitored by gas chromatography (Agilent 6890 N) equipped with a flame ionization detector with a DB-1 column (30 m \times 250 \mu m \times 1 \mu m). For acetylene reduction assay, 6 ml of acetylene was injected into headspace of each bottle when bacterial growth entered exponential phase. Ethylene was analysed by gas chromatography (Agilent 6890 N) equipped with a HP-PLOT/AL203 'S' Deactivated column (50 m \times 530 \mu m \times 15 \mu m). Ethylene production rate was determined from the slope of the linear regression of time versus ethylene concentration using software Origin version 7.5.

\textit{N}_2 fixation in \textit{D. tiedjei} was evidenced by bacterial growth and reductive dechlorination when \textit{N}_2 was the sole nitrogen source, although acetylene reduction activity was not observed in this organism. No growth or reductive.

![Figure 2. Growth optical density (OD\textsubscript{600}) or reductive dechlorinating product concentration and rate of acetylene reduction to ethylene.](image-url)

A. Growth of \textit{D. dechloracetivorans} under sulfate reduction conditions (32 mM sulfate + 10 mM lactate).
B. Reductive dechlorination of 2-chlorophenol (2-CP) to phenol with pyruvate (10 mM) as electron donor by \textit{D. dechloracetivorans}.
C. Fumarate respiration by \textit{S. multivorans} (10 mM fumarate + 10 mM pyruvate).
D. Reductive dechlorination of tetrachloroethene (PCE) to DCE with pyruvate (10 mM) as electron donor by \textit{S. multivorans}. Addition of 5.6 mM \textit{NH}_4\textsubscript{Cl} into culture resulted in complete suppression of acetylene reduction (data not shown). The error bars indicate the standard errors of the measurements. Symbols: \text{\Delta}, nitorgenase activity determined by the acetylene reduction with \textit{N}_2 as the sole nitrogen source; \text{\bullet}, addition of ammonia as nitrogen source; \text{\triangledown}, bacterial growth with \textit{N}_2 as the sole nitrogen source; \text{\blacktriangle}, addition of \textit{Ar} instead of \textit{N}_2 as negative control.
dechlorination was observed in *D. dehalogenans* under any growth conditions when ammonia was omitted, indicating that *nifH* is not functional in N₂ fixation in the bacterium. This result is consistent with the previous observation that diverse *nifH* homologues in cluster IV are not involved in N₂ fixation (Raymond et al., 2004; Mehta et al., 2005).

It is interesting to note that N₂ fixation gave rise to higher reductive dechlorination rates in *D. dechloracetivorans* and *D. tiedjei*, which were sustained over repeated feedings of chlorinated compounds as terminal electron acceptors. When N₂ was provided as the sole nitrogen source and pyruvate was used as electron donor in *D. dechloracetivorans*, dechlorination rate (4.24 μmol l⁻¹ h⁻¹) was the same as with ammonia (Fig. 2B). On the other hand, when N₂ was provided as the sole nitrogen source and acetate was used as electron donor, reductive dechlorination rate (3.94 μmol l⁻¹ h⁻¹) was higher than that (3.26 μmol l⁻¹ h⁻¹) of ammonia (Fig. 3A); under both growth conditions, biomass production was about 3 g of cells (dry weight) per mol of 2-chlorophenol dechlorinated. This suggests that acetate oxidation provides a more efficient and balanced carbon and nitrogen flux, and/or ammonia assimilation affects reductive dechlorination. Although we do not have convincing data to explain this phenomenon, it has significant implications in *in situ* bioremediation. In some cases, nitrogen input is necessary in bioremediation of haloorganics-contaminated sites, where nitrogen source is limited. Nitrogen input may lead to the dominance of bacterial populations other than dechlorinators, thereby decreasing their environmental function. In addition, excess nitrogen input may cause nitrate contamination in these sites. This finding not only may provide clues of how reductively dechlorinating bacteria take advantage of their diverse physiological features in natural environment, but also help us optimize nitrogen input and regulate the environmental function of dechlorinating bacteria in contaminated sites.

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction was executed to further detect the capability of N₂ fixation in dechlorinating bacteria. Anticipated RT-PCR products were amplified from *D. tiedjei* and *S. multivorans* cultures (Fig. 4C and D), further demonstrating the *nifH* expression and N₂ fixation in these bacteria. As predicted, no RT-PCR product was observed from *D. dehalogenans* cultures (data not shown). Interestingly, in *D. dechloracetivorans* an expression discrepancy of *nifH-1* and *nifH-2* was observed (Fig. 4A and B). Only *nifH-1* was expressed under sulfate reduction conditions; both *nifH-1* and *nifH-2* were expressed under pyruvate oxidation.

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**Fig. 3.** Reductive dechlorination and growth under various culture conditions.  
A. Reductive dechlorination of 2-CP to phenol with acetate (2.5 mM) as electron donor by *D. dechloracetivorans*.  
B. Reductive dechlorination of 3-chlorobenzoate (3-CB) to benzoate with lactate (10 mM) as electron donor by *D. tiedjei*. The error bars indicate the standard errors of the measurements. Symbols: ◆, addition of ammonia as nitrogen source; ▼, N₂ as the sole nitrogen source; ▲, addition of Ar instead of N₂ as negative control.
fermentation and reductive dechlorination conditions. Further elucidation of the nifH expression pattern in this organism should aid in our understanding of genetic regulation of N2 fixation in reductively dechlorinating bacteria.

The diversity and global distribution of reductively dechlorinating bacteria have been revealed in recent years, suggesting the significant role they play in the biogeochemical transformations of elements and ecosystem sustenance (Smidt and de Vos, 2004). This study reveals a heretofore unrecognized role for reductively dechlorinating bacteria in the anoxic ecosystem and in the global nitrogen cycle. Our results may also lead to a better understanding of the dynamics, the specific activities and the conditions required for environmental function of dechlorinating populations in in situ bioremediation.

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References


