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Evidence for Nitrogen Fixation by "*Dehalococcoides ethenogenes*" Strain 195▽

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Genome annotation of the chlorinated ethene-respiring "*Dehalococcoides ethenogenes*" strain 195 indicated the presence of a complete nitrogenase operon. Here, results from long-term growth experiments, gene expression, and $^{15}N_2$-isotope measurements confirm that strain 195 is capable of fixing atmospheric dinitrogen when a defined fixed-nitrogen source such as ammonium is unavailable.

"*Dehalococcoides ethenogenes*" strain 195 is the first isolated bacterium that is capable of reductively dechlorinating tetrachloroethene and trichloroethene (TCE) to vinyl chloride (VC) and ethene (22). Annotation of the 1.5-Mbp genome of strain 195 has identified 17 intact reductive dehalogenase (RDase) genes (25). The variety of RDases has essentially defined the metabolic capabilities of strain 195 and other *Dehalococcoides* strains for respiration of chlorinated ethenes (8, 9, 15, 23, 27) and other chlorinated compounds (1, 2, 6, 21), making them important participants in bioremediation processes (19). Expression of different putative RDase genes has been examined previously in pure culture (6) and in *Dehalococcoides*-containing enrichment cultures (3, 4, 13, 17, 24, 28).

Genome annotation of strain 195 has revealed the presence of a nitrogenase-encoding operon (*nif*) (DET1151-58) typical of those found in anaerobes (25). According to the published genome annotations of four strains of *Dehalococcoides*, strain 195 is the only one that contains a *nif* operon (16, 25; Joint Genome Institute, 2009, Integrated Microbial Genomes system [www.jgi.doe.gov]). A *nif* operon closely related to that in strain 195 has also been identified in a mixed *Dehalococcoides*-containing community (29); thus, the nitrogen-fixing function might be present in other unsequenced strains of *Dehalococcoides*.

Phylogenetically, the nitrogenase structural genes of strain 195 are clustered with diverse anaerobic *Bacteria*, including the molybdenum (Mo)-nitrogenase in *Clostridium pasteurianum*, as well as *Archaea*, including the Mo-nitrogenase in *Methanosarcina barkeri* (25, 30). In the genome of strain 195, the presence of an ABC transporter for molybdenum (DET1159-61) and a *nifV* gene (DET1614), which encodes homocitrate synthetase used in nitrogenase FeMo-cofactor biosynthesis, suggests that the nitrogenase is of the typical molybdenum-iron type (25). While strain 195 is the only sequenced *Dehalococcoides* isolate that contains a *nif* operon, Ju et al. (14) previously identified functional *nifH* genes in dechlorinating organisms from diverse genera such as *Sulfurospirillum multivorans*, *Desulfovibrio dechloracetivorans*, and *Desulfomonile tiedjei*.

Aquifers containing groundwater contaminated with chlorinated ethenes can potentially be limited in nutrients. For example, at the Wurtsmith Air Force Base, the chlorinated ethene-contaminated groundwater was found to contain less than 0.09 mM of ammonia, prompting ammonium amendment (26). Little is currently known about the potential effects of nitrogen limitation on reductive dechlorination in the environment, and the demonstration of nitrogen fixation in strain 195 was previously hindered by the use of an undefined medium (21). Here, we present results demonstrating that strain 195 is capable of fixing atmospheric dinitrogen and the physiological implications of the stress caused by nitrogen limitation.

Establishment of nitrogen-fixing *D. ethenogenes* strain 195. To induce nitrogen fixation, strain 195 was grown in 100 ml liquid medium as described by He et al. (7), except with the following modifications: (i) NH_4Cl and TES buffer [*N*-Tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid] were omitted, (ii) 0.2-μm-pore-filtered ultra-high-purity nitrogen gas was overpressurized into the bottle headspace, creating an $H_2/CO_2/N_2$ ratio of 44:11:45 under 12 lb/in^2 gauge pressure, and (iii) anoxic $Na_2MoO_4 \cdot 2H_2O$ was added to increase the Mo concentration to 2.7 μM. After inoculation with 2 ml of cells that had previously been grown in ammonium-containing medium (5.6 mM), the first subcultures took ~12 weeks to completely reduce the 40 μmol of TCE that was provided, coupling it with growth. Most of the incubation represented a lag period prior to growth. Subsequent subcultures (0.5% [vol/vol]) took 4 to 5 weeks for growth, indicating that cells had adapted to fixed-nitrogen limitation. Cultures of nitrogen-fixing strain 195 have now been growing in our laboratory for more than 3.5 years.

The purity of the nitrogen-fixing strain 195 cultures was assessed by microscopy and by sequencing the PCR products amplified with universal bacterial and archaeal primers (5, 10). In all cases, the archaeal primers returned no 16S rRNA band and the PCR products resulting from the bacterial primers generated sequences identical to the 16S rRNA gene of strain 195, demonstrating the purity of the culture.

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$^{15}N_2$ incorporation. The classic acetylene reduction assay (20) was not an applicable method to demonstrate nitrogen fixation in this study, as the diagnostic product ethene is a by-product of the respiratory activity of strain 195. Instead, the incorporation of labeled $^{15}N_2$ into biomass was measured (n = 3) using a GVI IsoPrime mass spectrometer along with a Eurovector elemental analyzer (EuroEA3028-HT). After completely reducing 40 μmol of TCE, the N-isotope ratio of the biomass that was incubated with a 16.4 ± 0.70 atom% ^{15}N headspace without ammonium amendment was 5.1 ± 0.69 atom% ^{15}N, compared to 0.36 ± 0.0001 atom% ^{15}N, close to natural abundance, for the biomass of control cultures grown with ammonium (5.6 mM). The biomass N-isotope ratio could be further enriched to 12.5 ± 0.67 atom% ^{15}N when reducing agent cysteine (0.2 mM) was eliminated from the growth medium. A mass balance of the ^{15}N in the headspace, medium, and biomass indicates that fixed atmospheric dinitrogen contributed approximately 29% and 75% of the nitrogen in the biomass pool in the presence and absence of cysteine, respectively. The significant difference in ^{15}N enrichment in the biomass compared with ammonium controls confirms that strain 195 can grow by fixing atmospheric dinitrogen and the extent of nitrogen fixation is governed by the availability of fixed-nitrogen sources including cysteine.

Growth and dechlorination activity while fixing nitrogen. After achieving stable growth of nitrogen-fixing strain 195 cultures, experiments were carried out to characterize the effects of nitrogen fixation on growth and activity. Figure 1A shows the growth and dechlorination profile of strain 195 with and without 5.6 mM ammonium amendment. For the cells challenged to fix nitrogen, two distinct growth phases were observed, an exponential phase (days 0 to 11) followed by a long stationary phase (days 11 to 25). Transition into stationary phase after day 11 when less than 20% of the TCE had been reduced to VC was accompanied by continued dechlorination, exhibiting decoupling between growth and TCE-dechlorinating activity and generating limited energy for cell maintenance. These growth and dechlorination characteristics were consistently observed in repetitions of this experiment. In contrast, when ammonium was present as a fixed-nitrogen source, exponential growth continued until almost all of the TCE had been dechlorinated to VC on day 11.

The cell density normalized rate of VC production and cell yield during exponential phase were higher in the presence of ammonium. The VC production rates during exponential phase in the presence and absence of ammonium were 10.0 ± 1.2 μmol VC/day/10^{10} cells and 6.7 ± 1.2 μmol VC/day/10^{10} cells, respectively, and yields were (4.7 ± 0.5) × 10^8 cells/μmol Cl^- and (2.8 ± 0.8) × 10^8 cells/μmol Cl^-. Furthermore, a sixfold difference in final cell density between the ammonium-free culture [(4.6 ± 1.0) × 10^7 copies of gene/ml] and the ammonium-amended culture [(2.8 ± 0.6) × 10^8 copies of gene/ml] was measured.

Expression of *nifD*, *tceA*, and 16S rRNA genes. In order to evaluate the regulatory impact of fixed-nitrogen limitation on strain 195, expression of the *nifD* gene as well as the 16S rRNA and *tceA* genes was measured in the presence and absence of ammonium amendment by multiplex reverse transcription-quantitative PCR (RT-qPCR) as previously described (12). Total RNA was isolated from frozen cell pellets using the RNeasy mini kit (Qiagen, CA). RNA standards were synthesized previously (12, 18) or according to previously outlined methods (18). The *nifD* gene (DET1155) encodes the nitrogenase molybdenum-iron protein α-chain and the primer and probe sequences are as follows: forward primer, 5′-AACTGGCCC AGGCTATAGAAGA; reverse primer, 5′-CCCACCGGACAGG TAGCAT; and probe 5′-6-carboxyfluorescein-ACCAGCTTTTCA AGCCCAAGACCATAGTTGT-6-carboxytetramethylrhodamine.

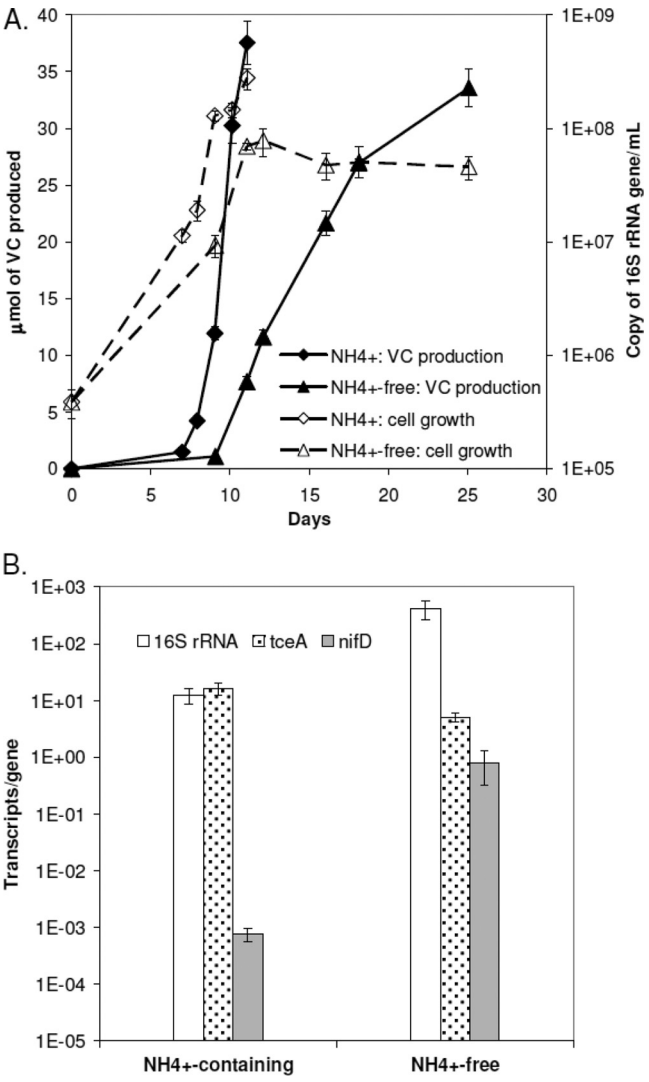

FIG. 1. (A) Dechlorination activity plotted as VC production and cell growth for strain 195 in medium with and without ammonium amendment. Cell growth data are averages of triplicate RT-qPCR analyses from each biological duplicate, and error bars represent 1 standard deviation. VC measurements are averages of biological duplicates, and error bars represent 5% uncertainty. The RT-qPCR method for 16S rRNA gene measurement has previously been described (11), and the chlorinated ethene concentrations were determined by gas chromatography-flame ionization detection (17). (B) Late-exponential-phase 16S rRNA, *tceA*, and *nifD* gene expression for cells growing with (day 10) and without (day 11) ammonium amendment. The results are averages of triplicate RT-qPCRs from each biological duplicate, and error bars represent 1 standard deviation.

Other primers and probe sequences have been published previously (11, 12).

Comparison of transcript quantities per gene during late exponential phase for the cultures shown in Fig. 1A shows that the *nifD* gene was upregulated 1,000-fold when ammonium was not provided (Fig. 1B), indicating that expression of the *nif* operon increases in response to fixed-nitrogen limitation and suggesting that cells were fixing nitrogen prior to stationary phase. In contrast, expression of the *tceA* gene was similar between the two conditions, while expression of 16S rRNA per gene copy was higher for cells that were fixing nitrogen, suggesting a high level of metabolic activity for these cells prior to stationary phase.

Effect of ammonium on cell growth, activity, and expression. Following observations that ammonium deprivation consistently caused reduced growth and activity, a study was carried out to determine the cellular responses of adding ammonium to cultures that were actively growing while fixing nitrogen (Fig. 2). As shown in Fig. 2, ammonium addition after nitrogen-fixing cells had just entered stationary phase (day 15) had an immediate stimulatory effect on both VC production activity (Fig. 2A) and cell growth (Fig. 2B). For VC production, rather than requiring 15 days to reduce the remainder of the TCE to VC after transitioning into stationary phase, only 5 days were required after ammonium was spiked into the nitrogen-fixing cultures (Fig. 2A). An equivalent experiment with ammonium addition on day 18, after cells were further into stationary phase, showed similar activity with increases in both VC production and cell growth.

The increase in dechlorination activity was accompanied by a return to exponential cellular growth (Fig. 2B), with cell densities increasing fivefold from $(2.3 \pm 0.4) \times 10^7$ copies of gene/ml to $(1.2 \pm 0.2) \times 10^8$ copies of gene/ml within 5 days of ammonium amendment. An equivalent cellular increase to $(1.4 \pm 0.07) \times 10^8$ copies of gene/ml was observed for the day 18 amendment. The yield of $(1.1 \pm 0.2) \times 10^8$ cells/μmol Cl^- after ammonium addition on day 15 was slightly lower than the yield of $(1.6 \pm 0.3) \times 10^8$ cells/μmol Cl^- during the first exponential phase, but nevertheless, demonstrated a return to active growth. As a comparison, in the culture that was not amended with ammonium, cell density changed by a factor of only 1.3 after day 15. The increases in growth after ammonium amendment are likely the result of cells diverting energy and electrons previously used for nitrogen fixation to other metabolic processes.

Ammonium amendment also resulted in changes in *nifD* gene expression (Fig. 3A). In the ammonium-free cultures, cells expressed *nifD* at quantities that were 3 orders of magnitude greater than cultures amended with ammonium, and *nifD* expression was relatively constant throughout the stationary phase. In contrast, cells that were grown initially ammonium free and amended with ammonium on day 15 exhibited a marked decrease in *nifD* expression in response to the amendment. A similar expression profile was exhibited by the day 18-amended cells. In the 24 h following the ammonium amendment, the *nifD* expression decreased by factors of 6 and 8 in the two ammonium-free cultures, with eventual downregulation reaching 2 orders of magnitude, suggesting that the *nif* operon is regulated according to the availability of ammonium as a fixed-nitrogen source. Conversely, the *tceA* and 16S rRNA genes were expressed at similar levels regardless of whether cells were provided with ammonium as a fixed-nitrogen source, with transcript number per gene declining as cells transitioned from exponential into stationary phase (Fig. 3B). Overall, no order-of-magnitude changes in gene expression were measured for *tceA* and the 16S rRNA genes.

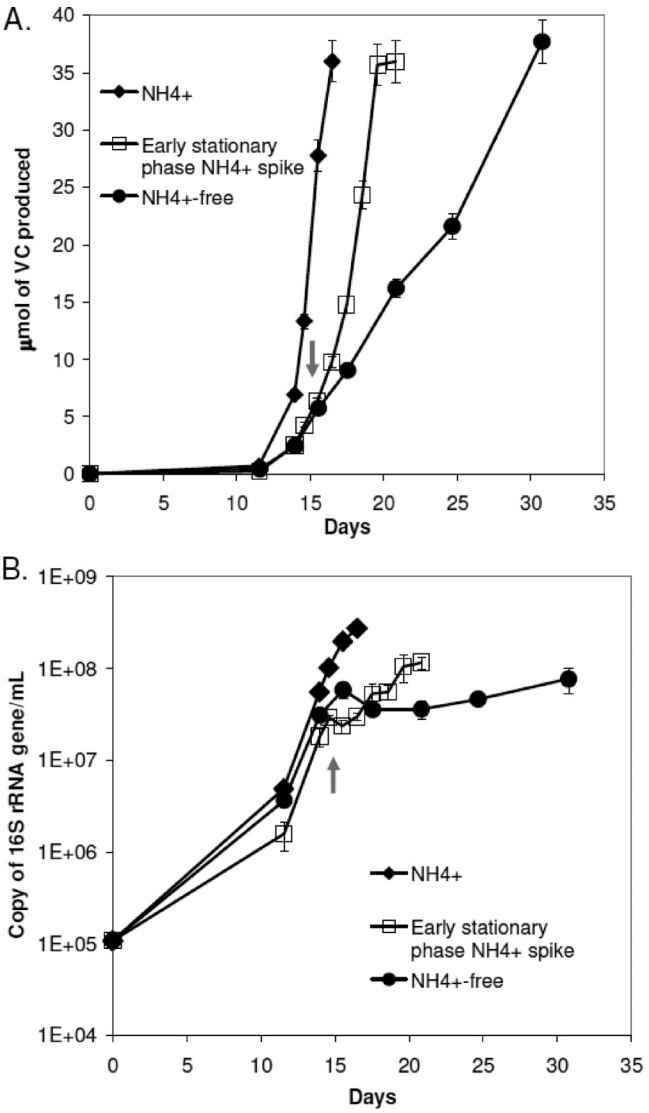

FIG. 2. Effects of ammonium addition on dechlorination activity (A) and cell growth (B) in ammonium-free cultures. The arrow indicates the time (day 15) when ammonium was added to the ammonium-free cultures.

Implications. The subsurface matrix at many contaminated aquifer sites is oligotrophic with low levels of carbon and nutrients. Here we have shown that while *D. ethenogenes* strain 195 can fix nitrogen to sustain growth, this activity is accompanied by decreased cell density and dechlorination activity that may adversely affect the performance of bioremediation processes. Hence, the concentrations of fixed nitrogen and expression of genes related to nitrogen metab-

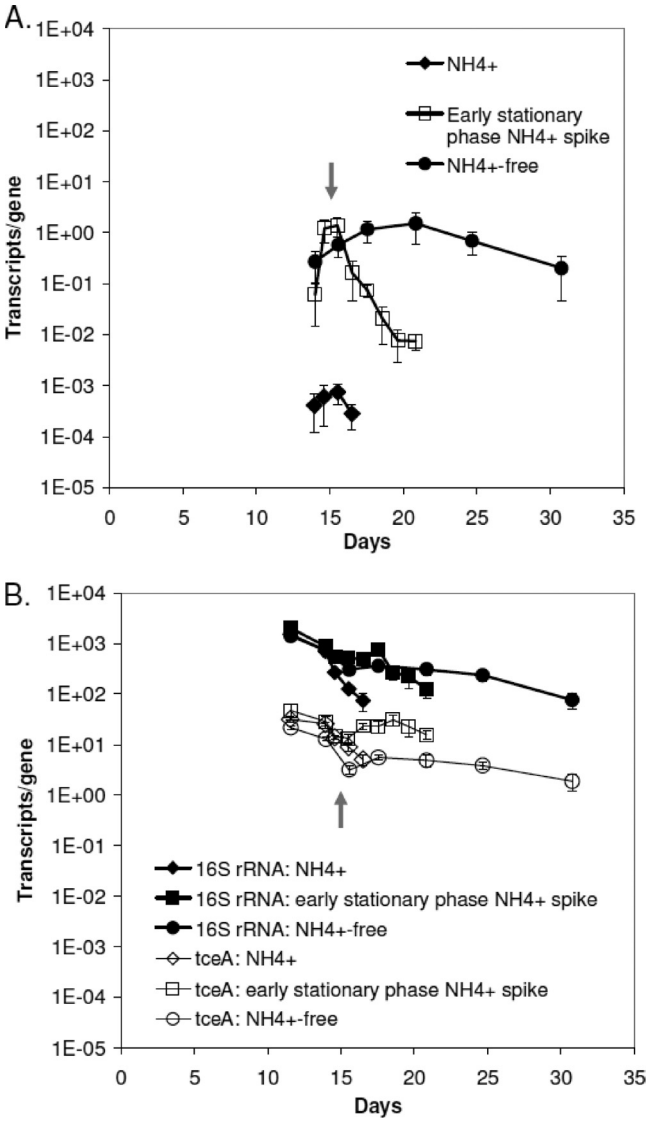

FIG. 3. *nifD* expression (A) and 16S rRNA gene and *tceA* expression (B) when cells were grown with or without ammonium amendment and the response to abrupt ammonium addition. The arrow indicates when the nitrogen-fixing cultures were amended with ammonium (day 15). No time zero point sample was collected for any transcription analysis, and *nifD* transcripts were unquantifiable at day 11.5 in all cultures. The analytical detection limit of *nifD* transcripts is 10^{-4} transcripts/gene as determined using the described RT-qPCR method and serial dilution of the quantified RNA standards.

olism could serve as useful biomarkers for effective bioremediation.

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