Culture-Dependent and Independent Identification of Denitrifying Bacteria in Areas 1 and 2 at the FRC

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An important strategy for bioremediation at the NABIR Field Research Center (FRC) consists of supplying an organic electron donor in situ to stimulate subsurface microorganisms responsible for nitrate reduction and uranium immobilization. The goals of this study were to characterize the microbial community structure of sediments from Areas 1 and 2 and to identify microorganisms whose growth may be stimulated during bioremediation with ethanol-amended groundwater. Push-pull tests were done in wells in Area 1 with ethanol-amended and unamended FW021 site groundwater (>130 mM nitrate) and in Area 2 with ethanol-amended and unamended GW835 site groundwater (<1mM nitrate). Community DNA was extracted from sediment adjacent to three Area 1 wells (two ethanol-stimulated and one control) and three Area 2 wells (two ethanol-stimulated and one control). Bacterial diversity and composition of samples were analyzed through cloning and sequencing of the 16S rRNA gene, and the denitrifying community composition of these samples was analyzed through phylogenetic analysis of cloned nitrite reductase genes, *nir*K and *nir*S. In Area 2, 16S rRNA clone libraries consisted of sequences related to the sulfate-reducing δ-Proteobacteria genus *Desulfovibrio*; and in Area 1, clones related to β-Proteobacteria genera *Alcaligenes* and *Burkholderia* were prevalent. Sequence analysis of *nir*K and *nir*S (nitrite reductase) clone libraries revealed a higher diversity for *nir*S than *nir*K. In Area 1 samples, one clone sequence (OTU 1K) dominated both ethanol-stimulated *nir*K clone libraries (70% of total clone sequences) but only made up a small portion (10%) of the nonstimulated *nir*K clone library. The sequence of OTU 1K is only 82% similar to the *nir*K gene sequence from *Alcaligenes xylosoxidans* and does not cluster with any previously reported *nir*K sequences. In an attempt to cultivate a representative of the dominant nitrate-reducer in Area 1, enrichment cultures were set up using Area 1 sediments, 100 mM nitrate, and 100 mM ethanol. Pure cultures were obtained by streaking serial dilutions of active enrichment cultures onto plates containing nitrate and ethanol. All isolates screened have the *nir*K gene, and sequences of these genes indicate that all isolates belong to OTU 1K from *nir*K clone libraries. Sequence analysis of the 16S rRNA genes revealed that all isolates are 97% similar to nitrate-reducing β-Proteobacterium *Alcaligenes defragrans*. Overall, both culture-independent and culture-dependent techniques suggest that a novel *Alcaligenes* species is important for in situ nitrate removal with ethanol in areas of the FRC with high nitrate concentrations.