Insights into environmental microbial denitrification from integrated metagenomic, cultivation and genomic analyses

INTRODUCTION

The advent of next generation sequencing platforms and the subsequent increased availability of genomic and metagenomic sequence data has revolutionized environmental microbiology. However, though our eyes have been opened to the vast genotypic and metabolic potential of microbial communities in nature, exploration of the role of specific microbial groups in ecosystem function still requires the application of cultivation-based approaches. In fact, the verification of microbial phenotypes through cultivation is arguably more critical than ever as metagenomic information now allows for the generation of boundless hypotheses based on the metabolic potential represented by complex microbial communities. Although the advances in cultivation-independent molecular analyses of microbial communities have been well advertised (e.g. high-throughput amplicon sequencing (e.g. Caporaso et al., 2011), metagenomics (e.g. Tringe et al., 2005), and metatranscriptomics (e.g. Poretsky et al., 2009), parallel advances in cultivation have also been made, including the use of lower organic carbon media, extended incubation, single-cell encapsulation approaches, and overall improved mimicking of natural conditions within a culture vessel (e.g. Bollmann et al., 2007; Kaeberlein et al., 2002; Zengler et al., 2002). Here, data from metagenomic sequencing and isolation, physiological testing and whole genome sequencing of denitrifying bacteria from the highly contaminated subsurface of the Oak Ridge Integrated Field Research Challenge (ORIFRC) site are considered, and the implications of this analysis on understanding the environmental distribution and ecological niche of denitrifying bacteria.

The ORIFRC Site

The ORIFRC site is highly contaminated with spent uranium and a wide variety of other contaminants (e.g. other radionuclides, heavy metals and volatile organic contaminants) as a result of long-term uranium enrichment for nuclear weapons, coupled with improper disposal in unlined ponds (S-3 ponds) (Brooks, 2001; Kostka and Green, 2011; NABIR, 2003; Watson et al., 2004). Although the ponds have been subsequently drained, much of the contaminant has migrated into the subsurface, where it serves to feed a plume migrating down-gradient across the site (Watson et al., 2005). Uranium is the priority contaminant of concern, though the nitrate in the near source zone (adjacent to the former S-3 ponds) reaches extraordinarily high concentrations (in the range of 10-1000 mM) due to the use of nitric acid in the processing of uranium. The high level of nitrate complicates remediation strategies at the site by inhibiting microbial reduction of soluble hexavalent uranium to an insoluble mineral form of tetravalent uranium (e.g. Finneran et al., 2002; Kostka and Green, 2011; Shelobolina et al., 2003). The moderately high acidity in the source zone (pH 3-4) also suppresses microbial activity and diversity (Fields et al., 2005; Hemme et al., 2010). Despite the restrictive conditions, there is evidence for significant nitrous oxide production in the near-source zone (Spalding and Watson, 2008). As the low pH is ameliorated down-gradient of the source zone, both nitrate, nitrous oxide and soluble uranium are attenuated without active remediation, due to both microbial and geochemical processes (Kowalsky et al., 2011).

The contaminant levels in the near source zone are alarming, and source-zone remediation strategies have been examined, with limited success (Wu et al., 2007). The extraordinary levels of nitrate must be removed before microbial reduction of U(VI) to U(IV) can proceed (Akob et al., 2008; Luo et al., 2005; Wu et al., 2010; Wu et al., 2006), and down-gradient remediation has been more effective as nitrate is essentially absent (e.g. Gihring et al., 2011). The presence of nitrous oxide in the source zone wells suggested the presence of in situ denitrification, and thus grew an interest in microorganisms capable of nitrate reduction at in situ pH, with the hope that stimulation of these native organisms could aid in the long-term removal of uranium from the site groundwater. Initial studies revealed significant diversity in nitrite reductase genes in groundwater at the site, including both genes encoding for copper-containing (nirK) and cytochrome (nirS) forms (Palumbo et al., 2004; Yan et al., 2003). Based on metagenomic analysis of acidic groundwater from the site, Hemme et al. (Hemme et al., 2010)hypothesized that denitrification comprised the predominant form of metabolism in the near-source zone microbial community due to the low oxygen and lack of fermentation genes observed there. The overabundance of nitrate/nitrate antiporters in the metagenome was interpreted as a further indication of the strong effect of the elevated nitrate on the source zone microbial community.

Prior to the metagenome sequencing of the acidic groundwater at the ORIFRC site, cultivation-independent molecular surveys had been performed to track denitrifying organisms. As the denitrification phenotype is a polyphyletic trait, and can be acquired readily via lateral gene transfer, ribosomal RNA gene sequencing is not suitable for identifying and

tracking denitrifying organisms. Functional genes assays –targeting nitrate, nitrite, nitric oxide, and nitrous oxide reductases – have been performed for this purpose. Yan et al. (Yan et al., 2003) and Palumbo et al. (Palumbo et al., 2004) performed site-wide surveys of nitrite reductase genes at the ORIFRC site. No clear pattern relating the composition and relative abundance of nitrite reductase genes with groundwater geochemical conditions was observed, however. For example, a principal components analysis of clusters of nirK (gene encoding for copper-containing nitrite reductase) sequences grouped all wells across the pH gradient together, with the exception of one high nitrate groundwater sample. In all wells, the most abundant nirK sequences were most similar to the nirK gene sequence derived from Hyphomicrobium zavarzinii, and all sequences were most similar to gene sequences derived from Proteobacteria. Thus, although a substantial diversity of nitrite reductase genes was observed, with many novel gene sequences recovered, more recent data from genome and metagenome sequencing indicates that the predominant denitrifiers were not detected in single gene surveys (Green et al., 2010; Green et al., 2012; Hemme et al., 2010).

Combined cultivation and direct molecular studies of denitrifying bacteria

The study of denitrifying microorganisms at the ORIFRC field site was approached with a multi-pronged approach, including (a) site-wide microbial community characterization using DNA extraction from sediment and groundwater, coupled with high-throughput bacterial ribosomal RNA (rRNA) gene amplicon sequencing, (b) quantitative PCR (qPCR) analyses of bacterial small subunit (SSU) rRNA and nitrite reductase (nirK) gene abundance in groundwater and sediment samples, (c) cultivation and physiological testing of denitrifying bacteria from sediment and groundwater, and (d) de novo whole genome sequencing of denitrifying isolates. Subsequently, gDNA samples from the site were re-analyzed with novel primers targeting unique nirK genes, and whole genome sequences were also recovered from non-denitrifying reference strains related to organisms isolated from the field site.

Bacteria from six distinct genera of denitrifiers were isolated, including strains of Hyphomicrobium (Alphaproteobacteria), Afipia (Alphaproteobacterium), Pseudomonas (Gammaproteobacteria), Rhodanobacter (Gammaproteobacteria), Bacillus (Firmicutes), and Intrasporangium (Actinobacteria) (Green et al., 2010). Under laboratory conditions, all strains were capable of growth with nitrate as the sole electron acceptor, though the Gram-positive strains produced only nitrous oxide as a terminal product, while Rhodanobacter produced a mixture of nitrous oxide and nitrogen gas. Physiological and genetic characterization of the isolates from the genus Rhodanobacter was prioritized, as these organisms had been detected in great abundance in acidic groundwater as well as sediments from the near-source zone (Green et al., 2010; Green et al., 2012). Bacteria from this genus were revealed to have extraordinarily high relative abundance in the near-source zone, over multiple sampling seasons, and were sometimes the only active organisms detected in RNA-based analyses of groundwater samples (Green et al., 2012). Highly similar strains were independently isolated from ORIFRC site sediment using a diffusion chamber approach (Bollmann et al., 2010), and in a metagenomic survey of acidic groundwater from the site, one of the dominant organisms detected (so-called FW106 γI) is clearly a member of the genus Rhodanobacter (Hemme et al., 2010). This organism contained a full denitrification pathway.

Despite the apparent numerical abundance of members of the genus Rhodanobacter in the acidic source zone, these organisms were not detected in prior molecular surveys of denitrification pathway genes at the ORIFRC site (Palumbo et al., 2004; Yan et al., 2003). Nor could PCR amplification of nirS (cytochrome cd 1-containing nitrite reductase), nirK or nosZ (nitrous oxide reductase) genes be achieved using standard primer sets (Green et al., 2010). Similar challenges were presented by the other isolated strains, excepting Afipia. For the Hyphomicrobium strain, a novel primer set targeting nirK was designed based on a reference gene available in GenBank, but no similar reference sequences were available for the other strains. Subsequently, metagenome sequence data from acidic groundwater acquired at the site (Hemme et al., 2010)was surveyed, and two novel nirK sequences were identified. Using these de novo assembled sequences, primer sets were developed that allowed the amplification of a nirK gene from the Rhodanobacter isolates and from putative Rhodanbacter organisms from environmental genomic DNA (Green et al., 2010; Green et al., 2012).

Quantitative PCR analysis was utilized to quantitate SSU rRNA and nirK gene abundance in groundwater from across the watershed, and this analysis revealed that nirK genes were present in abundance across the ORIFRC site, including nirK genes derived from Rhodanobacter (Green et al., 2012). Coupled with relative abundance measurements derived from qPCR of rRNA genes and from rRNA gene amplicon sequencing, this analysis revealed that Rhodanobacter were the most abundant organisms in the near source zone; that nirK genes most similar to those from Rhodanobacter strains were most abundant in the near-source zone; and that Rhodanobacter organisms were active, not just present in the near source zone. Coupled with in vitro analysis of the physiological capabilities of Rhodanobacter strains in pure culture,

these data led to the hypothesis that bacteria from the genus Rhodanobacter are the dominant near-source zone denitrifiers at the ORIFRC site. This hypothesis is supported by studies conducted in other ecosystems which demonstrate that Rhodanobacter spp. dominate under low pH, denitrifying conditions (e.g. van den Heuvel et al., 2010).

Direct PCR amplification of nitrite reductase genes from Rhodanobacter and other denitrifiers isolated from the site was not successful using standard primers, and subsequently, de novo shotgun genome sequencing and draft assembly of these bacterial denitrifiers was performed. The initial draft sequences of Rhodanobacter and Intrasporangium recovered complete nirK genes, and helped determine the cause of PCR amplification failure. First, the putative nitrite reductase genes from these organisms were highly divergent from many sequences present in gene databases, and the sequences contained a large number of mismatches with the most commonly used primer sets for targeting bacterial nirK genes (e.g. 10 and 11 mismatches, respectively, between primer R3Cu and first and second nirK gene of R. denitrificans 2APBS1; (Green et al., 2010; Hallin and Lindgren, 1999)). In addition, most Rhodanobacter spp. have two highly divergent nirK genes located in different positions in the genome (Green et al., 2010; Kostka et al., 2012). Two strains of Rhodanobacter independently isolated (Bollmann et al., 2010)similarly contain two nirK genes apiece, and both are nearly (>99% similar) or completely identical to nirK genes from R. denitrificans 2APBS1^T. Both forms of nirK are expressed under denitrifying conditions in R. denitrificans 2APBS1^T, but the purpose of two copies of the gene is not yet clear (Green et al., 2012). One copy of the gene, colloquially called “nirK-B” is most similar to nirK genes from certain Proteobacteria, including Betaproteobacteria from the genera Burkholderia and Ralstonia. The second copy, called “nirK-V” is most similar to the nirK gene from Opitutus terrae PB90-1, within the phylum Verrucomicrobia.

To examine this phenomenon on a broader phylogenetic scale, Green et al (2010) recovered complete nirK and nosZ genes from a number of microorganisms which had been sequenced by the Joint Genome Institute. These genes were aligned and primer binding sites were identified. This analysis revealed that the difficulty in amplifying nirK genes from ORIFRC site isolates is symptomatic of a broader difficulty in detecting denitrifying bacteria through single primer set amplification due to large numbers of mismatches between primer and gene sequences. The commonly-used primer sets (including quantitative PCR primer sets) target a relatively narrow range of organisms, primarily within the Proteobacteria (Green et al., 2010). Thus, molecular approaches that depend upon single primers, even heavily degenerate primers, cannot be used suitably to detect or quantify denitrifiers in environmental samples, and the true diversity and abundance of denitrifiers is most likely greatly underestimated from current surveys. Alternate approaches, which utilize the full availability of reference sequence data derived from de novo genome sequencing and from shotgun metagenome sequencing of environmental samples, must be developed to more fully assess the distribution of these important organisms.

Although the nitrite reductase gene is a particularly dramatic example, it is not unique in this regard, and other functional genes of significance to biogeochemical processes have shown similar levels of sequence diversity. The sequence diversity of nirK may be in part due to the multiple physiological roles for nitrite reduction (detoxification, respiration), different conditions under which the enzymes may be active (e.g. prior to anoxic conditions, after total anoxia) and multiple locations for nitrite reductases (periplasm, inner membrane), and for the different forms of the gene (copper nitrite reductase, nirK and cytrochrome-cd1 nirS). This broad sequence divergence but with retained function is present in other functional genes, including other genes in the denitrification pathway (e.g. nosZ; (Green et al., 2010; Jones et al., 2013; Sanford et al., 2012).

Although many Rhodanobacter spp. isolated from the ORIFRC site subsurface were capable of complete denitrification, some members of the genus were incapable of growth on nitrate. Similarly, in a survey of the literature regarding Rhodanobacter, most strains were identified as aerobic bacteria, incapable of nitrate reduction. Strains isolated independently from the ORIFRC site were observed to be acid-tolerant (arrest of growth was observed at pH 3.5-4), tolerant of high levels of nitrate (up to 250 mM) and moderately tolerant of various heavy metals, including uranium (Bollmann et al., 2010). The initial description of R. thiooxydans, the closest relative of R. denitrificans, indicated that the organism was capable of nitrate, but not nitrite, reduction (Lee et al., 2007). Subsequent work, however, demonstrated that these organisms are capable of complete denitrification from nitrate (Prakash et al., 2012; van den Heuvel et al., 2010). More recently, a novel species, R. caeni, was described as capable of nitrate reduction to nitrite, but no evidence for complete denitrification was demonstrated (Woo et al., 2012). Likewise, R. sp. strain A2-61, shown to form intracellular uranium-phosphate complexes, was unable to reduce nitrate (Sousa et al., 2013).

To understand the genetic basis of the differences in physiology with respect to denitrification, the genomes of five additional strains of bacteria from the genus Rhodanobacter were sequenced (Kostka et al., 2012). In total, three strains of denitrifying Rhodanobacter were sequenced (R. denitrificans 2APBS1^T, R. denitrificans 116-2, R. thiooxydans)

alongside three strains of apparent non-denitrifying (from nitrate) Rhodanobacter (R. fulvus Jip2 (Im et al., 2004), R. spathiphylli B39 (De Clercq et al., 2006), and R. sp. 115, isolated from the ORIFRC site, (Kostka et al., 2012). Preliminary analysis of the genomes of the six Rhodanobacter strains revealed that all members of the genus contained nearly complete denitrification pathways, including two copies of the nitrite reductase gene nirK (excepting R. spathiphylli, with only a single copy). All denitrifying isolates contained many genes in the dissimilatory denitrification pathway, but non-denitrifying isolates were missing several key genes involved in nitrate respiration, such as nitrate reductase genes (i.e. narG, narH, narJ and narI). The genomic context of these genes was further examined, and it was observed that the nitrous oxide genes (e.g. nosZ) showed the greatest synteny among all six genomes (Figure 1). Since relatively few organisms conduct nitrous oxide reduction alone, it may be supposed that the high level of synteny in this gene and the lower synteny in other parts of the denitrification pathway favors the hypothesis that the ancestral common ancestor of the bacteria within the genus Rhodanobacter likewise contained a full denitrification pathway, with subsequent re-arrangement of the genes in the pathway. Further clarity will be obtained with additional whole genome sequences of related organisms from the Xanthomonadaceae.

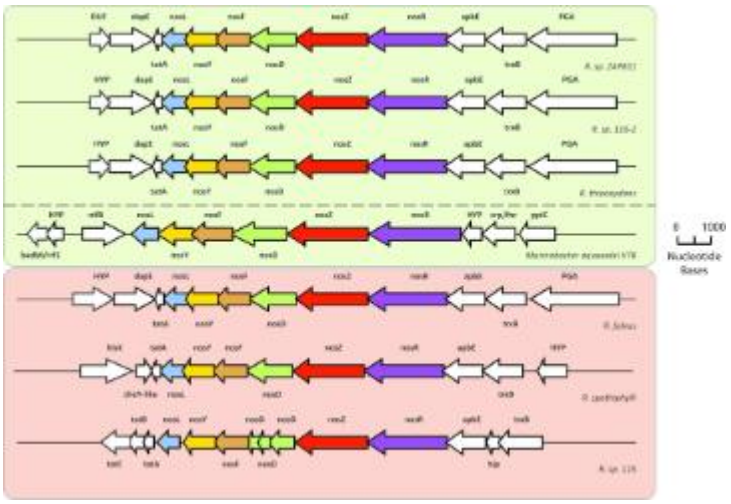

Figure 1: Gene order in the genomic region of the nitrous oxide reductase gene (nosZ) in denitrifying and apparent non-denitrifying strains of bacteria from the genus Rhodanobacter. Strong gene synteny is observed between denitrifying (highlighted in green) and apparent non-denitrifying lineages (highlighted in orange). Gene order in Marinobacter aquaeolei VT8 (Gammaproteobacteria, Alteromonadaceae), capable of anaerobic growth on nitrate, was included as an outgroup organism with a complete genome sequence.
Gene symbols: apbE - ApbE family lipoprotein; cheY-like - Two-component system sensor histidine kinase-response regulator hybridprotein; dapE - Succinyldiaminopimelate desuccinylase; DUF - Protein of unknown function DUF2165; hip - High potential iron-sulfur protein; hisK - Sensor histidine kinase; HYP - Hypothetical protein; nosD - Periplasmic copper-binding protein; nosF - ABC transporter related protein; nosL - NosL protein; nosR - Nitrous oxide expression regulator, NosR; nosY - ABC-type transport system involved in multi-copper enzyme maturation, permease component; nosZ - Nitrous-oxide reductase; PGA - Peptidase S45 penicillin amidase; tatA - Twin-arginine translocation protein, TatA/E; tatB - Twin arginine-targeting protein translocase TatB; tatC - Twin arginine targeting protein translocase subunit TatC; trxB - Thioredoxin reductase oxidoreductase; badM/Rrf2 - BadM/Rrf2 family transcriptional regulator; nifB - molybdenum cofactor biosynthesis protein A; ppiC - PpiC-type peptidyl-prolyl cis-trans isomerase.

Conclusions regarding Rhodanobacter. Bacteria from the genus Rhodanobacter appear to fill a relatively specific ecological niche, but under appropriate conditions, these organisms can dominate to an extreme extent. Conditions which appear to enable bacteria from the genus Rhodanobacter to dominate include low pH, high nitrate, low/variable oxygen concentrations, and heavy metal contamination. Although data in the literature are not particularly abundant for Rhodanobacter, what is present suggests that heavy metal tolerance is a common feature of these organisms. Bollmann et al (Bollmann et al., 2010) isolated two strains of Rhodanobacter that are tolerant of 200 micromolar uranium (as well as other heavy metals), and most recently Sousa et al. (Sousa et al., 2013) described R. sp. strain A2-61, tolerant of up to 500 micromolar uranium, under aerobic conditions. R. denitrificans strains are capable of tolerating 1 mm uranium (data not shown). Interestingly, R. sp. strain A2-61 was capable of forming intracellular uranium-phosphate complexes, presumably a detoxification strategy. In a survey of the genome of R. denitrificans 2APBS1^T, multiple genes involved in

metal resistance have been detected, and these genes are strongly associated with horizontal gene transfer as indicated by low lineage probability scores (LPI), anomalous nucleotide compositions and association with putative mobile genetic elements such as transposons and integrons (data not shown).

The presence of a near-complete denitrification pathway in “non-denitrifying” strains of bacteria from the genus Rhodanobacter suggests that denitrification capability is an inherent trait of all members of the genus but that denitrification by these organisms often requires nitrite rather than nitrate. Since nitrite is often available where there is nitrate, and a number of organisms are capable of nitrate to nitrite reduction, but cannot reduce nitrite further, the lack of a nitrate reductase may not be overly limiting for a facultative anaerobes such as members of the Rhodanobacter. For example, in a study of denitrification capabilities in bacteria from the genus Bacillus, most-probable number assays of a soil sample revealed nearly an order of magnitude greater abundance of organisms capable of nitrate-to-nitrite reduction relative to complete denitrifiers (Verbaendert et al., 2011). A further confounding observation is the presence of two putative nirK genes in almost all Rhodanobacter, including the non-nitrate reducers. It may be that the multiple nitrite reductases are involved in tolerance of high nitrate/nitrite conditions, stressful conditions that are further exacerbated by low pH (Spain and Krumholz, 2012). The nitrite reductases may also represent two different strategies relating to denitrification by Rhodanobacter under fluctuating aerobic/anaerobic conditions, such as those found in the ORIFRC site subsurface. As described by Bergaust et al. (Bergaust et al., 2011), bacteria can employ complex strategies to maximize energy generation, but provide insurance in case of sudden changes in environmental condition. Thus, while in the presence of oxygen, denitrifying bacteria (which are nearly always facultative anaerobes) will favor the use of oxygen as terminal electron acceptor, and repress nitrogen oxyanion reduction to avoid loss of ATP-generation capability through a truncated respiratory pathway, and “entrapment” under anoxic conditions without capability to continue respiration (Bergaust et al., 2011). It has been hypothesized that an earlier onset of denitrification (in terms of oxygen concentration) is an indication of the likelihood for nitrous oxide production by the strain (Bergaust et al., 2011; Zumft and Kroneck, 2007). This is consistent with the initial characterization of R. denitrificans, in which both nitrous oxide and dinitrogen accumulated during pure culture growth conditions in vitro, while other isolates from the site completed denitrification to dinitrogen (Afipia, Hyphomicrobium) or nitrous oxide only (Gram positives; Bacillus and Intrasporangium) (Green et al., 2010). Further work is needed to determine the regulatory strategy taken by Rhodanobacter in the subsurface under aerobic/microaerophilic/anaerobic conditions.

Are Rhodanobacter extremophiles? Based on the current data, it is not clear that they are. Although members of the genus can grow at pH values below pH 4, the optimum growth pH for R. denitrificans 2APBS1 is pH 6 (Bollmann et al., 2010; Prakash et al., 2012). However, even at circumneutral pH with excess organic carbon, growth by R. denitrificans is slow (generation time ~24 hr). This may represent another strategy by Rhodanobacter strains leading to dominance in contaminated/extreme environments, but low relative abundance in more ameliorated conditions. It appears most likely that Rhodanobacter retain a variety of physiological capabilities – anaerobic growth, metal tolerance and detoxification, denitrification phenotype, and broad carbon substrate utilization capability (including acetate) – that under specific environmental conditions provides them with the opportunity for dominance.

Conclusions regarding denitrification.The ORIFRC, with nitrate-replete groundwaters, represents an ideal natural laboratory for investigation of the microbial populations that mediate denitrification. Through a close coupling of cultivation-based and molecular approaches, characterization of denitrifying bacteria from the ORIFRC site has significant implications not just for broader characterization of denitrifying organisms, but also for the application of PCR-based approaches to characterize microbial functional groups. With specific reference to denitrification, it was observed that the most commonly used primers targeting functional genes within the dissimilatory denitrification pathway were highly biased to a select group of genes largely derived from bacteria within the Proteobacteria, and the genes from organisms outside this group could not conceivably be targeted with PCR due to the excessively large number of mismatches between primer and gene sequence. Thus, results generated from single gene primer (even degenerate) sets must be interpreted carefully. A similar finding has been obtained for nitrous oxide genes as well (Sanford et al., 2012). Since de novo genome and shotgun metagenome sequences generate gene sequences that are clearly identifiable as nitrite (or nitrous oxide) reductases but also impossible to target with common primers, new strategies must be developed to capture a broader collection of denitrifiers in the environment. As the organisms capable of denitrification are broadly distributed and are polyphyletic, functional gene analyses will continue to be essential to identify and quantitate denitrifying microorganisms and to characterize denitrifying microbial communities.

One of the essential extrapolations of these findings is that the true abundance of denitrification capability in bacterial lineages is underestimated due to two processes revealed in this study. First, the high sequence divergence present in

functional genes in the denitrification pathway limits the detection of denitrification genes from isolates through PCR and sequencing. Second, the partial pathway observed in Rhodanobacter strains suggests that when searching for denitrification capabilities, other electron acceptors besides nitrate should be tested. In a sense, cultivation approaches and physiological testing of Rhodanobacter strains have been partially misleading regarding the potential ecological niche for these organisms, and only when coupled with whole genome sequencing, has the putative in situ functional capability of these organisms been revealed. In an analysis of Bacillus isolate and culture-collection strains, Verbaendert et al. (2011) revealed that nitrate was not always a suitable electron acceptor for verification of denitrification capability, and that 20% of denitrifying strains could use nitrite but not nitrate to initiate denitrification. They opine that the true abundance of denitrifiers is underestimated because typically only nitrate is used as an electron acceptor when testing for denitrification capability, and this is consistent with observations of isolates of the genus Rhodanobacter. Remarkably, they also observed that growth conditions can also affect electron acceptor utilization, and this can further lead to missing identification of physiological capability. No doubt analogous situations for other genes, organisms and functions are with us, waiting to be identified. Thus, it seems clear that for more robust physiological characterization of bacterial strains, genome-guided physiological testing must be implemented. Such an approach will have profound implications for the assessment of the ecological role of bacteria taxa.

Prior to the acquisition of multiple genomes from the genus Rhodanobacter, the denitrification phenotype in Rhodanobacter strains was hypothesized to resulted from a relatively recent lateral gene transfer rather than from vertical transmission, as appears to be the case (Green et al., 2010). Hemme et al. (2010) also opined that the inferred lateral gene transfer events most likely occurred after the introduction of contamination at the site. With multiple genomes in hand, phylogenetic analysis of the nitrite reductase genes from the whole genome sequences of multiple Rhodanobacter strains revealed a phylogeny consistent with that of the rRNA genes from the same organisms. If there were lateral gene transfer events, these predated the last common ancestor of the genus Rhodanobacter, with the most parsimonious interpretation being that nitrate reduction capability was later lost from certain members of the genus. The evolutionary history of the full denitrification pathway, however, appears to be fragmented – for example, the nirK genes do appear to be derived from a lateral gene transfer, but this transfer is not recent and certainly is independent of the ORIFRC site. The Rhodanobacter nosZ genes are more consistent with other Gammaproteobacterial denitrifiers. It is possible, though entirely speculative, that Rhodanobacter previously had type (or class) I soluble periplasmic nitrite reductases, like those present in Pseudomonas denitrificans, and these have been subsequently replaced by type II cytoplasmic membrane nitrite reductases. The ecologic benefit derived from this is not clear yet, but may relate to activity under aerobic and anaerobic conditions, as has been observed for nitrate reductases (Bedzyk et al., 1999).

S U M M A R Y

A combination of approaches to the study of denitrifying bacteria in a contaminated subsurface environment, including cultivation and physiological testing of denitrifying bacteria, de novo whole genome sequencing, and shotgun metagenome sequencing, revealed key limitations to the application of more straightforward molecular approaches. Commonly used PCR primers targeting functional genes in the denitrification pathway are shown to be incapable of detecting a broad diversity of environmental denitrifiers. Likewise, some denitirifiers are incapable of nitrate reduction from nitrate, and may be mis-identified in routine physiological testing of bacterial isolates. Bacteria from the genus Rhodanobacter, which can be abundant in highly contaminated environments with low pH, appear to be native denitrifiers, while metal resistance genes appear to have been acquired via lateral gene transfer. Overall, Rhodanobacter dominate in certain environments with low pH, heavy metal contamination, and conditions favoring denitrification phenotype.

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