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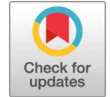

Capturing Compositional Variation in Denitrifying Communities: a Multiple-Primer Approach That Includes Epsilonproteobacteria

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ABSTRACT Denitrifying *Epsilonproteobacteria* may dominate nitrogen loss processes in marine habitats with intense redox gradients, but assessment of their importance is limited by the currently available primers for nitrite reductase genes. Nine new primers targeting the *nirS* gene of denitrifying *Epsilonproteobacteria* were designed and tested for use in sequencing and quantitative PCR on two microbial mat samples (vent 2 and vent 4) from the Calypso hydrothermal vent field, Bay of Plenty, New Zealand. Commonly used *nirS* and *nirK* primer sets nirS1F/nirS6R, cd3aF/R3cd, nirK1F/nirK5R, and F1aCu/R3Cu were also tested to determine what may be missed by the common single-primer approach to assessing denitrifier diversity. The relative importance of *Epsilonproteobacteria* in these samples was evaluated by 16S rRNA gene sequencing. *Epsilonproteobacteria* represented up to 75.6% of 16S rRNA libraries, but *nirS* genes from this group were not found with commonly used primers. Pairing of the new primer EPSnirS511F with either EPSnirS1100R or EPSnirS1105R recovered *nirS* sequences from members of the genera *Sulfurimonas*, *Sulfurovum*, and *Nitratifractor*. The new quantitative PCR primers EPSnirS103F/EPSnirS530R showed dominance of denitrifying *Epsilonproteobacteria* in vent 4 compared to vent 2, which had greater representation by "standard" denitrifiers measured with the cd3aF/R3cd primers. Limited results from commonly used *nirK* primers suggest biased amplification between primers. Future application of multiple *nirS* and *nirK* primers, including the new epsilonproteobacterial *nirS* primers, will improve the detection of denitrifier diversity and the capability to identify changes in dominant denitrifying communities.

IMPORTANCE Estimating the potential for increasing nitrogen limitation in the changing global ocean is reliant on understanding the microbial community that removes nitrogen through the process of denitrification. This process is favored under oxygen limitation, which is a growing global-ocean phenomenon. Current methods use the nitrite reductase genes *nirS* and *nirK* to assess denitrifier diversity and abundance using primers that target only a few known denitrifiers and systematically exclude denitrifying *Epsilonproteobacteria*, a group known to dominate in reducing environments, such as hydrothermal vents and anoxic basins. As oxygen depletion expands in the oceans, it is important to study denitrifier community dynamics within those areas to predict future global ocean changes. This study explores the design and testing of new primers that target epsilonproteobacterial *nirS* and reveals the varied success of existing primers, leading to the recommendation of a multiple-primer approach to assessing denitrifier diversity.

KEYWORDS *Epsilonproteobacteria*, denitrification, hydrothermal, *nirS*, primers

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Denitrification, the stepwise reduction of nitrate (NO_3^-) to dinitrogen gas (N_2), is a microbial process that removes nitrogen (N) from the pool that is available for assimilation into biomolecules (nucleic and amino acids) or for use in energy generation by dissimilatory redox processes. Only a small subset of microorganisms, the diazotrophs, are capable of utilizing N_2 gas to fulfill their nitrogen requirements. Nitrogen is often considered the primary limiting nutrient in marine ecosystems (1); therefore, pathways that remove N from the biologically available pool have important implications for marine biological productivity. Denitrification is favored when the availability of dissolved oxygen becomes limiting for microbial respiration and NO_3^- is available as an alternative electron acceptor (2). The reduction of NO_3^- to N_2 proceeds via nitrite (NO_2^-), nitric oxide (NO), and the greenhouse gas nitrous oxide (N_2O), but the process can also yield N_2O as the final product (2).

While denitrification rate process measurements can provide estimates for N loss, help understand spatial and temporal differences in measured rates, and forecast future ecosystem states, both require an understanding of the diversity and abundance of microbes involved in this process and community structural responses to environmental forcing. This interest is evidenced by the numerous studies of denitrifier functional gene diversity that have been undertaken in a variety of aquatic environments, including estuarine sediments (3–7), freshwater systems (8–12), coastal marine waters and sediments (4, 5, 13–15), oxygen minimum zones and anoxic basins (12, 16–23), hydrothermal vents (24, 25), and deep-sea sediments (26). The ability to denitrify exists across a wide breadth of bacteria and archaea (2) that may be favored under different environmental conditions. It follows then that greater diversity of denitrifiers and conditions permitting denitrification may translate to increased potential for denitrification across a wider range of environments. This has implications for biogeochemical modeling of marine productivity, nitrogen cycle processes, and N_2O emissions. Because oxygen limitation, a key factor that favors denitrification, is a growing phenomenon in marine coastal zones as a result of cultural eutrophication and is expected to worsen due to anthropogenic climate change (27), knowledge of the diversity and abundance of denitrifiers is crucial to predicting the potential for nitrogen loss from marine systems in this changing global ocean. The current global expansion of oceanic oxygen minimum zones adds to this urgency (28).

Aquatic denitrifier diversity studies (cited above) primarily target the functional marker genes encoding nitrite reductase, the enzyme that catalyzes the first committed step of denitrification (NO_2^- to NO), producing a gaseous intermediate (2). Two structurally different but functionally equivalent enzymes mediate the reduction of NO_2^- to NO: the copper-containing nitrite reductase encoded by the *nirK* gene and cytochrome cd_1-nitrite reductase encoded by the *nirS* gene (2). Primers specific to the *nirS* and *nirK* genes are used to amplify and sequence one or both of these functional genes in a community, and the resultant sequence data permit the evaluation of denitrifier diversity. While uncertainty exists regarding the true extent of the denitrifier diversity represented by these data, a design requirement in any microbial diversity study is to select amplification and sequencing primers that reveal as much of the target community as possible. Where genes are highly divergent, this can be accomplished using primers with degenerate bases, positions in the primer sequence in which proportions of the primer contain a different nucleotide, allowing more flexibility in the binding specificity of the primer.

The majority of the aforementioned aquatic studies have targeted the *nirS* gene using the primers nirS1F/nirS6R (nirS1F/6R), developed by Braker et al. (8), which were designed with degenerate bases that covered the six sequences of the *nirS* gene available from public databases at that time (1998). A search of public databases reveals that a wealth of additional *nirS* genes have since been sequenced, exhibiting high sequence divergence in the nirS1F/6R priming regions that is not addressed with the degenerate bases, thus decreasing the likelihood of successful primer binding, amplification, and sequencing of all genes in a given sample. The highly divergent nature of this gene at both the nucleotide and amino acid levels makes degenerate bases almost

a required feature of primers targeting the *nirS* gene. Throbäck et al. (29) assessed the usefulness of various primers for amplifying *nirS* genes from cultured and environmental denitrifying organisms and concluded that in spite of the inclusion of degenerate bases, no existing primer pair could be considered universal for all *nirS*-containing organisms investigated. They suggested that the primers cd3aF (30) and their own R3cd primer were the best pair for community analysis of *nirS* genes, based on the success of these primers at amplifying that gene from a wide range of tested organisms and samples. In spite of this important finding, the nirS1F/6R primers are still the most commonly used primer set for assessing denitrifier diversity in aquatic systems; therefore, many studies may have failed to detect an unknown proportion of the denitrifier community.

One group of denitrifiers that is unlikely to be identified by neither the nirS1F/6R nor the cd3aF/R3cd *nirS* primer pair is *Epsilonproteobacteria* belonging to the genera *Sulfurimonas*, *Sulfurovum*, and *Nitratifractor*. The *nirS* genes of species in these genera have little similarity to other *nirS* genes in public databases and 50% or fewer shared nucleotides with each of the four commonly used primer sequences. *Epsilonproteobacteria* have been found to be key components of microbial communities at hydrothermal vents (31–35) and in anoxic basins (36, 37), and several of the newly characterized epsilonproteobacterial isolates are denitrifiers. These include *Nitratiruptor tergarcus* and *Nitratifractor salsuginis* from hydrothermal vent chimneys (38), *Sulfurovum* sp. strain NBC37-1 and *Nitratiruptor* sp. strain SB155-2 from *in situ* samplers deployed on an actively venting sulfide mound (39), *Sulfurimonas denitrificans* from tidal flat sediment (40), and *Sulfurimonas gotlandica* (41) from the Baltic Sea redoxcline. Additionally, *Sulfurimonas autotrophica* strain OK10 from hydrothermal sediment (42) contains an *nirS* gene even though the isolate was found to grow with oxygen as the sole electron acceptor.

Recent studies have suggested an important role for denitrifying microbial communities in N cycling at deep-sea hydrothermal vents where steep redox gradients involving abundant reduced sulfur compounds favor zones of sulfur-driven autotrophic denitrification (43). Fluids discharging from diffuse vents and black smoker chimneys at several sites on the Juan de Fuca Ridge are hot spots for microbial denitrification, as shown from rate measurements (44) and the stable isotopic composition of NO_3^- and ammonium (45); these are further supported by measurements of denitrification gene abundances in GeoChip (46) and metagenomic (47) analyses. Abundant denitrification gene transcripts have also been reported in bacterial mat samples from chimneys on the Arctic Mid-Ocean Ridge (48).

However, in spite of these various lines of evidence supporting the importance of microbial denitrification in the hydrothermal environment, a recent study by Bourbonnais et al. (24) found low diversity and abundance of *nirS*-type denitrifiers in vent fluids from Axial Volcano and the Endeavor vent field, on the Juan de Fuca Ridge. Was this the result of the dominance of denitrifying *Epsilonproteobacteria* in their vent fluid samples, whose *nirS* genes were not detected by the standard nirS1F/6R primers employed in that study? These contradictory results call into question the practice of applying commonly used *nirS* primers in environments where *Epsilonproteobacteria* may represent a significant component of the microbial community and suggest that much of the denitrifier diversity is being overlooked in these systems.

In this study, we used two hydrothermal vent microbial mat samples from the Calypso vent field in the Bay of Plenty, New Zealand, to test this hypothesis (i.e., dominance by epsilonproteobacterial denitrifiers in sulfide-rich [hydrothermal] habitats) with a newly developed set of primers targeting epsilonproteobacterial *nirS* genes. We designed primers to target conserved regions of *Sulfurimonas*, *Sulfurovum*, and *Nitratifractor nirS* genes and produced a variety of amplicon sizes for diversity and abundance assessments using sequencing and real-time quantitative PCR (qPCR). The denitrifying epsilonproteobacterial genus *Nitratiruptor* was not targeted in the design of the primers, as it has an *nirS* sequence that is captured using one of the standard primer sets. Newly designed primers were tested on both environmental samples and

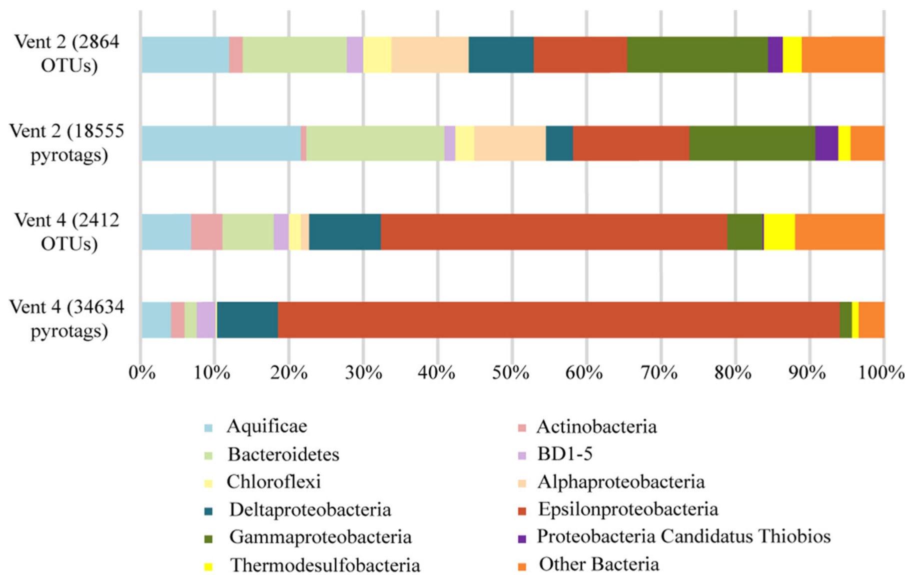

FIG 1 Relative abundance of 16S rRNA sequence tags and OTUs (97% definition) at the phylum and class levels. The category "other bacteria" includes groups that made up <1.5% of the total OTUs and belonged to *Acidobacteria*, *Caldiserica*, *Chlorobi*, *Chrysiogenetes*, *Cyanobacteria*, *Deferribacteres*, *Deinococcus-Thermus*, *Fibrobacteres*, *Firmicutes*, *Fusobacteria*, *Gemmatimonadetes*, *Lentisphaerae*, *Nitrospirae*, *Betaproteobacteria*, *Zetaproteobacteria*, unclassified *Proteobacteria*, *Spirochaetes*, *Tenericutes*, *Thermotogae*, *Verrucomicrobia*, and numerous candidate divisions.

cultured organisms. We also tested the success of commonly used *nirS* and *nirK* primers for assessing the diversity of denitrifiers in our hydrothermally influenced samples and compared relative abundances of standard (nonepsilonproteobacterial) *nirS* and epsilonproteobacterial *nirS* genes using qPCR.

RESULTS

Bacterial 16S rRNA gene Sanger and 454 sequences. To assess whole-community bacterial diversity, a total of 99,614 sequence tags encompassing the V1 to V3 variable regions of the bacterial 16S rRNA gene were recovered from the two microbial mat samples. After removal of low-quality and chimeric sequences, 18,555 and 34,634 high-quality sequence tags remained for further analysis from vents 2 and 4, respectively. Totals of 2,864 and 2,412 operational taxonomic units (OTUs) were identified in vent 2 and vent 4 samples, respectively, with only 380 OTU shared between the two samples. The dissimilarity of the two samples was evident even at the phylum and class levels (Fig. 1), with the highest proportions of vent 2 sequence tags belonging to *Aquificae* (21.6%), *Bacteroidetes* (18.5%), *Gammaproteobacteria* (16.9%), and *Epsilonproteobacteria* (15.7%), and vent 4 sequence tags were dominated by *Epsilonproteobacteria* (75.6%). Overall, vent 2 displayed a higher estimated diversity than the sample from vent 4 (Table 1) using both the Shannon and Simpson diversity indices. For the Shannon index, a higher number indicates greater diversity, and the Simpson index is on a scale of 0 to 1, with 0 indicating infinite diversity and 1 indicating a single species. In the vent 4 sample, the most highly represented group, the *Epsilonproteobacteria* were also the most diverse, containing 46.7% of the total OTUs, followed by *Deltaproteobacteria* with 9.7% of the OTUs. In contrast, the largest percentage of OTUs in the vent 2 sample (19.4%) did not belong to the most frequently recovered group, the *Aquificae*, but to the *Gammaproteobacteria*, followed closely by the *Bacteroidetes* (13.9% of OTUs) and *Epsilonproteobacteria* (12.9% of OTUs) (Fig. 1).

Using taxonomic assignments from comparison to the Silva nr_119 reference database, we identified 850 OTU belonging to 13 genera with known denitrifying species. These made up 12.5% and 25.1% of OTUs in vent 2 and vent 4 samples, respectively. Only 94 of the 850 OTU were present in both samples (data not shown). The vent 2 microbial mat had OTUs belonging to all 13 genera with denitrification potential, while

TABLE 1 Results of Sanger and 454 sequencing efforts

Gene	Characteristic	Vent 2	Vent 4[a]
16S rRNA[b]	No. of Sanger library clones (no. of OTUs)	145 (67)	98 (25)
	% *Epsilonproteobacteria* in Sanger libraries (% OTUs)	71 (52)	96 (98)
	No. of 454 sequence tags (no. of OTUs)	18,555 (2,864)	34,634 (2,412)
	% *Epsilonproteobacteria* in 454 tags (% OTUs)	15.7 (12.9)	75.6 (46.7)
	Shannon diversity index[c]	6.144 ± 0.03	4.891 ± 0.02
	Simpson diversity index[c]	0.012 ± 0.0005	0.033 ± 0.001
nirS[d]	No. of nirS1F/6R clones (no. of OTUs)	36 (9)	ND
	No. with <85% identity clones (no. of OTUs)	14 (5)	
	Library coverage (%)	94	
	No. of cd3aF/R3cd clones (no. of OTUs)	92 (16)	ND
	No. with <85% identity clones (no. of OTUs)	70 (10)	
	Library coverage (%)	95	
nirK[d]	No. of nirK1F/5R clones (no. of OTUs)	1 (1)	56 (3)
	No. with <85% identity clones (no. of OTUs)	0 (0)	0 (0)
	Library coverage (%)		98
	No. of F1aCu/R3Cu clones (no. of OTUs)	70 (19)	ND
	No. with <85% identity clones (no. of OTUs)	14 (6)	
	Library coverage (%)	94	

[a]ND, not detected, unsuccessful amplification with the listed primer pair.
[b]OTUs defined at 97% similarity.
[c]Diversity indices calculated based on 16S rRNA 454 sequence tags.
[d]OTUs defined at 85% similarity.

the vent 4 mat contained only 9 potential denitrifying genera (Table 2). At vent 2, the most abundant group of OTUs within potential denitrifying genera belonged to the genus *Persephonella* (*Aquificae*, 2.8% of total OTUs), but OTUs belonging to the four epsilonproteobacterial genera containing known denitrifiers (*Sulfurovum*, *Sulfurimonas*,

TABLE 2 Taxa detected in 16S rRNA tag sequences that contain known denitrifying species

Denitrifier-containing genus (class or phylum)	No. of sequence tags (% of total)	No. of OTUs (% of total)[a]
Vent 2		
Hydrogenivirga (*Aquificae*)	29 (0.2)	11 (0.4)
Persephonella (*Aquificae*)	2,049 (11.0)	80 (2.8)
Dinoroseobacter (*Alphaproteobacteria*)	20 (0.1)	2 (0.1)
Phaeobacter (*Alphaproteobacteria*)	32 (0.2)	1 (<0.1)
Roseobacter (*Alphaproteobacteria*)	128 (0.7)	12 (0.4)
Nitratifractor (*Epsilonproteobacteria*)	1,259 (6.8)	113 (3.9)
Nitratiruptor (*Epsilonproteobacteria*)	489 (2.6)	35 (1.2)
Sulfurimonas (*Epsilonproteobacteria*)	260 (1.4)	61 (2.1)
Sulfurovum (*Epsilonproteobacteria*)	341 (1.8)	27 (0.9)
Colwellia (*Gammaproteobacteria*)	4 (<0.1)	3 (0.1)
Pseudomonas (*Gammaproteobacteria*)	4 (<0.1)	2 (0.1)
Thiohalomonas (*Gammaproteobacteria*)	88 (0.5)	8 (0.3)
Nitrospira (*Nitrospirae*)	14 (0.1)	5 (0.2)
Total	4,717 (25.4)	360 (12.6)
Vent 4		
Hydrogenivirga (*Aquificae*)	24 (0.1)	16 (0.7)
Persephonella (*Aquificae*)	663 (1.9)	30 (1.2)
Roseobacter (*Alphaproteobacteria*)	1 (<0.1)	1 (<0.1)
Nitratifractor (*Epsilonproteobacteria*)	5,408 (15.6)	271 (11.2)
Nitratiruptor (*Epsilonproteobacteria*)	477 (1.4)	46 (1.9)
Sulfurimonas (*Epsilonproteobacteria*)	984 (2.8)	64 (2.7)
Sulfurovum (*Epsilonproteobacteria*)	8,677 (25.1)	173 (7.2)
Thiohalomonas (*Gammaproteobacteria*)	9 (<0.1)	5 (0.2)
Nitrospira (*Nitrospirae*)	1 (<0.1)	1 (<0.1)
Total	16,244 (46.9)	607 (25.2)

[a]OTUs defined at 97% similarity.

Nitratiruptor, and *Nitratifractor*) accounted for 8.1% of all OTUs recovered from the sample and collectively made up nearly 66% of the 850 potential denitrifier OTUs (Table 2). At vent 4, the OTUs belonging to the four epsilonproteobacterial genera accounted for nearly 23% of all OTUs sequenced from the sample and dominated the potential denitrifiers (91%) (Table 2).

Sanger sequence libraries provided longer sequence reads to confirm the classification of short 454 sequence tags into genera that were of interest for their denitrifying potential. We sequenced 145 and 98 clones that grouped into 67 and 25 OTU from vents 2 and 4, respectively (Table 1). Sixty-three sequence tag OTU were represented by Sanger sequences that were 715 to 1,428 bp in length. Thirty of these tag OTUs were assigned to the four denitrifying epsilonproteobacterial genera using Silva databases (see Materials and Methods) and were confirmed to be 91 to 98% related to cultured species belonging to those genera using BLAST (Table S1). While <2% of the OTUs in each sample were represented by Sanger sequences, this amounted to 8 and 36% of the vent 2 and vent 4 sequence tags recovered, respectively, and provided support for evaluations of denitrification potential from the shorter sequence tags.

***nirS* or *nirK* Sanger sequence libraries from standard primer sets.** The effectiveness of the primer sets frequently used in other studies for amplification of nitrite reductase genes varied considerably between the two mat samples (Table 1). In the vent 2 sample, *nirS* was successfully amplified using both the nirS1F/nirS6R and cd3aF/R3cd primer pairs and not amplified at all with either pair in the epsilonproteobacterium-rich vent 4 sample. In samples from both sites, the *nirK* gene was successfully amplified with only one of the two primer pairs, at vent 2 with the F1aCu/R3Cu primer pair and at vent 4 with the nirK1F/nirK5R primers. Successful *nirS* and *nirK* amplifications were cloned and sequenced from vent 2 (three libraries of between 9 and 19 OTUs, 85% amino acid similarity), along with a single *nirK* library from vent 4 with only 3 OTUs.

In the vent 2 sample, the four most abundant OTUs recovered using the nirS1F/nirS6R primers were also recovered using the cd3aF/R3cd primers; however, the most frequently recovered OTU (41 of 92 clones) in the cd3aF/R3cd library was not found in the nirS1F/nirS6R library. The closest match to this OTU was *Sulfurihydrogenibium subterraneum* (77% amino acid identity), which was originally isolated from a hot subsurface aquifer (49). OTUs shared between the two *nirS* libraries from vent 2 represented 67% and 35% of nirS1F/nirS6R and cd3aF/R3cd libraries, respectively. No sequences related to epsilonproteobacterial *nirS* were recovered from either clone library (Fig. 2).

Phylogenetic comparisons showed the majority of the *nirS* clone OTUs from vent 2 grouped into two clusters (clusters 1 and 2, Fig. 2), which were represented in both the nirS1F/6R and cd3aF/R3cd libraries and included all four of the OTUs that were shared between the two libraries. OTUs in these clusters generally had high percent identities (≥80%) to their closest relative, as determined by BLAST, with a few exceptions in cluster 2. These two clusters also contain the cultured denitrifiers that were used in the design and testing of the nirS1F/6R and cd3aF/R3cd primers by Braker et al. (8) and Throbäck et al. (29). In contrast, clusters 3 and 4 were uniquely assembled around the cd3aF/R3cd library and included the most frequently recovered OTU in that library. OTUs falling into these clusters had lower identities (63 to 81%) to their closest database relatives. Clusters 3 and 4 contained sequences from phylogenetically distant members of the phyla *Deinococcus-Thermus*, *Proteobacteria* (class *Epsilonproteobacteria*), and *Aquificae*. A comparison of the *nirS* sequences of the representative denitrifying species of *Oceanithermus*, *Thermus*, *Hydrogenivirga*, *Hydrogenobacter*, and *Nitratiruptor* (the only *Epsilonproteobacteria* whose *nirS* gene groups separately from the others) with the primer sequences for nirS1F and nirS6R revealed that they have 6 to 8 nucleotide differences compared to the forward primer and 1 to 4 nucleotide differences compared to the reverse primer.

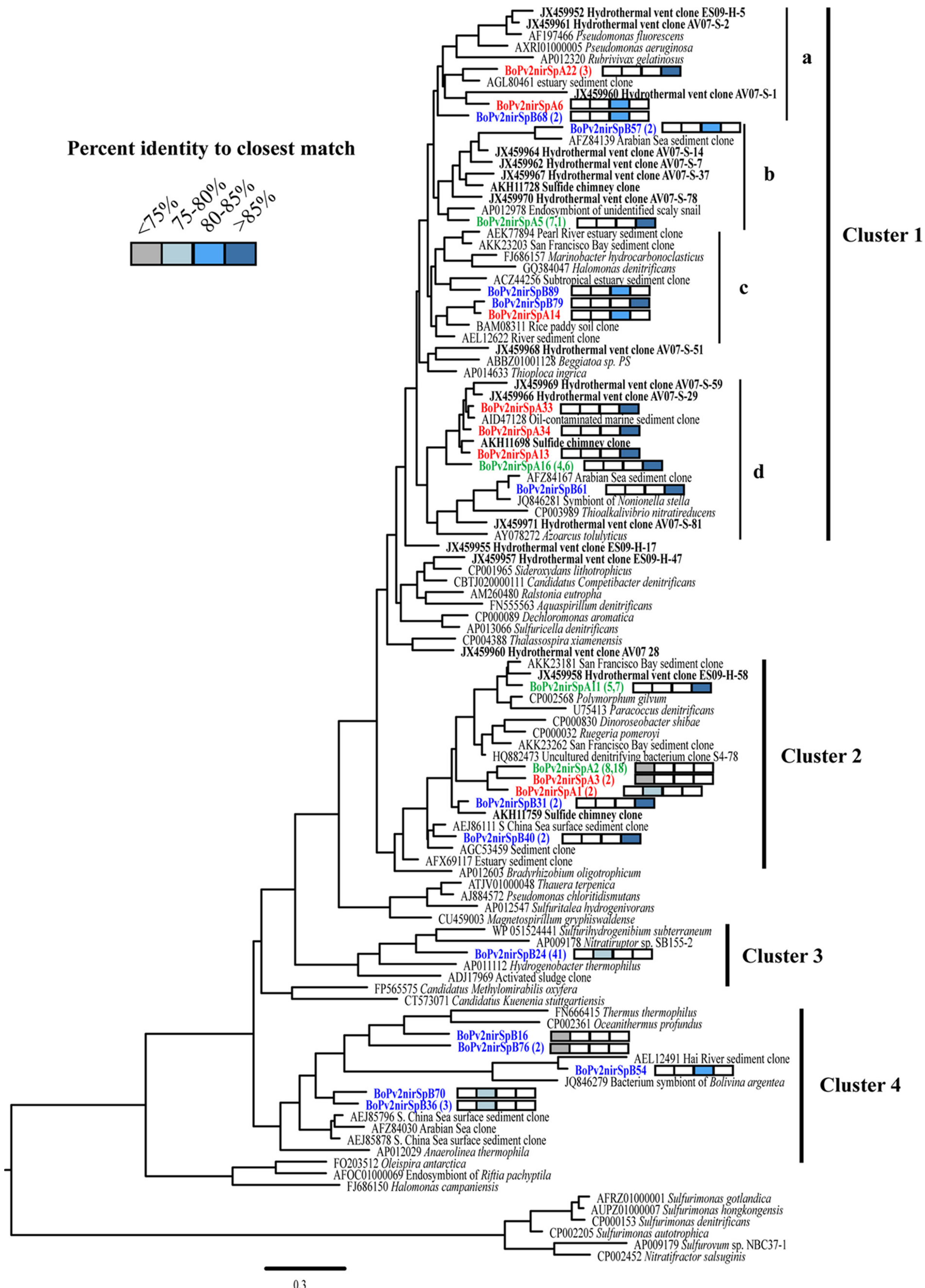

FIG 2 Maximum likelihood tree of 343 deduced amino acid positions of vent 2 *nirS* sequences from clone libraries produced using primer sets nirS1F/6R (v2nirSpA clones, in red) and cd3aF/R3cd (v2nirSpB clones, in blue). Clones in green are OTUs that were identified by both primer sets.

(Continued on next page)

TABLE 3 Percent amino acid similarity of clones to cultured denitrifying *Epsilonproteobacteria* using different primer combinations

	% amino acid similarity by vent and primer combination (no. of clones sequenced)					
	EPSnirS511F/EPSnirS1100R		EPSnirS511F/ EPSnirS1105R	EPSnirS103F/ EPSnirS1607R	EPSnirS103F/EPSnirS1492R	
BLAST result	Vent 2 (*n* = 27)	Vent 4 (*n* = 37)	Vent 4 (*n* = 37)	Vent 4 (*n* = 12[a])	Vent 2 (*n* = 40)	Vent 4 (*n* = 94[b])
Nitratifractor salsuginis	—[c]	95–96	96	94	85–88	86–88
Sulfurimonas autotrophica	93–100	98–100	90–100	—	—	91
Sulfurimonas gotlandica	97	—	96–97	—	—	—
Sulfurovum sp. NBC37-1	86–94	86–96	85–94	—	82–91	77–85
Sulfurimonas denitrificans	—	—	—	—	—	—

[a]The primer pair 103F/1607R recovered only two high-quality sequences, and only one was *nirS*.
[b]Sampling effort spread over 3 cloning attempts using different PCR annealing conditions.
[c]—, no clones related to that species were recovered.

Despite covering nearly the same region of the *nirK* gene (*Alcaligenes faecalis* positions 526 to 1040 and 568 to 1040) the two *nirK* primer sets exhibited unequal specificities (Table 1). From the vent 2 sample, we obtained only weak amplification with nirK1F/5R primers that resulted in a single clone related to *nirK*, as well as several that were not *nirK*. Using the F1aCu/R3Cu primers, we sequenced 70 clones that grouped into 19 OTUs (85% amino acid similarity). From the vent 4 sample, we sequenced 94 clones with nirK1F/5R primers. Thirty-eight of these were not *nirK*, and the remaining 56 grouped into 3 OTUs, with 52 of those sequences belonging to a single OTU. A true comparison of the two primer sets is not possible, as neither sample amplified well with both primer sets; however, it is worth noting that the single *nirK* clone from vent 2 produced using the nirK1F/5R primers was not found in that sample using the F1aCu/R3Cu primers but was 99% identical to the most frequently recovered clone from vent 4 using the nirK1F/5R primers.

Epsilonproteobacterial *nirS* Sanger sequence libraries. Three of the four pairs of newly designed primers successfully amplified environmental *nirS* from the epsilon-proteobacterial genera from which they were designed (Table 3); however, the primer pair EPSnirS103F/1607R did not perform well on our Bay of Plenty samples. For this pair, we attempted steadily lower PCR annealing temperatures on both samples but only achieved weak amplification of the vent 4 sample using a 46°C anneal. Cloning and sequencing of the product resulted in a single *nirS* sequence with 94% similarity to *Nitratifractor salsuginis*. All other sequences were poor quality or had a BLAST result other than *nirS*.

The primers EPSnirS1100R and EPSnirS1105R, which targeted nearly identical regions, were both paired with EPSnirS511F. The 511F/1105R pair was only cloned and sequenced from one sample to assess its performance relative to the 511F/1100R pair. Both pairs performed well when tested on the vent 4 sample by producing clones with BLAST results related to three to four of the five species for which the primers were designed (Table 3 and Fig. 3). The 511F/1100R pair did not recover any clones related to *Sulfurimonas gotlandica*, and this could simply be due to sampling effort (only 37 clones sequenced). *Sulfurimonas denitrificans* was not amplified by any primer set in either sample, which was not unexpected considering that this species was not seen in our 16S Sanger sequence libraries.

The primer pair EPSnirS103F/1492R was potentially the most useful, as it targets the largest fragment of the *nirS* gene. However, it seemed to show preferential amplification of the genus *Sulfurovum* (58 of the first 80 clones sequenced from both samples

FIG 2 Legend (Continued)
Numbers preceding organism names are accession numbers. Numbers in parentheses after clones denote the size of nonsingleton OTUs. Numbers after shared OTUs (green) are given as the size of the OTU in the nirS1F/6R library followed by the size of the OTU in cd3aF/R3cd library). Other hydrothermal vent *nirS* clones are in bold type. Bootstrap values from 100 replicates indicated only at major nodes. Scale bar represents 0.3 substitutions per site.

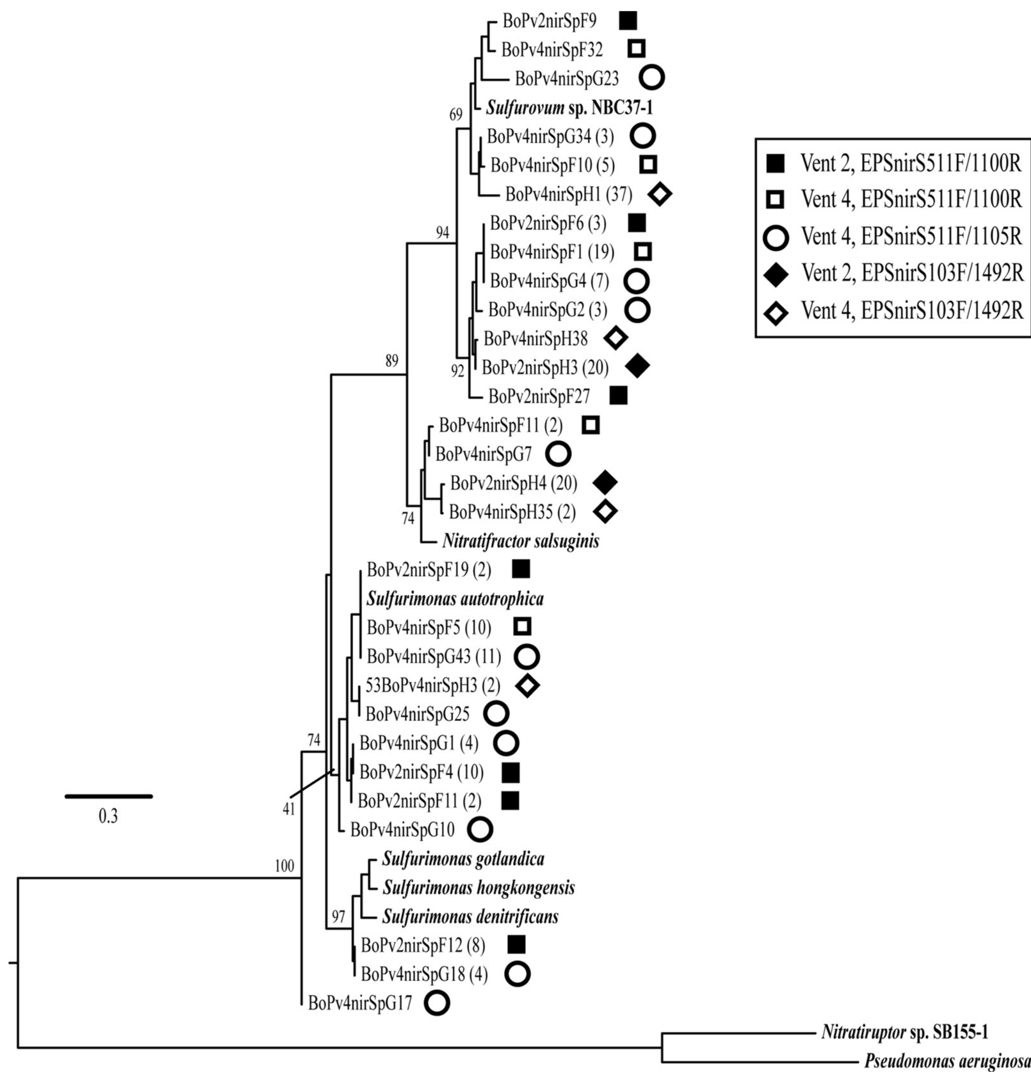

FIG 3 Maximum likelihood tree of 347 deduced amino acid positions of *nirS* genes from *Epsilonproteobacteria* and related Bay of Plenty clones. Numbers in parentheses after clones denote the size of nonsingleton OTUs. Filled symbols are clones from vent 2; open symbols are clones from vent 4. Symbol shape indicates the primer set used (see key). Bootstrap values from 100 replicates are indicated only at major nodes. Scale bar represents 0.3 substitutions per site.

using a 56°C annealing temperature). For this reason, we tested decreasing PCR annealing temperatures using the vent 4 sample to look at differences in the *nirS* genes recovered. We sequenced 40, 31, and 23 clones from the 56°C, 53°C, and 50°C annealing PCR products, respectively. At all three temperatures, the most frequently recovered sequence was related to *nirS* from *Sulfurovum* sp. NBC37-1 (38, 28, and 21 clones, respectively), with ≤2 sequences related to *Nitratifractor salsuginis*. Only two sequences related to *Sulfurimonas autotrophica* were recovered from the 53°C-annealed product, which seems more an effect of limited clone picking than PCR conditions.

Quantitative PCR. Of the three epsilonproteobacterial *nirS* primer pairs that were tested for quantitative PCR, the EPSnirS103F/530R pair outperformed the others by successfully amplifying both environmental DNA and pure-culture DNA from all five of the culture collection species. The primer pair EPSnirS1084F/1492R amplified standards and environmental samples well but did not amplify DNA from *Sulfurimonas gotlandica* or *Sulfurovum lithotrophicum* (data not shown). The primer pair EPSnirS511F/698R was tested using a range of annealing temperatures from 55 to 63°C but was unable to amplify DNA from any environmental or culture DNA tested. Melt curves performed with pure-culture DNA exhibited genus-specific melt peaks as follows: *Sulfurimonas*,

TABLE 4 Abundance and standard errors of 16S rRNA and *nirS* genes in Bay of Plenty microbial mat samples

Gene	Copies/ng DNA		% bacteria	
	Vent 2	Vent 4	Vent 2	Vent 4
Bacterial 16S	1.72×10^5 ($\pm 1.91 \times 10^4$)	8.27×10^5 ($\pm 8.91 \times 10^4$)		
nirS	3.14×10^3 ($\pm 4.82 \times 10^2$)	5.41×10^1 ($\pm 1.99 \times 10^1$)	1.82 (±0.28)	0.01 (±0.002)
Epsilonproteobacteria nirS	3.38×10^2 ($\pm 5.46 \times 10^1$)	4.96×10^3 ($\pm 3.16 \times 10^2$)	0.20 (±0.03)	0.60 (±0.04)

79°C; *Sulfurovum*, 82°C; and *Nitratifractor*, 86°C. This was seen as multiple peaks in the melt curves of the environmental samples and seemed to track the GC% of the *nirS* gene in the different genera (*Sulfurimonas*, 37.3 to 38.3 mol%; *Sulfurovum*, 39.0 mol%; and *Nitratifractor*, 53.3 mol%). EPSnirS103F/530R was used for further quantitative measures of denitrifying *Epsilonproteobacteria* in Bay of Plenty microbial mat samples.

Gene copies of *nirS* and epsilonproteobacterial *nirS* were generally two orders of magnitude less abundant than bacterial 16S rRNA genes (Table 4), with standard *nirS* genes being more abundant at vent 2 and epsilonproteobacterial *nirS* more abundant at vent 4. In both cases, however, these groups made up less than 2% of the bacterial community, assuming 1 copy of the 16S rRNA gene per cell. Bacteria are known to possess as many as 15 copies of the 16S rRNA gene (50), with an average number of 4.2 copies per genome (51), yet *nirS* genes exist as single copies in a cell (2). Even if we consider the total bacterial abundance to be 4.2 times less than our measured copies per nanogram of DNA, *nirS* genes would still account for less than 10% of the bacterial population in both samples. An additional caveat to these estimates comes from variability in the efficiencies of the qPCRs between bacterial 16S and *nirS* genes. While bacterial reactions typically had efficiencies of around 100%, reaction efficiencies for *nirS* genes tended to be between 58% and 91%.

DISCUSSION

Comparing the diversity of denitrifying bacteria within and between natural systems is complicated by the existence of two forms of nitrite reductase as targets for diversity studies and multiple-primer options for analyzing them, each with its own PCR biases (52). While a standard approach is needed, it must permit the reliable detection of spatial or temporal shifts in denitrifier populations and their relationship to environmental change. Failure to amplify the *nirS* or *nirK* gene from a sample may indicate absence of these genes and therefore conditions unfavorable to denitrifying organisms, but it may also be attributable to poor primer choice, an issue we have addressed in this study.

While primer selection based on precedent allows a comparison of results to those of previous studies, it falls short of being optimal if the selected primers only anneal to a small fraction of organisms containing the gene of interest. Such is the case for the nirS1F/6R primers frequently used to assess denitrifier diversity. Our findings from the Calypso field vent 2 microbial mat support those of Throbäck et al. (29), namely, that the primers cd3aF/R3cd capture a wider diversity of *nirS*-containing denitrifiers than nirS1F/6R. The cd3aF/R3cd primer pair captured members of the same clusters as the nirS1F/6R primers, as well as several OTUs that represented potentially novel denitrifiers, not likely discovered using nirS1F/6R due to large numbers of sequence mismatches in the priming region.

It is worth noting that other studies have produced more consistent results using both of these *nirS* primer sets. Braker et al. (53) found comparable sequence variability using nirS1F/6R and cd3aF/R3cd primers in soil samples, which harbor many of the organisms the nirS1F/6R primers were designed to target. Similarly, the same *nirS* clusters were identified using both primer sets on water column samples from the northeastern Black Sea (22); however, nirS1F/6R amplified throughout the suboxic zone, while the cd3aF/R3cd primers were successful only in the lower suboxic zone. Therefore, while using only one of these primer sets can produce skewed diversity assessments, it is not always the case and may be environmentally dependent.

These two existing primer sets can completely fail in environments favoring denitrifying *Epsilonproteobacteria*, as evidenced by our inability to amplify *nirS* from the vent 4 sample using standard primers. Both 16S rRNA sequencing and qPCR results showed that *Epsilonproteobacteria* vastly outnumbered standard *nirS*-containing denitrifiers in this sample, but *nirS* genes were successfully amplified only using our newly designed primers. Even in the less extreme case of the vent 2 sample, where *nirS* genes were amplified using standard primers, 16S rRNA sequencing revealed that 66% of the denitrifier OTUs belonged to epsilonproteobacterial genera, indicating that ignoring the *nirS*-containing *Epsilonproteobacteria* in this environment would have meant ignoring the bulk of denitrifier diversity.

Three of the four sets of epsilonproteobacterium-specific primers designed for amplification and sequencing were considered successful based on recovery of clones related to all three genera for which they were designed. The pair EPSnirS511F/1105R slightly outperformed EPSnirS511F/1100R by recovering clones related to all target *Epsilonproteobacteria* with relatively even frequency and creating a 594-bp amplicon. For sequencing of a larger portion of the gene (1,389 bp), the EPSnirS103F/1492R pair worked moderately well but seemed to have some bias toward amplification of *Nitratifractor* and *Sulfurovum.* Interestingly, clones from the two libraries created using the EPSnirS103F/1492R primers had lower relatedness to the cultured epsilonproteobacterial *nirS* genes than that from the other two successful primer pairs. This may be indicative of novel denitrifying species that inhabit microbial mats in this environment and which are captured preferentially by the EPSnirS103F/1492R primer pair.

Quantitative PCR tests indicated that the EPSnirS103F/530R primer pair was best suited to quantification of all five target *Epsilonproteobacteria*. Using these primers and cd3aF/R3cd, we found low ($<$2%) copy numbers of both standard *nirS* and epsilonproteobacterial *nirS* relative to bacterial 16S rRNA genes. Determining the true proportion of bacteria capable of denitrification in environmental samples is complicated by varied copy numbers of the 16S rRNA gene used to quantify bacteria and by the different efficiencies of 16S rRNA and *nirS* primers. Nonetheless, our implementation of a multiple-primer approach that included the denitrifying *Epsilonproteobacteria* allowed us to quantify differences in the dominant denitrifiers between the two Calypso vent field mat samples.

Discrepancies between functional measures of denitrification, such as rate measurements, isotopic composition, and gene transcripts, as well as diversity and abundance measures of denitrifiers have been reported for both hydrothermal vents and anoxic basins. Bourbonnais et al. (24) found low diversity and an abundance of *nirS*-containing bacteria in diffuse hydrothermal vent fluids that were reportedly dominated by *Epsilonproteobacteria*, despite finding other evidence for substantial denitrification (44, 45). Similar discrepancies between rate measurements (54–56) and *nirS* gene studies (12, 20, 21) have been reported for the Baltic Sea, where *Epsilonproteobacteria* of the genus *Sulfurimonas* are prominent (57, 58), yet *nirS* community analysis found no genes related to this group (21). In both of these locations, the dominant *Epsilonproteobacteria* were not recovered using standard primers for diversity assessment of *nirS* genes, suggesting a need for a multiple-primer approach to better understand the structural dynamics of denitrifying communities in environments that favor autotrophic denitrification.

While we did not focus on *nirK* in this study, our amplification and cloning results for this gene suggested a potential bias in the two *nirK* primer pairs we investigated. Recent research by Helen et al. (59), supporting the presence of two distinct clades of *nirK*-containing denitrifiers, highlights the divergent nature of these gene sequences and the inadequacies of currently available *nirK* primer sets for assessment of their diversity in nature. This suggests the need for rigorous testing of *nirK* primers similar to that applied here for *nirS* in order to determine specific biases toward the two recognized clades of *nirK* denitrifiers.

This study has demonstrated that a multiple-primer approach can better resolve structural shifts in denitrifying community composition than can the standard single-

primer approach or rate measurements of denitrification, especially under conditions that favor autotrophic epsilonproteobacterial denitrifiers. The addition of our newly developed epsilonproteobacterial primers to the suite of commonly used primer sets will not provide a complete and comprehensive assessment of denitrifier diversity. However, it does markedly increase the breadth of denitrifiers that can be monitored using PCR-based assessments of diversity. Despite the decreasing cost of metagenomic analyses, PCR-based marker gene analyses are highly suited for rapid (and low-cost) assessment of functional redundancy and changes in microbial community diversity related to specific rate processes in time-series and spatial surveys. While a hydrothermal vent setting was used to demonstrate the effectiveness of this approach, our findings are applicable to a wider environmental context wherever oxygen limitation combines with sulfidic conditions to favor autotrophic denitrification by *Epsilonproteobacteria*.

MATERIALS AND METHODS

Sample collection. The Calypso hydrothermal field is located in the offshore section of the Taupo Volcanic Zone in the Bay of Plenty, off the North Island of New Zealand. Detailed descriptions of the Calypso vents can be found elsewhere (60–62) but briefly, venting in this area is characterized by large bubble plumes of gaseous CO_2 and H_2S, high levels of liquid and gaseous hydrocarbons, and large anhydrite mounds. The presence of numerous small patches of microbial mats is also a common feature of the venting areas. Microbial mat samples were collected from vents 2 and 4 of the Calypso hydrothermal field using a suction sampler on the remotely operated vehicle *ROPOS* during cruise SO-192/2 of the RV Sonne in April and May 2007. Vents 2 and 4 were located at 185 m depth in the southwestern and southeastern vent fields, respectively, which lie ~2 km apart. Mat samples were allowed to settle for 1 to 2 h in the suction jars at 4°C. Overlying water was decanted off, and the mats were transferred into sterile 50-ml tubes and stored at −80°C until DNA extraction.

DNA extraction and amplification. Samples of microbial mat were thawed in a 40°C oven for 10 min and centrifuged for 5 min at 3,000 × *g*, and the supernatant was removed before DNA extraction using a soil extraction kit (Mo Bio Laboratories, Inc.). The wet weights of microbial mat material were 5.5 and 2.0 g from samples R1040-003 (vent 2) and R1040-010 (vent 4), respectively. DNA extracts were cleaned using a QIAquick PCR purification kit (Qiagen), and DNA yields were measured on a TD700 fluorometer (Turner Designs) using the Quant-iT PicoGreen double-stranded DNA (dsDNA) assay (Invitrogen).

Bacterial 16S rRNA genes were amplified using the primers 8Fb (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492Rb (5′-GGTTACCTTGTTACGACTT-3′) (Eurofins MWG Operon). *nirS* and *nirK* genes were each amplified using two different primer pairs: nirS1F/nirS6R (8) and cd3aF/R3cd (29, 30) for *nirS*, and nirK1F/nirK5R (8) and F1aCu/R3Cu (63) for *nirK*. Each 20-μl amplification reaction mixture consisted of 1× buffer (2.5 mM $MgCl_2$; Promega), 0.2 mmol each deoxyribonucleoside triphosphate (dNTP), 0.25 μmol each primer, 1 U of GoTaq polymerase (Promega), 1 to 2 μl of DNA, and DNase-free water to final volume. Amplification reactions were carried out on a iCycler (Bio-Rad) and consisted of an initial denaturation step of 94°C for 2 min, followed by 27 cycles of 94°C for 30 s, either 54°C (16S), 56°C (*nirS*), or 52°C (*nirK*) for 45 s, and 72°C for either 2 min (16S) or 1 min (*nirS* and *nirK*), followed by a final extension step at 72°C for 10 min.

Amplification products were visualized using a Gel Doc EZ (Bio-Rad) under UV light in a 1% agarose gel stained with SYBR Safe DNA stain (Invitrogen). In order to minimize the effects of PCR bias in any given reaction, four replicate reactions were performed on every sample. Amplification products were reconditioned to minimize heteroduplexes (64) by using 2 μl of PCR product as the template in a fresh PCR cocktail and running an additional 3 cycles of amplification, including initial denaturing and final extension steps.

Clone library construction and Sanger sequencing. Replicate reaction mixtures were combined and concentrated using a MinElute PCR purification kit (Qiagen) and cloned using the pGEM-T vector system (Promega). The insert sizes of individual clones were verified by PCR using vector-specific M13 primers. Inserts were sequenced at the University of Washington's High Throughput Genomics Center (Seattle, Washington) on an ABI 3730xl DNA analyzer using the same primers as the initial amplification. Sequence reads were manually trimmed using Sequencher version 4.7 (Gene Codes Corporation, Ann Arbor, MI), and database searches for closest match and closest cultured match were performed using the BLAST tool available on the National Center for Biotechnology Information (NCBI) website. BLASTn was used for nucleotide 16S rRNA gene searches, and BLASTx was used for translated nucleotide *nirS* and *nirK* sequences. Sequences were grouped into OTUs using DOTUR (65), with 97% nucleotide and 85% amino acid identities for 16S rRNA and the *nirS* or *nirK* gene, respectively. Library sequencing coverage was determined according to the formula coverage $= 1 - (n/N)$, where n is the number of OTUs represented by only one clone and N is the total number of clones sequenced (66).

454 sequencing and analysis. A separate aliquot of genomic DNA from each sample was sent to the Laboratory for Advanced Genome Analysis (Vancouver Prostate Centre Core Facility, Vancouver, Canada) for bacterial 16S rRNA gene tag sequencing of the V1 to V3 hypervariable regions for whole-community comparison between the two samples. Amplicon libraries were generated using fusion primers (Integrated DNA Technologies) consisting of the 5′ Roche A and B adapters for forward and reverse primers, respectively, followed by a multiplex identifier (MID) in the forward primer only and the template-specific

TABLE 5 Epsilonproteobacterial *nirS* primers designed and optimum PCR conditions used

Primer or primer combination	Sequence or annealing temp (°C)[a]	*S. denitrificans* positions or amplicon length (bp)
EPSnirS103F	5′-AAAGAGTGYCARGGKTGTC-3′	103–122
EPSnirS511F	5′-GGWTTYGCKGTWCACGTMAC-3′	511–530
EPSnirS530R[b]	5′-GTKACGTGWACMGCRAAWCC-3′	511–530
EPSnirS698R[b]	5′-CCHGCCAKNASRTATTTDCC-3′	679–698
EPSnirS1084F[b]	5′-AAGCCVCACCCMGGWCARGG-3′	1084–1100
EPSnirS1100R	5′-TGWCCKGGGTGBGGCTT-3′	1084–1100
EPSnirS1105R	5′-AACCYTGWCCKGGGTGBGG-3′	1087–1105
EPSnirS1492R	5′-CATGGTTAGCAGGCTCAGC-3′	1474–1492
EPSnirS1607R	5′-GARTAWGTRAANGTMGGWG-3′	1589–1607
EPSnirS103F/EPSnirS1492R	56	1,389
EPSnirS103F/EPSnirS1607R	46–56 tested[c]	1,504
EPSnirS511F/EPSnirS1100R	56	589
EPSnirS511F/EPSnirS1105R	56	594
EPSnirS103F/EPSnirS530R[b]	58	427
EPSnirS511F/EPSnirS698R[b]	55–63 tested[c]	187
EPSnirS1084F/EPSnirS1492R[b]	58	408

[a]Degenerate base codes: Y, C/T; R, A/G; K, G/T; W, A/T; M, A/C; S, G/C; H, A/C/T; D, A/G/T; V, A/C/G; B, C/G/T; N, A/G/C/T.
[b]For use in qPCR.
[c]No optimum condition was found.

sequences 63F and 519R (67). Emulsion PCR and sequencing were set up according to Roche's protocols for Lib-L type libraries, and sequencing was carried out on a 454 GS FLX+ Titanium system (Roche/454 Life Sciences). The results were analyzed with Roche software (GS Run Processor, version 2.9), with signal processing set to shotgun sequencing.

The mothur software package version 1.32.1 (68) was used to quality filter the raw reads, according to the recommendations by Huse et al. (69), and to cluster sequence reads into operational taxonomic units (OTUs) using a 97% similarity threshold. Sequence processing was performed according to the analysis example "454 SOP" on the mothur webpage (http://mothur.org/wiki/454_SOP). Taxonomic identification of OTUs was determined in mothur using the Silva nr_119 database as a reference. Near-full-length bacterial 16S rRNA gene Sanger sequences were trimmed to the length of 454 sequence tags and clustered into OTUs with 454 sequences, and then a BLAST result of the near-full-length sequence was used to verify taxonomic identifications of short sequence OTUs. Shannon and inverse Simpson diversity estimators were also calculated in mothur using the 97% OTU definition.

Design and testing of epsilonproteobacterium-specific *nirS* primers. Primers targeting the *nirS* gene of the *Epsilonproteobacteria* were designed using the aligned *nirS* nucleotide sequences of *Sulfurimonas denitrificans* DSM 1251, *Sulfurimonas gotlandica* GD1, *Sulfurimonas autotrophica* DSM 16294, *Sulfurovum* sp. NBC37-1, and *Nitratifractor salsuginis* DSM 16511. Potential primer regions were identified using the Design Probes function in ARB (70) and Primaclade (71). Degeneracies were introduced into the primer sequences as needed to allow coverage of all five species (Table 5), and primers were synthesized by Invitrogen (Carlsbad, CA).

Optimal annealing temperatures were determined for each primer pair by running parallel PCRs along a temperature gradient during the annealing step of each cycle and selecting the highest temperature at which there was strong amplification. The specificities of primer combinations listed in Table 5 were evaluated using *nirS* PCR, cloning, and sequencing of the Bay of Plenty microbial mat DNA as described above, with the following modification: a 45-s extension time was used for PCR cycling using the EPSnirS511F/1100R and EPSnirS511F/1105R primer pairs to mitigate the shorter amplicon size. The primer pair EPSnirS103F/1492R was amplified and cloned using three different annealing temperatures (56°C, 53°C, and 50°C) with the vent 4 sample to test for variability in the specificity of the primers with decreasing reaction stringency.

Phylogenetic analysis of *nirS* genes. Representatives of each *nirS* OTU from the nirS1F/6R and cd3aF/R3cd libraries (vent 2) and the five epsilonproteobacterial *nirS* libraries (both samples) were translated into amino acids and aligned, along with their closest database relatives, using TranslatorX (72). Maximum likelihood trees were constructed with GARLI (73) under the LG+G+F model of protein evolution, as chosen by ProtTest (74) using the Akaike's information criteria (AIC) for selecting both the *nirS* and epsilonproteobacterial *nirS* data sets. One hundred bootstrap replicates were performed and a consensus tree was produced using PAUP* version 4.0a146 (75).

Real-time qPCR. The abundance of *nirS* genes was determined using the cd3aF/R3cd primer pair (see "DNA extraction and amplification, above), and the primer pairs EPSnirS103F/530R, EPSnirS511F/698R, and EPSnirS1084F/1492R (Table 5) were evaluated for their ability to quantify the abundance of epsilonproteobacterial *nirS* genes. Success was measured by the ability of each primer pair to amplify both environmental and pure culture epsilonproteobacterial DNA obtained from the German Culture Collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ]). Pure-culture DNA was tested from the five species used to design the primers (see "Design and testing of epsilonproteobacterium-specific *nirS* primers," above), with the exception of *Sulfurovum lithotrophicum*,

which was used instead of *Sulfurovum* sp. NBC37-1. Total abundance of bacterial 16S rRNA genes was determined by qPCR using the primers BAC331F (5′-TTCTACGGGAGGCAGCAGT-3′) and BAC797R (5′-GGACTACCAGGGTATCTAATCCTGTT-3′) (76).

Plasmid DNA was extracted from bacterial, *nirS*, and epsilonproteobacterial *nirS* clone libraries described above using a miniprep plasmid extraction kit (Qiagen) and further prepared as described in reference 77, beginning with DNase treatment. Three standards were prepared by combining equal amounts of two to three plasmids each from bacterial, *nirS*, or epsilonproteobacterial *nirS*-containing clones. A 10-fold dilution series of each plasmid mixture, ranging from 10^7 to 10^1, was used to produce standard curves with R^2 values of >0.995. Each 10-μl reaction mixture consisted of 5 μl of SsoFast EvaGreen Supermix (Bio-Rad), 0.5 μM each forward and reverse primer, 2 μl of DNase-free water, and 1 μl of DNA template. Reactions were performed in triplicate on a CFX96 real-time PCR detection system (Bio-Rad) and consisted of an initial denaturation for 2 min at 95°C, followed by 45 cycles of 95°C for 5 s and 58°C for 5 s, and concluding with a melt curve from 65 to 95°C. Each run included a standard curve and a no-template control (NTC). A dilution series of Bay of Plenty DNA was analyzed to test for inhibitory reaction effects, which resulted in all successive reactions being run using a 1:10 dilution. The CFX Manager 2.0 software (Bio-Rad) was used to analyze the results. Gene copies per nanogram of DNA were calculated based on concentrations of the raw extracts that were measured at 43.7 and 28.8 ng/μl for the vent 2 and vent 4 samples, respectively.

Accession number(s). Representative clones from each bacterial 16S rRNA, *nirS*, *nirK*, and epsilonproteobacterial *nirS* OTU were deposited in GenBank under accession numbers KU242430 to KU242565. Bacterial V1 to V3 sequence tags were submitted to the NCBI Sequence Read Archive under accession no. SRP075636.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02753-16.

TEXT S1, PDF file, 0.1 MB.

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