

Spatial Distribution of Total, Ammonia-Oxidizing, and Denitrifying Bacteria in Biological Wastewater Treatment Reactors for Bioregenerative Life Support

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Bioregenerative life support systems may be necessary for long-term space missions due to the high cost of lifting supplies and equipment into orbit. In this study, we investigated two biological wastewater treatment reactors designed to recover potable water for a spacefaring crew being tested at Johnson Space Center. The experiment (Lunar-Mars Life Support Test Project—Phase III) consisted of four crew members confined in a test chamber for 91 days. In order to recycle all water during the experiment, an immobilized cell bioreactor (ICB) was employed for organic carbon removal and a trickling filter bioreactor (TFB) was utilized for ammonia removal, followed by physical-chemical treatment. In this study, the spatial distribution of various microorganisms within each bioreactor was analyzed by using biofilm samples taken from four locations in the ICB and three locations in the TFB. Three target genes were used for characterization of bacteria: the 16S rRNA gene for the total bacterial community, the ammonia monooxygenase (*amoA*) gene for ammonia-oxidizing bacteria, and the nitrous oxide reductase (*nosZ*) gene for denitrifying bacteria. A combination of terminal restriction fragment length polymorphism (T-RFLP), sequence, and phylogenetic analyses indicated that the microbial community composition in the ICB and the TFB consisted mainly of *Proteobacteria*, low-G+C gram-positive bacteria, and a *Cytophaga-Flexibacter-Bacteroides* group. Fifty-seven novel 16S rRNA genes, 8 novel *amoA* genes, and 12 new *nosZ* genes were identified in this study. Temporal shifts in the species composition of total bacteria in both the ICB and the TFB and ammonia-oxidizing and denitrifying bacteria in the TFB were also detected when the biofilms were compared with the inocula after 91 days. This result suggests that specific microbial populations were either brought in by the crew or enriched in the reactors during the course of operation.

A mission of the Advanced Life Support (ALS) Project is to develop bioregenerative life support systems to enhance the International Space Station and ultimately sustain a lunar base or a voyage to Mars. This necessitates creating a combination of physicochemical and biological systems that can regenerate resources (or materials) needed to sustain life for long time periods, possibly without the potential of resupply. Since bacteria perform recycling of various elements on Earth, it is reasonable to assume that a bioregenerative ALS system for a space vehicle or colony may also rely on bacterial activity. Understanding the microbial ecology in a biological treatment system is important for designing and operating it at optimal efficiency and to ensure reliability. In 1997, two fixed-film biological wastewater treatment reactors were tested by the National Aeronautics and Space Administration (NASA) at the Johnson Space Center (JSC) during a 91-day simulated long-term manned space mission. This experiment (Lunar-Mars Life Support Test Project—Phase III) consisted of four crew members confined in a test chamber. The system utilized an immobilized cell bioreactor (ICB) for organic carbon removal and a trickling filter bioreactor (TFB) for ammonia removal, supplemented by additional physicochemical devices for reclaiming potable water.

In this report, we characterize the microbial community structures in the ICB and the TFB. Previously, many studies have demonstrated the existence of diverse groups of microorganisms in activated sludge and in fixed-film treatment systems (7, 8, 14, 20, 22, 23, 28, 31). However, the Phase III test at JSC represents a unique opportunity in which the microbial populations responsible for treating the wastewater (and a human crew) were allowed to develop in isolation for a 3-month period. The major questions addressed in this research include (i) which microorganisms were present in the JSC bioreactors and whether they differ from those previously reported and (ii) which microorganisms, if any, were brought in by the crew or enriched during the course of the Phase III test.

To address these questions, we characterized the total, ammonia-oxidizing, and denitrifying bacterial communities in the system by using three target genes, i.e., the 16S rRNA, ammonia monooxygenase (*amoA*), and nitrous oxide reductase (*nosZ*) genes, respectively. The *amoA* gene was chosen to identify bacteria responsible for ammonia removal (24, 25), and the *nosZ* gene (26, 27) was used to detect bacteria responsible for loss of fixed nitrogen from the wastewater treatment systems. These two functional groups (nitrifiers and denitrifiers) are essential for waste processing and resource recovery of nitrogen. A functional gene approach was selected for the N-cycling groups because of concerns regarding specificity when a 16S rRNA gene approach is used for nitrifiers

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TABLE 3. Number of terminal restriction fragment peaks of three target genes in the two bioreactors

| Bioreactor layer | No. of terminal restriction fragment peaks | | |
|----------------------|--|-------------------|-------------------|
| | 16S rRNA genes | <i>amoA</i> genes | <i>nosZ</i> genes |
| ICB inoculum | 129 | NA ^a | NA |
| Top | 75 | 8 | 56 |
| Middle | 62 | 7 | 43 |
| After air injection | 110 | 5 | 50 |
| Before air injection | 111 | 4 | 57 |
| TFB inoculum | 43 | 3 | 38 |
| Top | 35 | 7 | 30 |
| Middle | 35 | 5 | 19 |
| Bottom | 28 | 5 | 34 |

^a NA, not available due to the unsuccessful PCR amplification.

as described previously (6, 12) with *TaiI* (MBI Fermentas, Amherst, N.Y.) for *amoA* and with *HinPI* (New England Biolabs) for *nosZ* fingerprints (27). (The restriction endonucleases were selected to give maximal resolution of terminally labeled fragments to known target sequences in the database.) All digestions were done in accordance with the manufacturer's instructions for 12 h. After ethanol precipitation, T-RFLP analysis was also carried out on an ABI 310 genetic analyzer with Genescan software and an internal size standard. The comparative T-RFLP data analyses were carried out by using the Combinatorial Polythetic Agglomerative Hierarchical clustering package (3; <http://www.es.umb.edu/edgwebp.htm>).

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study have been deposited in the GenBank database and assigned accession numbers AF390906 to AF390962 and AF390116 to AF390135.

RESULTS

T-RFLP analysis of the reactors. Initial characterization of the microbial communities at various places in the reactors relied on T-RFLP fingerprinting. Fluorescent amplifications were successful for nearly all of the samples, with the exception of *amoA* and *nosZ* for the ICB inoculum. It may be that ammonia-oxidizing and denitrifying bacteria were a small portion of the total community in this sample and were below the detection limit of the PCR assay (17, 18). An alternative explanation (PCR inhibitors) was not deemed likely given the 16S rRNA amplifications from the ICB inoculum did not demonstrate appreciable product suppression, and numerous attempts to eliminate any such inhibitors were unsuccessful.

In the T-RFLP fingerprints of the microbial communities, total peak numbers varied by a factor of 5 (range, 28 to 129) for SSU genes and by a factor of 3 for the functional genes (ranges, 3 to 8 for *amoA* and 19 to 57 for *nosZ*) (Table 3). The middle of the ICB contained the lowest number of T-RFLP peaks for the SSU and *nosZ* genes, while the anaerobic part of the ICB (before) harbored the lowest number of *amoA* peaks. In the TFB, the bottom and middle regions also enclosed the lowest numbers of SSU genes and *amoA* and *nosZ* genes, respectively. Not only were changes in absolute numbers of peaks discernible, but marked differences in profiles could also be seen between the inoculum and the various biofilm samples taken throughout the reactor at the end of the test (Fig. 2). Here, a

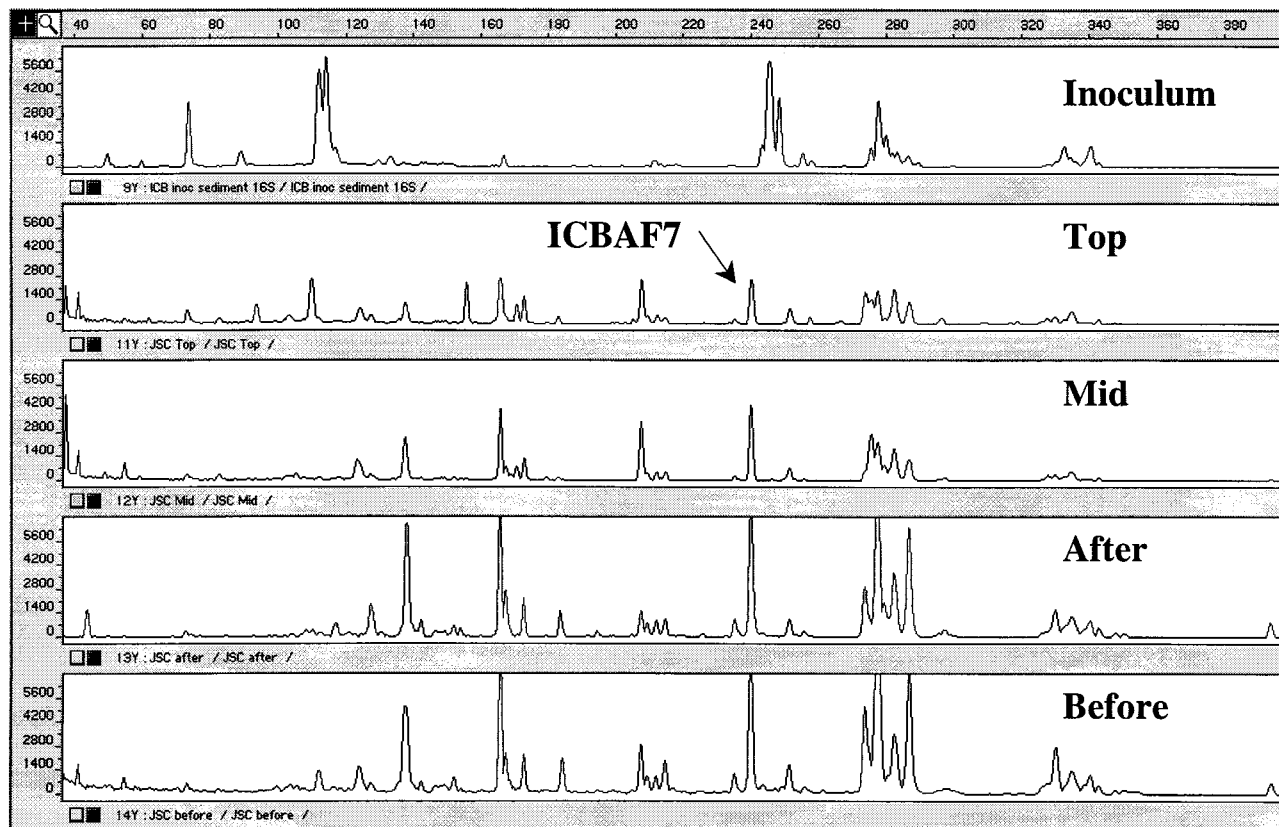


FIG. 2. T-RFLP profiles of 16S rRNA genes from biofilm samples of the ICB after digestion with *MnlI*. A unique peak (clone ICBAF7) not detectable in the inoculum but found in the final samples is indicated.

TABLE 4. Comparison of the 16S rRNA genes from the clonal libraries in the ICB to the T-RFLP fingerprint of the inoculum and to sequences in the Ribosomal Database Project

| Taxon and clone | Peak length (bp) ^a | Most similar organism | Sab ^b | I ^c | T ^d | M ^d | A ^d | B ^d |
|---------------------------------|-------------------------------|---|------------------|----------------|----------------|----------------|----------------|----------------|
| <i>Proteobacteria</i> | | | | | | | | |
| Alpha group | | | | | | | | |
| ICBTD9 | 124 | <i>Holospira obtusa</i> | 0.716 | + | ++ | -- | -- | -- |
| ICBTE8 | 211 | <i>Sphingomonas</i> sp. strain BRW2 | 0.773 | + | ++ | -- | -- | -- |
| ICBMF2 | 288 | Marine snow-associated bacterium Adriatic83 | 0.712 | + | -- | ++ | -- | -- |
| Beta group | | | | | | | | |
| ICBBA5 | 132 | Diazotroph strain BA27 | 0.938 | + | -- | -- | -- | +- |
| ICBTA10 | 164 | <i>Thauera terpenica</i> strain 58 | 0.918 | + | ++ | ++ | -- | ++ |
| ICBTC9 | 215 | <i>Brachymonas denitrificans</i> strain AS-P1 | 0.748 | + | ++ | -- | -- | -- |
| ICBBD2 | 214 | <i>B. denitrificans</i> strain AS-P1 | 0.704 | + | -- | -- | -- | ++ |
| ICBBB10 | 208 | Unidentified beta proteobacterium sewCLONE2 | 0.836 | + | -- | -- | -- | ++ |
| ICBBD7 | 277 | Type 0803 filamentous bacterium strain Ben04B ^e | 0.799 | + | -- | -- | -- | ++ |
| ICBTF8 | 276 | Unidentified bacterium strain rA1 ^e | 0.824 | + | ++ | -- | -- | -- |
| ICBTF7 | 276 | Uncultured bacterium clone KC208 | 0.784 | + | ++ | -- | -- | -- |
| ICBBC5 | 279 | Unidentified bacterium strain rA1 ^e | 0.789 | + | -- | -- | -- | ++ |
| ICBBA10 | 279 | Uncultured bacterium clone KC208 | 0.792 | + | -- | -- | -- | ++ |
| ICBMD10 | 278 | Strain SBR 2090 ^e | 0.864 | + | -- | ++ | -- | -- |
| ICBAA12 | 278 | Uncultured bacterium SJA-10 | 0.717 | + | ++ | -- | ++ | -- |
| Gamma group | | | | | | | | |
| ICBMF3 | 54 | <i>Acinetobacter lwoffii</i> strain A391 | 0.837 | + | ++ | ++ | -- | -- |
| ICBTE5 | 90 | Iron-oxidizing lithotroph ES-1 | 0.664 | + | ++ | -- | -- | -- |
| ICBBE7 | 100 | Denitrifying Fe(II)-oxidizing bacterium strain BrG3 | 0.766 | + | ++ | -- | -- | ++ |
| ICBTB11 | 126 | <i>Serratia fonticola</i> strain DSM4576 | 0.900 | + | ++ | ++ | -- | -- |
| Epsilon group | | | | | | | | |
| ICBBA6 | 136 | <i>Arcobacter cryaerophilus</i> ATCC 49615 | 0.839 | + | -- | -- | -- | ++ |
| ICBAA7 | 139 | Unidentified bacterium clone T55 ^a | 0.890 | + | ++ | -- | ++ | ++ |
| ICBAF5 | 141 | <i>A. cryaerophilus</i> ATCC 49615 | 0.908 | + | -- | -- | ++ | -- |
| ICBTE4 | 142 | <i>A. cryaerophilus</i> ATCC 49615 | 0.878 | + | ++ | -- | ++ | -- |
| ICBTA1 | 142 | <i>A. cryaerophilus</i> ATCC 49615 | 0.941 | + | ++ | ++ | ++ | ++ |
| ICBTG2 | 289 | Unidentified bacterium clone T55 ^e | 0.804 | + | ++ | ++ | ++ | ++ |
| ICBTC5 | 288 | <i>Arcobacter butzleri</i> CCUG 10373 | 0.742 | + | ++ | -- | -- | -- |
| ICBTD7 | 289 | <i>A. butzleri</i> CCUG 10373 | 0.803 | + | ++ | -- | -- | ++ |
| ICBTC7 | 289 | <i>A. butzleri</i> CCUG 10373 | 0.724 | + | ++ | -- | -- | ++ |
| <i>Cytophagales</i> | | | | | | | | |
| ICBTA3 | 44 | Uncultured eubacterium WCHB1-29 | 0.621 | + | ++ | -- | -- | -- |
| ICBAF7 | 241 | Uncultured eubacterium WCHB1-69 | 0.639 | - | -- | -- | ++ | -- |
| Low-G+C gram-positive bacteria | | | | | | | | |
| ICBBB9 | 125 | <i>Clostridium hastiforme</i> DSM 5675 | 0.731 | + | -- | -- | -- | ++ |
| ICBBB7 | 133 | <i>Lactosphaera pasteurii</i> strain KoTa2 | 0.856 | + | -- | -- | -- | +- |
| ICBA14 | 155 | <i>Acetonema longum</i> strain AP0-1 | 0.638 | + | -- | -- | ++ | -- |
| ICBBC4 | 170 | <i>Clostridium hastiforme</i> DSM 5675 | 0.774 | + | -- | -- | -- | ++ |
| ICBTA2 | 252 | <i>Clostridium viride</i> strain T2-7 | 0.531 | + | ++ | -- | -- | -- |
| ICBTG1 | 297 | Bacterium ASF500 | 0.650 | - | ++ | -- | -- | -- |
| <i>Verrucomicrobia</i> , ICBTG4 | 187 | <i>Verrucomicrobiales</i> PB90-1 | 0.635 | + | +- | +- | -- | -- |
| <i>Planctomycetes</i> , ICBMG6 | 384 | <i>Isosphaera</i> sp. strain Schlesner 666 | 0.646 | - | -- | +- | -- | -- |
| Unclassified, ICBMG3 | 187 | Unidentified eubacterium | 0.801 | + | -- | +- | -- | -- |

^a Base pair length of terminal restriction fragment peak expected based on sequence.^b Sab value in the Ribosomal Database Project database; a value of 0.9 or greater indicates a close match to a sequence in the database.^c I, inoculum. No clonal library was generated. A plus or minus indicates the presence or absence of a terminal restriction fragment peak at this length.^d T, top; M, middle; A, after air injection; B, before air injection (layers in the ICB). Within each pair, plus or minus on the left means that sequence is present or absent in the clonal library; a plus or minus on the right indicates the presence or absence of a terminal restriction fragment peak at this length.^e Sequence from activated sludge reported by other researchers.

dominant T-RFLP peak is shown that corresponds to a clone (ICBAF7) not found in the inoculum (Table 4; see below). For the nitrifying population, the observed peaks in the inoculum were only of the lengths associated with *Nitrosomonas*-like *amoA* genes (177 and 282 bp), while those in the reactor represented both *Nitrosomonas*- and *Nitrospira*-like *amoA* gene peaks (453 bp) (data not shown). Differences in the *nosZ* fingerprints between inocula and samples were observed. Some *nosZ* peaks matched the following sequences in the database: *Pseudomonas* sp.-*Sinorhizobium meliloti* (152 bp), *Achromobacter cycloclastes*-*Bradyrhizobium japonicum* (199 bp), *Paracoccus denitrificans* (153 bp), and *Rhodobacter sphaeroides* (203 bp).

Cluster analysis of the various T-RFLP fingerprints verified much of this visual inspection. The inocula always grouped outside the samples from the various places within the reactor (Fig 3) for all three target genes. This result suggests that many members of the community are lost during reactor operation or that most of the microbial inhabitants of the reactors are induced from undetectable levels in the inocula or introduced in the wastewater feed. Total communities based on 16S T-RFLP patterns demonstrated similarities ranging from 35 to 75% (Fig. 3) for the ICB and from 28 to 67% for the TFB, with much of the difference in similarity based on very small peaks (data not shown). The clustered profiles of the 16S rRNA genes demonstrated a separation between the bacterial communities in the ICB and the TFB, implying that different microbial populations had developed despite the recycling of effluent between the reactors.

The distributions of the ammonia-oxidizing bacteria based on the *amoA* profiles also showed a clustering pattern similar to that of the 16S rRNA genes. For the ICB, the after- and before-air injection layers clustered together (89%) and the top and middle layers clustered together (80%), with the three TFB samples also clustering (83%) (Fig. 3). In the before- and after-air injection zones of the ICB and all three zones of the TFB, the peaks of both *Nitrosomonas*-like and *Nitrospira*-like *amoA* genes were identified; however, in the top and middle parts of the ICB, only *Nitrosomonas*-like *amoA* genes were found (data not shown). The similarity values of *nosZ* were lower than those of the other two target genes, ranging from 47 to 58% for the ICB and from 27 to 34% for the TFB. In addition, the T-RF peak profiles clustered differently from the other two genes. In the ICB, the middle-layer *nosZ* populations grouped with the before and after samples rather than with the top layer sample. In the TFB, the top and bottom samples grouped together and the middle sample was more distant.

Clonal analysis of 16S rRNA genes. To gain a better understanding of the microbial populations in the reactors, clonal libraries of 16S rRNA genes were constructed from all four layers of the ICB and from the middle layer of the TFB as described previously (12, 25). Due to the similarities of the T-RFLP profiles, only two samples from the ICB and the middle sample from the TFB were analyzed for *amoA* or *nosZ* genes. Of the 384 16S rRNA clones obtained from the ICB, 39 unique partial 16S rRNA gene sequences (541 bp) were identified. Comparison of these ICB SSU sequences to the Ribosomal Database Project database indicated low homology, with similarity (Sab) values ranging from 0.621 to 0.941 (Table 4),

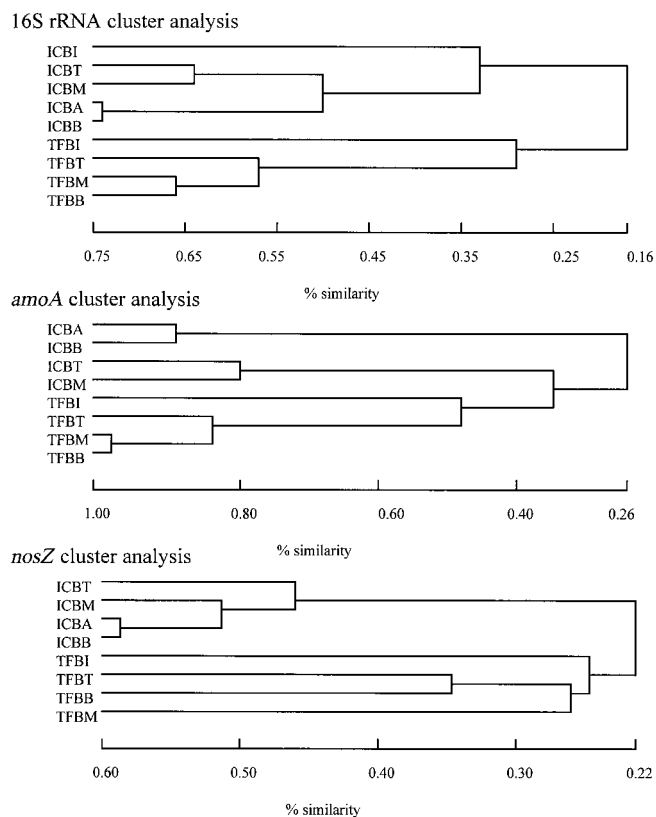


FIG. 3. Cluster analyses of the T-RFLP profiles in the ICB and TFB based on the 16S rRNA, *amoA*, and *nosZ* genes. The samples from the ICB are indicated by the following suffixes: I, inoculum; T, top layer; M, middle layer; A, after-air injection layer; B, before-air injection layer. For the TFB, the following similar suffixes are used: T, top layer; M, middle layer; B, bottom layer. The cluster analyses were performed with the Combinatorial Polythetic Agglomerative Hierarchical clustering package (COMPAH96; <http://www.es.umb.edu/edgwebp.htm>).

suggesting that many of the SSU genes found in the ICB have not been described in previous studies of waste treatment systems. Only five sequences showed high levels of homology (Sab values of >0.9) to sequences in the database. A total of 28 SSU clones were affiliated with the *Proteobacteria*. Low-G+C gram-positive and *Cytophagales*-like 16S rRNA gene sequences were also detected in many of the libraries. No distinct spatial distribution patterns were discerned based on the SSU genes in the different layers of the ICB.

In the TFB middle layer, analysis of 96 clones revealed 18 unique 16S rRNA gene sequences (Table 5). A total of 14 proteobacterium-like 16S rRNA gene sequences were detected (Sab values of 0.791 to 0.973), with 12 demonstrating high levels of similarity (Sab values of >0.9) to sequences in the Ribosomal Database Project. The remaining six clonal sequences were loosely related to the *Cytophaga-Flexibacter-Bacteroides* group, *Planctomycetales*, and *Nitrospira*.

Ammonia-oxidizing bacteria in the ICB and TFB based on the *amoA* genes. Among the 288 clones, a total of 27 different *amoA* genes (14 in the ICB and 13 in the TFB) were observed. Most shared high levels of sequence similarity to sequences in the database, ranging from 99.1% for *Nitrospira* sp. strain

TABLE 5. Comparison of the 16S rRNA genes from the middle layer of the TFB to the T-RFLP fingerprints from the inoculum and other parts of the reactor and sequences in the Ribosomal Database Project

| Taxon and clone | Peak length (bp) ^a | Most similar organism | Sab ^b | I ^c | T ^c | M ^c | B ^c |
|------------------------------|-------------------------------|---|------------------|----------------|----------------|----------------|----------------|
| <i>Proteobacteria</i> | | | | | | | |
| Alpha group TFBMC2 | 252 | <i>Ochrobactrum tritici</i> strain SCII24 | 0.973 | + ^d | – | + | + |
| Beta group | | | | | | | |
| TFBMD9 | 208 | <i>Dechlorisoma suila</i> strain PS | 0.935 | + | + | + | + |
| TFBMF4 | 165 | Unidentified bacterium strain rJ13 ^e | 0.833 | + | + | + | + |
| TFBME9 | 164 | <i>Ralstonia eutropha</i> strain CH34 | 0.931 | + | + | + | + |
| TFBMA4 | 219 | <i>Alcaligenes</i> sp. strain NKNTAU | 0.904 | – | – | – | – |
| TFBMA5 | 132 | Diazotroph strain BA27 | 0.938 | – | – | – | – |
| Gamma group | | | | | | | |
| TFBMH1 | 126 | <i>Enterobacter agglomerans</i> strain AH16 | 0.964 | – | – | + | + |
| TFBMF5 | 126 | <i>Enterobacter</i> sp. | 0.933 | – | – | + | + |
| TFBMB11 | 126 | <i>Enterobacter agglomerans</i> strain AH16 | 0.791 | – | – | + | + |
| TFBMH12 | 126 | <i>Enterobacter agglomerans</i> strain AH16 | 0.907 | – | – | + | + |
| TFBMH2 | 101 | <i>Stenotrophomonas nitritireducens</i> strain L2 | 0.925 | – | – | – | – |
| TFBME10 | 237 | Unidentified gamma proteobacterium strain R2A30 | 0.962 | – | – | – | – |
| TFBME4 | 279 | <i>Pseudomonas</i> sp. strain CRE 12 | 0.937 | – | – | – | – |
| TFBMC1 | 279 | <i>Pseudomonas</i> sp. strain CRE12 | 0.905 | – | – | – | – |
| <i>Cytophagales</i> , TFBMF3 | 97 | Unidentified bacterium Riz1026 | 0.708 | – | – | – | – |
| <i>Nitrospira</i> , TFBMG11 | 50 | Unidentified eubacterium | 0.802 | + | + | + | + |
| <i>Planctomycetes</i> | | | | | | | |
| TFBME6 | 111 | <i>Gemmata obscuriglobus</i> UQM446 | 0.554 | – | + | – | – |
| TFBMI5 | 386 | <i>Isosphaera</i> sp. strain Schlesner 666 | 0.659 | – | – | – | – |

^a Length of terminal restriction fragment peak.

^b Sab value in the Ribosomal Database Project; a value of 0.9 or greater indicates a close match to a sequence in the database.

^c I, inoculum; T, top; M, middle; A, after air injection; B, before air injection (layers in the ICB).

^d A plus indicates that a terminal restriction fragment peak was found at this length; a minus indicates that none found.

^e Sequence from activated sludge reported by other researchers.

Np39-19 to 100% for *N. europaea* and were considered proof that these nitrifying microorganisms inhabited the bioreactors. However, four novel *amoA* genes were obtained that were more distantly related to *amoA* genes in the database. Two sequences (clones ICBTG2 and ICBBA2) appeared to be affiliated with the *N. europaea* cluster as defined in references 22 and 29 (Fig. 4), showing 93.5 and 93.9% similarity at the nucleotide level and 98.8 and 98.2% similarity at the amino acid level to *N. europaea*, respectively. Two other novel sequences (clones ICBBC11 and ICBBG2) grouped with the *Nitrosospira multififormis* lineage, with 92.7 and 97.1% similarity at the nucleotide level and 97.1% similarity for both at the amino acid level to *N. multififormis*, respectively. No clones were affiliated with the *Nitrosomonas eutropha* or other *Nitrosospira* branches (e.g., *N. briensis* and *N. tenuis*).

In the TFB, a total of four unique *amoA* sequences (TFBMH12, TFBME3, TFBMH4, and TFBMB10) were identified (Fig. 4). The clone TFBMH12 sequence had 94.5 and 99.4% identity with the *N. europaea amoA* gene at the nucleotide and amino acid levels, respectively. The other three sequences were close to the *N. multififormis amoA* gene, with similarities ranging from 89.9 to 92.1% and from 96.4 to 97.7% at the nucleotide and amino acid levels, respectively. The clone TFBMB10 sequence, however, appeared to be more closely related to the clone ABIAC5 sequence (97.3% identity at the

nucleotide level and 98.8% identity at the amino acid level), which was present in an ammonia biofilter reported previously (25). Many of the *amoA* genes found in these bioreactors clustered at the periphery of the known groups of nitrifying bacteria.

Denitrifying bacteria in the ICB and TFB based on the *nosZ* genes. In the ICB, nine novel partial *nosZ* genes (489 bp) were obtained from the clonal libraries generated from the top and before-air injection layers, and their affiliation is shown in a reconstructed phylogenetic tree in Fig. 5. Various degrees of similarity were observed between the clonal *nosZ* gene sequences from the bioreactors and known cultured denitrifying bacteria (*Pseudomonas* sp., *Achromobacter* sp., and *Sinorhizobium* sp.) and environmental *nosZ* sequences in the GenBank database. The similarities ranged from 76.7 to 94.2% at the nucleotide level and from 68.0 to 94.2% at the amino acid level. One *nosZ* sequence, ICBBE12, was nearly identical to an environmental clone from marine sediments (696O; 99.7% identity at the nucleotide level and 100% identity at the amino acid level) (26). In the TFB, three unique *nosZ* genes were identified in the middle-layer clonal library (Fig. 5) and the clone TFBB2 sequence appeared to be related to the *P. stutzeri nosZ* gene, with similarities of 89.6% (nucleotide level) and 88.9% (amino acid level). Clone TFBD2 showed sequence similarities to the *P. fluorescens nosZ* gene of 86.5% (nucleo-

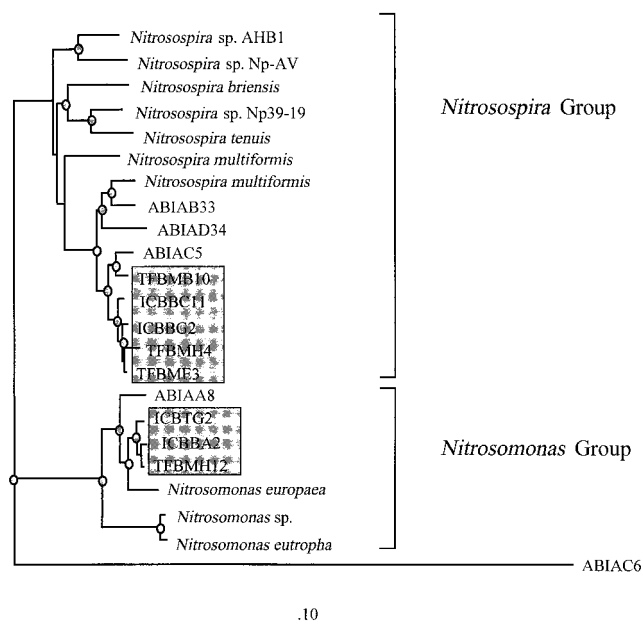


FIG. 4. Phylogenetic tree based on the partial *amoA* genes (513-bp alignment) in the ICB and TFB reconstructed by the neighbor-joining method. Nodes with more than 50 bootstrap iterations (of 100) are highlighted by circles. Clonal sequences obtained in this study are highlighted. The accession numbers of the *amoA* genes are as follows: *Nitrosomonas europaea*, L08050; *Nitrosomonas eutropha*, U51630; *Nitrosomonas* sp., AB031869; *Nitrosospira briensis*, U76553; *Nitrosospira multififormis*, AF042171; *Nitrosospira multififormis*, U89833; *Nitrosospira* sp. strain AHB1, X90821; *Nitrosospira* sp. strain Np39-19, AF006692; *Nitrosospira* sp. strain NpAV, AF016003; *Nitrosospira tenuis*, U76552; *Nitrosococcus oceani*, U96611; bioreactor samples with an ABIA prefix, AF070983 to AF070987.

tide level) and 88.9% (amino acid level). Clone TFBC2 was affiliated with the *P. denitrificans* clade at 94.7% (nucleotide level) and 94.2% (amino acid level) similarity.

DISCUSSION

The Phase III test at JSC demonstrated an integrated biologically-physicochemically based system capable of treating and recycling wastewater for consumption by a human crew (19). This analysis was carried out to obtain baseline information on the microorganisms in the reactors associated with organic carbon degradation, nitrification (ammonia oxidation), and denitrification being developed at JSC and to test whether these microbial populations are similar to those of other wastewater treatment facilities. In this study, three eubacterial divisions were found in both bioreactors: *Proteobacteria* (α , β , and γ groups), *Cytophagales*, and *Planctomycetales*. The taxonomic divisions observed in this study concur with the findings of other studies associated with wastewater (reviewed in reference 11) in finding *Proteobacteria* (α , β , and γ groups), *Cytophagales*, *Planctomycetales*, *Verrucomicrobium*, and low-G+C gram-positive bacteria. However, sequences affiliated with high-G+C gram-positive bacteria and the δ group of *Proteobacteria* reported in other wastewater studies were not detected in the JSC bioreactors. Conversely, sequences belonging

to the ϵ group of *Proteobacteria* were found in the ICB in our study but not in the previous studies.

With respect to the spatial distribution of ammonia-oxidizing bacterial populations, the ICB top and middle layers contained only *Nitrosomonas*-like *amoA* genes and the before- and after-air injection sections harbored both *Nitrosomonas*- and *Nitrosospira*-like *amoA* genes, consistent with other reports for a bioreactor (25) and the environment (10, 24, 22, 29). The detection of *Nitrosospira*-like *amoA* may not be a surprise, in contrast to its "sporadic" detection in other wastewater treatment systems, as noted in reference 22, since biofilms may provide a greater diversity of niches compared with activated sludge. However, questions have been raised about whether a T-RFLP approach can adequately resolve *amoA* sequences, especially when using the Holmes priming set as we have in this study (22).

For this fingerprinting exercise, we used the enzyme *TaiI*, in contrast to previous work using *TaqI*, to distinguish *amoA* genes (10). Although no restriction enzyme is able to resolve all of the possible target genes for T-RFLP analysis, the *TaiI* enzyme can give peaks to diagnostic groups such as the *Nitrosomonas*-like (177 and 282 bp) and *Nitrosospira*-like (483 bp) groups. Improved resolution with T-RFLP approaches will require multiple digestions to fully resolve the various components of the different clusters. As for the Holmes priming set, in reference 22 it is reported that there exist up to six mismatches for members of the *N. europaea*-like cluster, which implies that no PCR product will be obtained when this priming set is used. However, in this and a previous study of biofilters (25), *N. europaea*-like genes have been amplified and characterized. Although these primers contain mismatches,

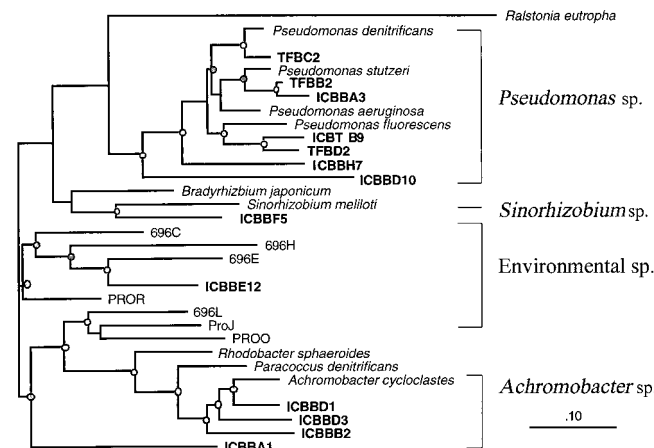


FIG. 5. Phylogenetic tree based on the partial *nosZ* genes (489-bp alignment) in the ICB and TFB reconstructed by the neighbor-joining method. Nodes with more than 50 bootstrap iterations (of 100) are highlighted by circles. Designations of clonal sequences obtained in this study start with ICB or TFB. The accession numbers of the *nosZ* genes are as follows: *Achromobacter cycloclastes*, AF047429; *Bradyrhizobium japonicum*, AJ002531; *Paracoccus denitrificans*, X74792; *Pseudomonas aeruginosa*, X65277; *Pseudomonas denitrificans*, AF016059; *Pseudomonas fluorescens*, AF056319; *Pseudomonas stutzeri*, M22628; *Ralstonia eutropha*, X65278; *Rhodobacter sphaeroides*, AF125260; *Sinorhizobium melliloti*, U47133; 696C, AF119951; 696E, AF119949; 696L, AF119927; 696H, AF119947; ProJ, AF119934; ProO, AF119933; ProR, AF119937.

these do not appear to significantly inhibit the detection of *N. europaea*-like *amoA* genes from these bioreactor samples.

Additionally, the question arises of whether these *amoA* genes belong to different species of nitrifying bacteria inhabiting these bioreactors or represent divergent copies of *amoA* genes from previously described nitrifying bacteria. Purkhold et al. suggested a threshold value of 80% similarity for *amoA* genes from different species of nitrifiers based on an extensive comparison of known strains using DNA-DNA hybridization, 16S rRNA similarity, and *amoA* similarity. None of the *amoA* genes described in this study meet this threshold criterion for novel nitrifying species (similarities in these data ranged from 90 to 97%). Although we cannot unequivocally rule out the possibility that these *amoA* genes represent novel nitrifying bacteria, it is more likely that the bioreactors harbor nitrifying strains closely related to *Nitrosomonas* and *Nitrospira* cultures that have been characterized.

As for the denitrifying populations, the spatial distribution profiles of *nosZ* genes in the TFB differed slightly from those of the 16S rRNA and *amoA* genes, with the top and bottom portions of the reactor clustering together. Based on the *nosZ* T-RFLP profiles, the ICB appeared to harbor a more diverse group of denitrifying bacteria than the TFB. As with the 16S rRNA T-RFLP profiles, this finding is expected because of the higher total organic carbon concentrations (180 mg/liter on average, ranging from 74 to 500 mg/liter) and pH values (7.37 to 8.48) in the ICB influent than in the TFB influent (TOC of 16.6 mg/liter on average, ranging from 9 to 56 mg/liter, and pHs of 6.07 to 7.37) (19). The clonal analysis of *nosZ* genes suggests that the denitrifying bacteria that inhabit the bioreactors are generally related to cultured denitrifiers (*Pseudomonas-Paracoccus-Achromobacter* group); however (as with the *amoA* genes), the *nosZ* sequences all cluster at the periphery of the various known denitrifying microorganisms. This is in contrast to an environmental study in which few of the *nosZ* genes discovered were associated with any cultured denitrifying bacteria (26). In spite of this, three sequences were identified that were not closely affiliated with any known groups of *nosZ* genes. More attempts should be made to identify those microorganisms possessing unique *nosZ* genes from bioreactors and other samples to gain a better understanding of denitrifying bacteria in these systems.

In conclusion, the bioreactors at JSC were found to harbor unique heterotrophic (ϵ -*Proteobacteria*, *Cytophagales*, and *Planctomycetales*) and denitrifying populations of bacteria, but the ammonia-oxidizing populations were generally found to be closely related to known strains of nitrifiers from other wastewater treatment systems (22). This information on microbial populations is important to establish and enhance the reliability of biological water recovery systems for long-duration space flight missions. The next phase of research should focus on measuring gene expression of total and specific microorganisms, such as ammonia-oxidizing and denitrifying bacteria, to provide information about active populations in a given system (e.g., a wastewater treatment reactor). Combination of these data and quantitative data (e.g., fluorescence in situ hybridization) for these organisms with physical and chemical measurements of processes within the reactors may begin to describe the workings of what has been, until now, a "black box" system

and eventually improve the design and operation of these biological systems.

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