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Complete Detection of the β-subclass of the Ammonia-Oxidizing Proteobacteria in a Sequence Batch Reactor Requires a Multi-Probe Approach

JAMIE P. FINLEY

Department of Microbiology and Immunology, UBC

During the operation of a sequencing batch reactor to study the nitrification process, poor correlation in data generated from the chemical analyses and molecular probes initiated an investigation into the potential mismatches between the probes and a 16S rDNA library. Analysis of 98 clones generated four (8%) positive clones that hybridized with the Nso190 and Nso1225 probes. These probes are used to identify the ammonia oxidizing β-proteobacteria thought to be responsible for the conversion of ammonia to nitrite in the sequencing batch reactor. Only one of these positive clones was observed to be positive for both probes. Sequencing of the positive clones showed mismatches that were found to be consolidated in the near center of a slightly destabilizing nature and the terminal ends of the target sites in 16S rDNA sequences. As the Nso190 and Nso1225 probes were found to target multiple genera within the β-proteobacteria division, a multi-probe approach is required to identify all potential ammonia oxidizers to better reduce the discrepancy in the activity seen in the SBR. On a technical note, the observation of the singular orientation insertion of 16S rDNA sequences into the selected vector by Topoisomerase I in transformed *Escherichia coli* cells has not been seen in 16S rDNA analysis using similar cloning systems used previously in similar studies.

The nitrification process of converting ammonia (NH_4^+) to nitrite (NO_2^-) then to nitrate (NO_3^-) is mediated by two groups of bacteria, typically named ammonia oxidizers and nitrite oxidizers respectively (9, 13, 14). In wastewater treatment systems, chemolithoautotrophic ammonia oxidizing bacteria (AOB) such as *Nitrosomonas* are responsible for the conversion of ammonia to nitrite while autotrophs such as *Nitrobacter* are responsible for the conversion of nitrite to nitrate (14). The *Nitrosomonas* genus is part of the β-proteobacteria division that is often used in wastewater treatment systems for the reduction of nitrogen (ammonia) before discharge (14) and is being intensively studied.

Current research in developing treatment systems to investigate the potential of the so-called "nitrate shunt", where nitrite oxidizers are inhibited to reduce substrate competition between AOB and nitrite autotrophs, is fraught with poor correlation in data generated by chemical analyses and molecular probes (R. Simms and W. Ramey, personal communication). Chemical analyses are based on principles that are well understood and do not have as many potential issues as seen with molecular probes. For example, the poor correlation may be due to multiple factors such as the use of incorrect or insufficient probes, presence of novel organisms that have not been yet detected but contribute to the process of nitrification, and/ or the selected probe has too many mismatches that may prevent correct matching with the target organisms, i.e. the use of the Nso190 and/ or Nso1225 probes to identify ammonia oxidizers among others (2, 14). The potential of using probes that appeared insufficient was investigated using molecular biological methods. Mismatches within PCR amplified 16S rDNA sequences isolated from a sample in a sequencing batch reactor (SBR) were investigated for their impact upon hybridizing with selected probes identifying *Nitrosomonas* strains within the SBR.

MATERIALS AND METHODS

Reactor samples. A supplied sample dated March 30, 2003 from a sequencing batch reactor (SBR) was provided by the Department of Civil Engineering pilot plant at the University of British Columbia. On March 2, 2003 the original seed for the SBR came from the University of Cape Town process at the pilot plant. The SBR was operated at moderate ammonia loading for approximately two to three days and the influent ammonia was increased to give a free ammonia concn of >10 mg/l at the anoxic/aerobic cycle start. The increase in ammonia resulted in a loss of nitrite oxidation during the aerobic phase that was accompanied by nitrite accumulation. On March 30, 2003 the SBR was accumulating nitrite in the aerobic phase. The nitrite oxidation eventually recovered on April 2, 2003 (R. Simms, personal communication).

DNA extraction. A vol of 5 mL of the SBR sample was centrifuged at 1500 x *g* for 5 minutes. The supernatant was then removed and the pellet flash frozen with dry ice and put into storage at -80 ºC until needed. Before DNA extraction, the pellet was taken out of storage and put into a -20

°C freezer overnight. Nucleic acids were released from the cells in the pellet by means of mini-bead beating (Mini-BeadBeater, BioSpec Products) and precipitation of impurities with ammonium acetate as previously described (20) with one modification in step eight: dissolve the nucleic acids in 300 µL of 10 mM Tris 1 mM EDTA (TE) plus 33 µL of 3M pH 7 sodium acetate. A vol of 30 µL of the crude nucleic acids solution was treated with 1 µL of 10 mg/mL DNase-free RNase A for 30 min at 37 °C.

PCR amplification and cloning. The resultant RNase A treated DNA was PCR amplified with the generic bacterial primers (synthesized by Nucleic Acids Protein Services Unit (NAPS) of the Biotechnology Laboratory at the University of British Columbia (UBC)), 27f and 1542r (5'-GAGTTTGATCCTGGCTCAG-3' and 5'AGAAAGGAGGTGATCCAGCC-3', corresponding to positions 9-to-27 and 1525-to-1542 of the *Escherichia coli* 16S rDNA respectively) (18). The primers were included with the AccuPrime™ SuperMix II kit (Invitrogen) by following the manufacturer's recommendations with the following modification in the cycle steps: 94 °C for 2 min (1 cycle) then 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1.5 min (30 cycles) then 72 °C for 5 min (1 cycle) based on reference (20) in the Gene Cycler Thermal Cycler (Bio-Rad). The PCR amplified products were directly inserted into the pCR®2.1-TOPO® cloning vector with Topoisomerase I and transformed into chemically competent TOP10 *E. coli* cells supplied in the TOPO TA cloning kit (Invitrogen) as described in the manufacturer's instructions (Version Q dated July 21, 2003). Selected white colonies were plated triplicate into a grid with two blue colonies as a negative control.

Colony hybridization. The colonies in the grid were transferred onto nitrocellulose membranes and lysed. The denatured DNA was then baked onto the membrane as described previously (8). Before labeling with the radioactive probe, the membranes were transferred into a roller bottle containing approximately 7 mL of hybridization buffer-S (prepared in the following order: 3 mL 10% polyvinylpyrrolidone (PVP), 3 mL 10% Bovine Serum Albumin (BSA), 3 mL 10% Ficoll 400, 2.2 mL 10 mg/mL salmon sperm DNA, 15 mL 3.0 M NaCl 0.3 M sodium citrate (20x SSC), 1.5 mL yeast RNA, and add sufficient sterile distilled H_2O to a volume of 150 mL) (8) and prehybridized for a period of two to three hours at 45 °C. The Nso190 and Nso1225 ammonia-oxidizing β-proteobacteria probes (9, 13, 14) (5'-CGATCCCCTGCTTTTCTCC-3' and 5'-CGCGATTGTATTACGTGTGA-3' respectively) synthesized by NAPS at UBC were each labeled as directed by the manufacturer's recommendations with [γ-^{32}P]ATP at the 5'end by a phosphorylation reaction with bacteriophage T4 polynucleotide kinase supplied by a KinaseMax™ 5'end-labeling kit (Ambion). The labeled probes were then further purified using the QIAquick Nucleotide Removal kit (Qiagen). Then a volume of the labeled probe containing about 20 million CPM (counted by a Beckman LS 6000TA) was transferred into the roller bottle containing the incubating membrane. The incubation temperature was determined by calculating the melting temperature ($T_m = 16.6 \log[Na^+] + 0.41[P_{gc}] + 81.5 - P_m - 675/L - 0.65[P_f]$) as explained in reference (8). The determination of a signal-to-noise ratio that is optimal for each probe was not attempted since the determination has to be empirically determined if no reference can be found for such probes. The resultant mixture in the roller tube was left to hybridize overnight at the incubation temperature. The hybridization solution was discarded and the membrane was washed with 60 mL of 1x SSC for 30 min (1st wash) and 40 min (2nd wash). The membranes were air-dried for 30 min, mounted onto Whatman 3MM filter paper, covered with mylar plastic then autoradiographed. The exposed x-ray film was developed to identify colonies that hybridized with the probes.

Sequencing and analysis. Plasmids of colonies that hybridized with either probe were isolated using the Concert High Purity Miniprep System (Life Technologies) as directed by the manufacturer's protocol. The 1.5 kb 16S rDNA inserts in the pCR2.1-TOPO cloning vector were sequenced by NAPS with an Applied Biosystems PRISM 377 automated sequencer and Applied Biosystems BigDye™ v3.1 Terminator chemistry using the –21M13 and M13R primers. Sequences were aligned using the 'BLAST 2 Sequences' tool as described by reference (16). The nearly complete 16S rDNA sequences were compared for similarity with others found in the NCBI public database (1) using BLAST and further verified by the European Ribosomal RNA database (19) and the Ribosomal Database Project II (7). A phylogenetic analysis was generated by ClustalW (version 1.82) at EBI (17) as described in reference (6) and further illustrated by Phylodendron (version 0.8d).

RESULTS

DNA extraction and PCR amplification. A total of 0.256 mg nucleic acid was successfully isolated from the single sample taken from the SBR (Figure 1). PCR amplification of the 1.5 kb 16S rDNA from the RNase A treated nucleic acid mixture was successful with and without bovine serum albumin (BSA) which has been suggested elsewhere as necessary to be included in the PCR buffer for successful amplification (20).

Cloning and colony hybridization. A total of 98 white clones (with 2 blue clones as a negative control) were isolated and plated in triplicate in a grid-like fashion. After hybridization with the Nso190 and Nso1225 probes separately, four clones (pCR2.1-TOPO-JF25, -JF31, -JF32, and -JF52) had hybridized with the Nso190 probe and two clones (pCR2.1-TOPO-JF31, and -JF79) had hybridized with the Nso1225 probe. Only one clone (pCR2.1-TOPO-JF31) was able to hybridize with both probes.

Sequencing and analysis. All five 16S rDNA sequences cloned in the pCR2.1-TOPO vector were oriented such that the 27f and 1542r primer was always beside the M13R and -21M13 primers of the pCR2.1-TOPO vector respectively. This suggests that the 16S rDNA sequences are destructive in the reverse orientation (positions 1542 to 9 of the *E. coli* 16S rDNA) as transcripts in transformed TOP10 *E. coli* cells.

BLAST similarity indicated that pCR2.1-TOPO-JF31, -JF32, -JF52, and -JF79 had the highest sequence similarity to the 16S rDNAs of *Nitrosomonas oligotropha*, an uncultured bacterium SJA-62 associated with the β-proteobacteria division, *Diaphorobacter nitroreducens* associated with the β-proteobacteria division, and *Thauera terpenica* associated with the β-proteobacteria division respectively (Figure 2).

Mismatches in the Nso190 and Nso1225 sites within the aligned 16S rDNA sequence of each positive clone are shown in Table I. Probe Nso190 had at least two mismatches with each of the clones, A:G and C:T in *N. oligotropha* (pCR2.1-TOPO-JF31), G:T, A:G, and A:G in uncultured bacterium SJA-62 (pCR2.1-TOPO-JF32), and G:T, A:G, A:G, and G:T in *D. nitroreducens* (pCR2.1-TOPO-JF52). Probe Nso1225 had only one mismatch (C:G)

with two of the clones (*N. oligotropha* and *T. terpenica*). Thus BLAST similarity verified what was seen in the hybridization experiment with one exception: pCR2.1-TOPO-JF25 did not contain any similarity to the Nso190 target site, generating a false positive which was verified by a BLAST result of a *Rhizobium* sp. X59, which is in the α-Proteobacteria genus. This false positive may be due to the non-optimized conditions in the hybridization reaction and the single sequence result (amplified by the M13R primer only) caused by so-called neighbourhood effects.

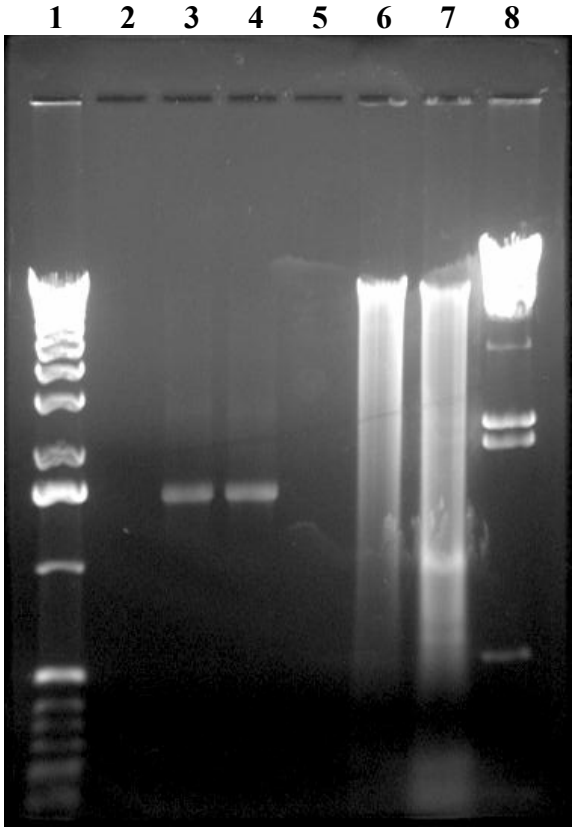

Figure 1. Agarose gel electrophoresis (1% agarose, 0.2 µg/mL EtBr) of total nucleic acids and PCR samples loaded at a 4 µg load level. The 1 kb DNA ladder and λDNA-H*ind*III standards were loaded in lanes 1 and 8. Lanes 6 and 7 represents the crude nucleic acid isolation sample digested with and without RNase A. The PCR samples: BSA only (no RNase A treated nucleic acids), BSA and RNase A treated nucleic acids, RNase A treated nucleic acids without BSA, and a negative control (no RNase A treated nucleic acids) were loaded in lanes 2-5 respectively.

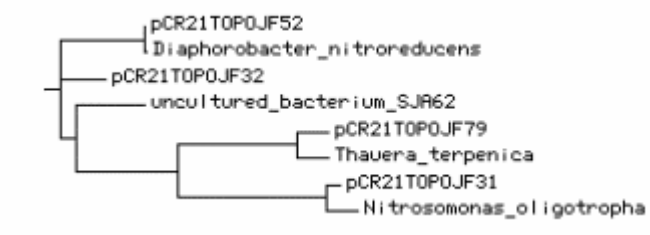

Figure 2. A phylogenetic tree generated with ClustalW and Phylodendron tools.

Table 1. Observation of the location and number of mismatches within probe regions in the PCR amplified 16S rDNA segment inserted in the cloned pCR2.1-TOPO plasmids.

Source	Sequence[a]
Nso190	3'-CCT CTT TTC GTC CCC TAG C-5'
target site	5'-GGA GAA AAG CAG GGG ATC G-3'
pCR2.1-TOPO-JF31	5'-· · · · G · · · · T · · · · · · · · · -3'
pCR2.1-TOPO-JF32	5'-· · · TG · · · · · G · · · · · · · · -3'
pCR2.1-TOPO-JF52	5'-· · · TG · · · · · G · · · · · · · T-3'
Nso1225	3'-AGT GTG CAT TAT GTT AGC GC-5'
target site	5'-TCA CAC GTA ATA CAA TCG CG-3'
pCR2.1-TOPO-JF31	5'-· · · · · · · · · · · · · · · · · G · · · -3'
pCR2.1-TOPO-JF79	5'-· · · · · · · · · · · · · · · · · G · · · -3'

[a] The periods in the sequences indicate the nucleotides are identical to that in the target site. Mismatches in the sequences are shown by the replacement nucleotides below the sequence of the target site of the probe. pCR2.1-TOPO-JF25 was not included since it was only able to generate one sequence with the M13R primer.

DISCUSSION

DNA extraction and PCR amplification. BSA was not necessary for successful amplification with the 27f/1542r primers (Figure 1). This observation contradicts the previous result of amplifying with the same primers using activated sludge biomass treating waste water from a pulp and paper mill (20). This discrepancy might be due to the differences in the PCR reaction mix. The AccuPrime SuperMix II contains anti-Taq DNA polymerase antibodies and a thermostable AccuPrime protein. The anti-Taq DNA polymerase antibodies act to inhibit polymerase activity ("hot start"). The thermostable AccuPrime protein enhances the hybridization of the primer-template during every cycle of the PCR as explained by the manufacturer (AccuPrime SuperMix II manual, Invitrogen). The PCR reaction mix used by Yu and Mohn (20) did not contain both anti-Taq DNA polymerase antibodies and a thermostable AccuPrime protein; rather, BSA was used to limit inhibitors of the Taq polymerases in solution (12). Therefore, the use of anti-Taq antibodies that inhibit (and stabilize) the Taq polymerases does not require the stabilized effect of BSA in the PCR.

Cloning and colony hybridization. The observation that only one clone was able to hybridize with both probes is clear evidence that a single probe is insufficient to reveal all strains within the β-proteobacteria division even though the Nso190 and Nso1225 probe were both designed to reveal or detect all characterized ammonia-oxidizers in β-proteobacteria. However, the determination of a signal-to-noise ratio that is optimal for each probe was not attempted. It is known that the stringency of the hybridization between the target site in the cloned plasmid and the probe depends on the hybridization buffer, the temperature, and the time of hybridization (3), all which can impact the results obtained in a colony hybridization experiment. The selected hybridization buffer (8) was chosen since a full description of the hybridization buffer was available while other hybridization buffers used in journal articles were not thoroughly explained and were difficult to prepare. Although other articles (13) can be found that describe dissociation profiles of nucleic acid probes, these articles often used a hybridization buffer differed from that used in this study. Hence, it was decided to follow the recommendations for the hybridization protocol used (8).

Sequencing and analysis. The unexpected singular orientation of the PCR amplified 16S rDNA insertion into the pCR2.1-TOPO vector is contrary to previous observations of fragments of interest inserted in both orientations aided by Topoisomerase I (5, 15). This contradictive observation might be due to the destructive nature of the reverse order (positions 1542 to 9 of the *E. coli* 16S rDNA) transcripts when expressed in the transformed TOP10 *E. coli* cells. For example, a promoter within the LacZα gene in the pCR2.1-TOPO vector could induce transcripts including the cloned 16S rDNA fragments in the reverse order which may be destructive to *E. coli* ribosomal processing. As well, one needs to also consider the interaction of Topoisomerase I upon ligation of the PCR

amplified 16S rDNA fragments. Topoisomerase I is known to interact with the DNA sequence (T/C)CCTT, along with six nucleotides upstream, and two nucleotides downstream of the (T/C)CCTT sequence when being covalently bound to substrates or vectors such as pCR2.1TOPO (4, 11). As the recognition site of Topoisomerase I include the sequences of the 27f and 1542r primers used to amplify the 16S rDNA fragments, the 27f and 1542r sequences might potentially influence the singular orientation insertion. Interestingly, a previous study looking at the phylogeny of all recognized species of ammonia oxidizers made no mention of the contradictive observation despite using the identical TOPO TA cloning kit (14) with PCR primers that differ slightly (positions 8-to-27 and 1529-to-1542 for the forward and reverse primers respectively) from the 27f and 1542r sequences. In conclusion, due to the observed singular orientation insertion, the frequency of the positive clones in the colony hybridization experiment should be doubled, i.e. 4 / 98 x 100% = 4% x 2 = 8%.

BLAST results of the 16S rDNA sequences indicate that the cloned sequences are not the same. This observation suggests that the ammonia oxidizers in the SBR exist as different genus of the same division, the β-proteobacteria (Figure 2). Interestingly, the only genus identified by both probes was *Nitrosomonas*, specifically *N. oligotropha* (pCR2.1-TOPO-JF31). It should be noted that only one clone (pCR2.1-TOPO-JF31) was closely related to an ammonia oxidizer in the *Nitrosomonas* genus while the rest of the clones were related to other genera in the β-proteobacteria division. This indicates that several possible explanations exist for this observation: the probes are not specific enough, as they are able to target other genera in the β-proteobacteria division along with *Nitrosomonas*. Secondly, other novel organisms may potentially exist along with *Nitrosomonas* as ammonia oxidizers. Lastly, the cloning system used appears to limit the identification of the 16S rDNA sequences of *Nitrosomonas*.

Mismatches between the probes and target sites in the 16S rDNA sequences are observed (Table I), which is not surprising since mismatches are typically mutations due to the evolutionary process (3). Interestingly, Probe Nso190 has mismatches such as G:A, G:T, and C:T, known to be slightly less destabilizing than mismatches such as A:A, T:T, and C:A (3). The mismatches seen with the Nso190 hybridization are within the multiloop secondary structure seen in *E. coli* 16S rRNA (10). Probe Nso1225 has only one G:C mismatch near the terminal end of the target site for both clones, where it is known that such mismatches are less destabilizing (3) and within the multiloop secondary structure seen in *E. coli* 16S rRNA (10). Despite the presence of mismatches, the hybridization results of the two probes with the four cloned 16S rDNA sequences shows evidence that the probes are not necessarily matched with ammonia oxidizers in the β-subdivision of the proteobacteria. Rather, the results show that a multiprobe approach is required to better identify the ammonia oxidizers responsible for the activity measured in the SBR using chemical analyses. For example, *amoA* probes could be included with 16S rDNA probes to further aid in identifying the ammonia oxidizers (14).

FUTURE EXPERIMENT

To resolve the singular orientation contradictive observations of the 16S rDNA insertions, future experiments should be developed to test whether reversed 16S rDNA sequences create destructive transcripts when expressed in the transformed TOP10 *E. coli* cells. Perhaps experiments be set up to cleave the 16S rDNA insertions from the pCR2.1-TOPO vector, undergo a purification step, then inserted back into the pCR2.1-TOPO vector and other selected cloning vectors. This approach will confirm whether the 16S rDNA fragments can only be inserted in one orientation.

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