Journal of Experimental Microbiology and Immunology (JEMI) Vol. 10:1-5

Attempts to Identify Planctomycete Ammonium Oxidizing Bacteria in a Sequence Batch Reactor Sample

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Modern biological nitrogen removal processes focus on the use of anaerobic ammonium oxidation (anammox), where ammonium is converted to nitrogen gas under anoxic conditions. The discovery of anammox bacteria in many different environments has led to applications such as the single-reactor high-activity ammonia oxidation over nitrite (SHANON) and completely autotrophic nitrogen-removal over nitrate (CANON) reactors. Molecular approaches based on 16S rRNA, such as PCR and FISH, have overcome the limitations presented due to the slow growth rate of anammox organisms. This experiment aims to detect and identify any potential planctomycete anammox organisms that may be present in biofilm obtained from a sequence batch reactor, previously shown to contain various ammonium and nitrate oxidizing bacteria. DNA extracted from the biofilm was amplified using primers specific for the 16S rRNA of anammox planctomycetes. The pAA064 clone was created from the 1500bp amplified fragment, and the clone was digested with restriction enzymes and sequenced to identify the insert. Restriction enzyme digests indicated that the sequence did not belong to a known planctomycete anammox organism. The BLAST results showed that the sequence likely belongs to a spirochete or to a planctomycete anammox organism that is yet to be sequenced. One reason for the inconclusive identity of this organism is the large number of sequences from uncultured organisms or unidentified sequences from environmental samples. However, screening multiple clones to cover a wider range of organisms that may be present in the sample may overcome limitations in sequence databases.

Due to the large quantities of nitrogen compounds in effluents from industries, many biological nutrient removal processes have been developed to treat wastewater. Conventional systems have relied on nitrification, where ammonia and nitrite are oxidized, and denitrification, where nitrate or nitrite is reduced to gaseous forms of nitrogen (N_2, NO, N_2O). Anaerobic ammonium oxidation (anammox) is another process for converting nitrogen compounds to nitrogen gas, without relying on denitrification (8). Here, ammonium is oxidized by nitrite via hydrazine and hydroxylamine intermediates under anoxic conditions (3). The existence of this mechanism was hypothesized based on nutrient profiles and thermodynamic calculations as early as 1965 (7, 8). After the initial discovery of anammox bacteria, in a pilot plant in the Netherlands treating wastewater from a yeast producing company, similar nitrogen losses, which could only be explained by the anammox reaction, were reported in many other settings (5). Anammox bacteria have been found in the anoxic zone of the Black Sea, coastal shorelines, oceanic oxygen-minimum zone, and sediments of inner harbour and estuaries (4, 16). Anammox bacteria have also been found to play a role in landfill leachate treatment plants in Germany as well as semitechnical wastewater treatment plants in Australia and Japan (8).

The anaerobic ammonium oxidation process has recently been applied in setting up better wastewater treatment facilities. One such example is the single-reactor high-activity ammonia oxidation over nitrite (SHARON) process where conditions are set up to select for the growth of ammonium oxidizing bacteria over nitrite oxidizers, and the accumulating nitrate can then react with the ammonia produced by anammox bacteria (18). Anammox organisms have also been utilized to treat side streams of sludge handling units, which contain high ammonia concentrations, using the CANON (completely autotrophic nitrogen-removal over nitrate) system (17).

One critical limiting factor in understanding the functioning of anammox bacteria is the inability to culture these organisms due to a very slow growth rates, which, even with enrichments, is at best 11 days per doubling time (13,14). This limits the use of traditional identification and enumeration techniques, including immunofluorescence detection by serological distinction, which have been used previously to study nitrifying bacteria in soil, sewage and marine environments (1, 20, 21). Thus, advantages offered by molecular techniques have led to recent advances being made using PCR and fluorescence in situ hybridization (FISH) based on 16S rRNA methods (8). PCR allows

the amplification of specific sequences from only a few copies of the target DNA, offering a significant advantage over hybridization methods (19). The detection and identification of active anammox bacteria in environmental samples along with information of environmental conditions can provide potential biomass sources for use as inoculum in laboratories, semitechnical or full-scale anammox reactors, as well as provided insights into the niche differentiation of anammox organisms (8). For some planctomycete anammox species, including *Candidatus Kuenenia stuttgatiensis* and *Candidatus Brocadia anammoxidans*, small subunit rRNA molecules, and various other genes, have been completely sequenced (10) whereas others, such as *Candidatus Scalindua brodae* and *Candidatus Scalindua wagneri* are relatively newer species with partially sequenced genes (4,11).

This experiment aims to detect and identify any potential planctomycete anammox organisms that may be present in biofilm obtained from a sequence batch reactor, which has been shown to contain various ammonium and nitrate oxidizing bacteria (Wayne Lo, unpublished data).

MATERIALS AND METHODS

Cell Samples. The biocarrier sample was obtained from a lab scale hybrid sequence batch reactor run by the Environmental Laboratory, Department of Civil Engineering at the University of British Columbia. The biofilm was present in the walls of the biocarrier (Kaldnes, K3 type, 500m^2/m^3, diameter 20 mm, height 15mm), a round plastic disc, as shown in Figure 1a. The round disc was aseptically transferred into a sterile Petri dish and a small part was cut with a sterile scalpel (figure 1b). Cells from the biofilm were resuspended in TE Buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.0) in the Petri dish and then transferred to a 2 ml microfuge tube to concentrate the cells for lysis. The piece of plastic was also added to the centrifugation tube.

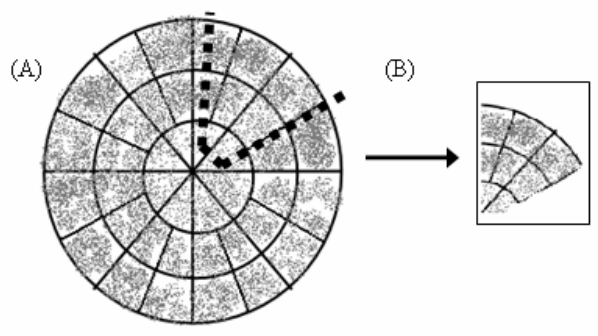

Fig 1. (A) A biocarrier containing biofilm (shaded). (B) Fragment of biocarrier used for DNA isolation.

Cell Lysis and DNA Extraction. The cell pellet was resuspended in 400 µl of Lysis Buffer (100 mM NaCl, 500 mM Tris pH 8.3, 10% SDS and 4µl of 0.02mg/ml Proteinase K) and the reaction was incubated at 65°C for 2 hours. One volume of TE saturated phenol at pH 8.0 was added and the mixture was vortexed for 30 seconds before centrifuging at 12,000 rpm for 5 minutes at room temperature. The aqueous upper layer was transferred to a new tube and 1 volume of 1:1 TE saturated phenol: chloroform solution was added, followed by another round of centrifugation at 15,000 rpm for 5 minutes at room temperature. The upper aqueous layer was again transferred into a clean tube and 2 ½ volumes of ice-cold salted ethanol (1:9 3 M sodium acetate in 95% ethanol) was added, and incubated overnight at -20°C. The DNA was pelleted by centrifugation at 15,000 rpm for 15 minutes at 4°C. The pellet was washed with 70% ethanol to remove any salts, followed by another centrifugation at 15000rpm, 4°C for 15 min. The supernatant was removed and the pellet was air dried before resuspension in 200 µl of sterile, distilled water.

DNA quantification. DNA was diluted 10 times and 100 times in sterile distilled water and an absorption spectrum scan was performed from 230nm to 320nm. Using the conversion coefficient of 50 µg/ml, the absorbance at 260nm (A_{260}) was used to determine the quantity of DNA. This value was averaged for the 10-times diluted and 100-times diluted samples to give the average yield of DNA obtained. The ratio of A_{260} to A_{280} values indicated the relative purity of the DNA. DNA quality was assessed by performing electrophoresis on a 1% agarose gel.

Primer Design. A planctomycete specific forward and an anammox specific reverse primer were selected and modified from previously published sources (8). Each primer was aligned with the five 16S rRNA sequences from different planctomycete anammox organisms (Table 1) using MegAlign software (Lasergene 7 software, DNASTAR, Madison, WI). The planctomycete specific forward primer, S-P-Planc-0046-a-a-18 (or Pla46) aligned exactly with all 5 sequences and so was left unchanged (5'-GAC TTG CAT GCC TAA TCC -3') (6). S*-Amx-1240-a-A-23 was previously used as an oligonucleotide probe for RNA hybridization and binds to 1240-1262bp positions in the 16S rRNA sequence of *B. anammoxidans* (9). By aligning this primer with the five 16S rRNA sequences, it was found that by changing a few nucleotides, this primer would bind to all five sequences. This designed reverse primer, AA01 (5'-CTCGGGAACATATTCACC- 3'), when paired with Pla46, was expected to yield a PCR product of approximately 1350 bp. Primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA) and purified by standard desalting.

Table 1. Size and accession numbers of 16S rRNA genes for various anammox bacteria.

Organism	GenBank Accession ID	Gene Size (bp)
Candidatus Kuenenia stuttgartiensis	AF375995.1	1538
Candidatus Brocadia anammoxidans	AF375994.1	1543
Candidatus Scalindua sorokinii	AY257181.1	1387
Candidatus Scalindua brodae	AY254883.1	1529
Candidatus Scalindua wagneri	AY254882.1	1525

PCR Amplification. Two microliters of diluted DNA (1, 1/10, 1/100 and 1/1000) was added to each 50 µl PCR reaction containing 0.25 µM of each primer (Pla46 forward and AA-01 reverse), 10X Buffer, 12.5 mM dNTPs, 100 mM $MgCl_2$ and 1U of Taq DNA polymerase (Fermentas, Burlington, ON). Samples were mixed on ice and a simple hot start was provided by an initial denaturation at 94°C for 3 minutes. This was followed by 40 cycles of 1 minute each at 94°C, 55°C and 72°C, with a final extension of 10 minutes at 72°C and then rest at 4°C. 10 µl of PCR product, along with 2 µl of 6X gel loading solution (3 ml glycerol, 2 mg bromophenol blue and 2 mg xylene cyanole in 10 ml distilled water) was run on a 1% agarose gel in TBE Buffer (10.8 g/L Tris base, 5.5 g/L boric acid, 0.744 g/L EDTA) at 105V, along with Fermentas MassRuler DNA ladder (Cat # SM1283) to assess the presence and size of the PCR product. Gels

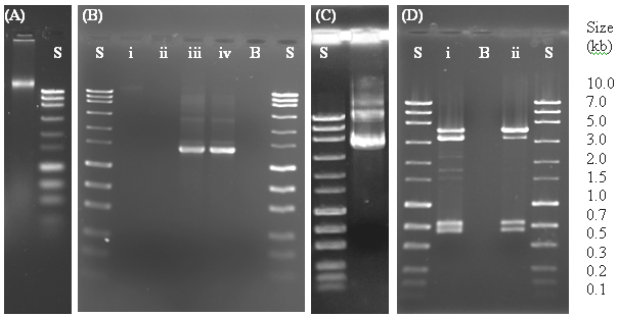

Fig 2. Gel electrophoresis images and ladder sizes. (A) DNA extracted from biofilm. (B) PCR product of (i) undiluted, (ii) 10X diluted, (iii)100X diluted and (iv) 1000X diluted DNA. (C) Plasmid DNA from pAA064. (D) pAA064 DNA digested with (i) *EcoR*1 and *Mbo*1 and (ii) with *EcoR*1 alone. S: Fermentas MassRuler Ladder (Cat # SM1283). B: unloaded lanes.

were stained in 0.2mg/ml ethidium bromide bath for 1 hour, destained in water for up to 4 hours and then viewed using AlphaEase FC system (Alpha Innotech Corporation, San Leandro, CA).

Cloning and Transformation. PCR products were cloned into the pCR2.1 TOPO vector using TOPO Cloning Kits for sequencing using manufacturer's protocol (Invitrogen Corporation, Burlington, ON). TOP10 competent *Escherichia coli* were used to express the plasmid and transformed cells were selected by spread plating on Luria Bertani (LB) agar plates (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl, 15g/L agar in water) supplemented with 100 µg/ml of ampicillin and pre-spread with 80µl of 20mg/ml X-gal substrate.

Plasmid Isolation from Clones. Isolated colonies of suspected positive clones were grown in 5 ml of LB broth containing 100µg/ml of ampicillin. Plasmid DNA was isolated from each culture using the GeneJet Plasmid Miniprep Kit (Fermentas, Burlington, ON) (Cat # K0501) based on manufacturer's instructions. Plasmid DNA was quantified by absorbance readings at 260 nm and gel electrophoresis.

Restriction Enzyme Digest. A virtual digest was performed for the 5 anammox sequences listed in Table 1 using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/). Restriction enzymes that yielded different fragment sizes and different number of fragments for most of the 5 sequences were deemed suitable for the *in vitro* digest. In a 40µl reaction, 1.345 µg of plasmid DNA was incubated overnight at 37°C with 4 µl of 10X React2 buffer, 1 unit of *Eco*R1 and 1 unit of *Mbo*1 (Invitrogen Corporation, Burlington, ON). The digests were run on a 1% agarose gel at 90V. The gel was stained in 0.2 mg/ml ethidium bromide solution for 1 hour and destained in water for up to 4 hours.

Sequencing and BLAST. Clone pAA064 was sent for sequencing to the Nucleic Acid Protein Service Unit (NAPS) at the University of British Columbia (Vancouver, BC). Primers T7 promoter and M13 reverse were used for bidirectional sequencing. The resulting sequences were edited using VecSreen to remove any plasmid region that was sequenced (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The SeqMan program was used to assemble the 2 sequences into a single consensus sequence (Lasergene 7 Software, DNASTAR, Madison, WI). BLASTN and mega BLAST were then used to identify the sequence.

RESULTS

DNA yield and purity. The extraction from the biofilm on the disc yielded 66 µg of DNA which had an $A_{260/280}$ ratio of 1.5 and was of good quality (Fig 2A). Undiluted DNA gave no PCR product but PCR of 100 and 1000 times diluted DNA yielded fragments that were approximately 1400 bp in size (Fig 2B). Plasmid miniprep of pAA064, a TOPO clone of the PCR product, yielded 13.45 µg of plasmid DNA and showed appropriately sized bands (Fig 2C, D). Digestion of the clone with both *EcoR*1 and *Mbo*1, yielded fragments that are approximately 650 bp, 750 bp, 1500 bp, 1750 bp, 2400 bp, 3500 bp, and 4000 bp long (Fig 2D, lane i). When 0.673 µg of DNA was digested with *EcoR*1 alone, it yielded fragments of 650 bp, 750 bp, 3500 bp and 4000 bp (Fig 2D, lane ii).

Table 2. Common BLAST results from BLASTN and mega BLAST.

Accession	Description
AF050549.1	Uncultured eubacterium WCHB1-40 16S ribosomal RNA gene, partial sequence
AB244312.1	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone:LCFA-B05
AY214182.1	Uncultured bacterium clone ZZ12C6 16S ribosomal RNA gene, partial sequence
AF050550.1	Uncultured eubacterium WCHB1-91 16S ribosomal RNA gene, partial sequence
AF050551.1	Uncultured eubacterium WCHB1-30 16S ribosomal RNA gene, partial sequence
AY133081.1	Uncultured spirochete clone ccslm209 16S ribosomal RNA gene, partial sequence
AY780558.1	Uncultured spirochete clone KB-1 16S ribosomal RNA gene, partial sequence
AY695841.1	Spirochaetes bacterium SA-10 16S ribosomal RNA gene, partial sequence
AJ009481.1	uncultured bacterium SJA-102 16S rRNA gene, clone SJA-102
AY695839.1	Spirochaetes bacterium SA-8 16S ribosomal RNA gene, partial sequence

Table 3. Top 5 matches with BLASTN limited to all planctomycete bacterial sequences, sorted by total score.

Accession	Description	Total Score	Query coverage	E-value	Max identity
CP000124.1	Burkholderia pseudomallei 1710b chromosome I, complete sequence	2350	57%	4e-48	100%
CT574327.1	Uncultured bacterium partial 16S rRNA gene from clone 055A09_P_DI_P58	1011	66%	2e-68	95%
DQ269110.1	Uncultured planctomycete clone DPC183 16S rRNA gene, partial sequence	803	55%	7e-62	90%
AM285341.1	Candidatus Brocadia sp. 40 partial 16S rRNA gene, clone 40	802	58%	1e-54	100%
AM056027.1	planctomycete A-2 partial 16S rRNA gene, isolate A-2	732	63%	4e-79	95%

Table 4. BLAST results obtained when query of unknown sequence was limited to 5 accession numbers.

Accession	Description	Total Score	Query Coverage	E-value	Max Identity
AF375994.1	Candidatus Brocadia anammoxidans 16S rRNA gene, complete sequence;	781	46%	3e-37	100%
AY254883.1	Candidatus Scalindua brodae clone EN 8 16S rRNA gene, partial sequence	755	51%	3e-62	100%
AY257181.1	Candidatus Scalindua sorokinii 16S rRNA gene, partial sequence	751	50%	3e-62	100%
AF375995.1	Candidatus Kuenenia stuttgartiensis 16S rRNA gene, complete sequence;	719	50%	4e-55	100%
AY254882.1	Candidatus Scalindua wagneri clone EN 5 16S rRNA gene, partial sequence	699	53%	2e-41	100%

Sequence Analysis. Sequencing with the M13R and T7 promoter primers yielded fragments that were 973 bp and 977 bp respectively. After using VecScreen to remove the plasmid DNA sequence from the ends of these sequences, the two sequences were aligned in SeqMan and yielded a 1246 bp consensus sequence. The consensus sequence was then used to query the

non redundant (nr) database at the NCBI using BLASTN and mega BLAST, limiting the search to bacterial sequences. Common sequences found in the top 11 BLASTN and mega BLAST results list are listed in Table 2. Both results were sorted by maximum score and all hits had an E-value of 0. While BLASTN gave query sequence coverage ranging from 83-90%, mega BLAST gave query sequence coverage of 93% for all the same matches.

To refine the BLAST search, the entrez query was limited to "planctomycete" bacterial sequences. This showed that there are several short conserved regions between the queried sequences and a variety of planctomycetes, most of which are uncultured. The conserved regions have an 80-95% identity, have no gaps and approximately span from 30-78bp, 509-579bp, 252-428bp, 693-816bp, 871-987bp and 1045-1385bp in the query sequence. The top 5 matches, sorted by the highest total score, are shown in Table 3 below. The BLAST search was also repeated with the entrez query limited to "spirochetes" since 4 of the top 10 hits in the general BLASTN search belonged to this group. From the results of this BLAST search, the top scoring sequences had 82-89% coverage with the unknown sequence with 90-95% sequence identity. In another BLASTN search, the Entrez query was limited to 5 accession numbers, each representing 16S rRNA sequence from different planctomycete ammonium oxidizing bacteria, the same sequences used to design the PCR primers. The maximum identity, score and sequence coverage for each are summarized in Table 4.

DISCUSSION

Overall, a sufficiently large quantity of DNA was obtained from the small amount of biofilm used as starting material. As seen in Fig 2A, most of the DNA was larger than 10kb and does not appear to be sheared by a great extent. The extracted DNA likely contains PCR inhibitors from the environmental sample since no PCR product was seen for the undiluted and even the 10X diluted DNA samples (Fig 2B). Adding a control template and control primers (provided in TOPO kit) to the PCR mixture containing the undiluted DNA did not produce any amplified fragments either (data not shown). This is indicative of the presence of inhibitors such as humic acid and other related compounds that usually co-purify with DNA in other environmental samples (12, 19). Diluting the DNA to dilute out PCR inhibitors can also result in the complete removal of target DNA (15), which raises the possibility of the amplification of a non target sequence. However, if the quantity of target DNA was small enough to be diluted to extinction, it is likely that these planctomycetes do not play a significant role the anammox process in this system.

The pattern of fragments seen by the restriction enzyme digest was unexpected. Within the TOPO plasmid, *EcoR*1 sites flank both sides of the insert region, thus excising out the insert upon digest with *EcoR*1 (TOPO TA cloning user's manual, Invitrogen, Burlington, ON). The virtual digest with *EcoR*1 of the 5 different anammox sequences confirmed that no *EcoR*1 sites were present in any of the sequences besides *Candidatus Kuenenia stuttgartiensis*, which contains one *EcoR*1 cut site at position 188. Thus, the *EcoR*1 single digest should have yielded a 3900bp plasmid fragment and the 1500bp insert fragment, but instead fragments of 650bp, 750bp, 3500bp and 4000bp were seen (Fig 2D). The additional fragments indicate that the insert sequence contains some *EcoR*1 sites. When the sequence of the insert was virtually digested with Webcutter 2.0, the fragments were shown to contain 3 *EcoR*1 cut sites (at positions 40, 677 and 1396) that would have yielded fragments of sizes 40, 552 and 618bp on complete digestion (data not shown).

*Mbo*1 was used to digest within the insert and was expected to yield 2 to 4 fragments in combinations of 100, 275, 600, 700, 1000, 1200, or 1350bp, adding up to approximately 1500bp. However, comparing the fragments between the *EcoR*1 single digest and the *EcoR*1 and *Mbo*1 digest, the fragments produced by *Mbo*1 digestion seem to be 1500, 1750 and 2400bp in size. This seems to suggest that some Mbo1 recognition sites exist in the plasmid and it may be likely that the insert does not contain any recognition sites. Upon checking the restriction map for

pCR2.1-TOPO, the plasmid was found to contain 25 different recognition sites within its 3931bases (pCR2.1 TOPO Map, Invitrogen Burlington ON). Partial digestion of these fragments would have resulted in the fragment bands seen on the gel with the *EcoR*1 and *Mbo*1 digest. Thus while the restriction enzyme digest indicates that the plasmid contains an insert, we are unable to determine the size of the insert and the restriction enzyme fragments do not contain a pattern suggestive of any of the five anammox organisms that are suspected to be present in the reactor sample.

BLAST results indicate that the insert does not belong to a planctomycete organism. As seen from Table 2, the insert shares greater sequence identity and sequence coverage with various spirochetes or uncultured bacteria. When the insert sequence was compared to planctomycete sequences, only short regions within the sequences shared high sequence identity (typically >90%) and the overall sequence had poor similarity. However, when the insert sequence was compared to other spirochaetes sequences, a much larger region of the sequence shared high sequence identity (>90%). When the insert sequence was queried against the 5 anammox organisms used to design the

primers, again only 50% of the sequence was covered, even though the sequence identity here was 100% in each case. This instance reflects that while 16S rRNA is suitable for providing long-term evolutionary relationships, it fails to discriminate among close relatives, such as species within a given group, due to its highly conservative nature (1).

However, the BLAST tool also has its limitations. For instance, when the entrez query was limited to planctomycetes, the highest scoring match was *Burkholderia pseudomallei*, which actually belongs to the β-proteobacteria class of bacteria, not to the planctomycetes class. Also, of the approximately 300 BLAST results obtained when the entrez query was limited to planctomycetes, 284 hits were to 16S genes of uncultured planctomycetes. Similarly, when BLASTN was performed without any limits, most of the resulting hits were uncultured spirochetes. Due to the vague description of the organisms to which the insert sequence is similar, it is only possible to conclude that the insert probably belongs to a spirochete organism. However, based on the very high (~100%) sequence identity seen with planctomycete organisms, there is still the possibility that the sequence may be from a planctomycete organism that has yet to be sequenced.

FUTURE EXPERIMENTS

From these experiments, one can not positively ascertain whether planctomycetes do play any role in ammonium oxidization in this bioreactor. Here, only one clone containing extracted DNA was screened. It is important to screen a wide range of clones to get a better idea of the diversity of organisms in the sample. Organisms could be collected from the entire biocarrier to better estimate the population diversity and increasing the likelihood of identifying planctomycetes, if present. Since bioreactor conditions may differ from region to region, collecting organisms from multiple biocarriers in different sections of the reactor may also be important. Other genes known to be specifically present in ammonium oxidizing bacteria, such as *amoA* or *amoB*, can be used for PCR amplification instead of, or in conjunction with, 16S rRNA to detect the presence of anammox planctomycetes.

ACKNOWLEDGEMENTS

I gratefully acknowledge all the support assistance and guidance provided by Dr. William Ramey and Jennifer Sibley, as well as my MICB 447 classmates who assisted with various issues in the laboratory. This work was graciously funded by the University of British Columbia, Department of Microbiology and Immunology. The generosity of DNASTAR to use their licensed software is also appreciated.

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