Chapter 2

Materials and Repetitive Methods

2.1. Materials

2.1.1. Culture media

2.1.1.1. Modified Alexander medium for enrichment of nitrifying bacteria contains (per liter) 0.5 g $(NH_4)_2SO_4$, 0.5g K_2HPO_4, 0.5g $NaHCO_3$, 0.5g $MgSO_4.7H_2O$, 11.9g N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), and 1ml trace element solution (5 g/l $CaCl_2.2H_2O$, 2g/l $MnSO_4.4H_2O$, 5 g/l Fe-EDTA, 0.1g/l $CuSO_4.5H_2O$, 0.1g/l $ZnSO_4.7H_2O$, 0.05 g/l $Na_2MoO_4.2H_2O$, and 0.001 g/l $CoCl_2.6H_2O$) (Liu *et al.*, 2000). The salinity level is adjusted using sea salt to be 25 ppt equal to the average salinity values in shrimp ponds (from survey of this study as shown in Chapter 3, part 1).

2.1.1.2. Artificial wastewater for cultivation of nitrifying bacteria was adapted from Liu *et al.* (2000) by adding sea salt to provide 25 ppt salinity and 0.5 g/l $(NH_4)_2SO_4$. Others chemicals were (per liter), 0.2 g $MgSO_4.7H_2O$, 9 g $Na_2HPO_4.2H_2O$, 1.5 g K_2HPO_4 , 0.5 g $NaHCO_3$, and 1ml trace element solution. The pH was adjusted to 8.0 by adding 1 M K_2CO_3.

2.1.1.3. Artificial seawater. The composition of artificial seawater was (g/l): NaCl, 25; $MgCl_2.7H_2O$, 5; $CaCl_2$, 1.0; KCl, 1.0 in distilled water (Stephen *et al.*, 1996).

2.1.1.4. Modified Watson's medium for ammonia oxidizing bacteria (AOB) cultivation, prepared in artificial seawater, with the pH of the medium being adjusted to 7.5 by adding sterile 5% $NaCO_3$ after autoclaving (121°C for 15 min). The medium contained: $(NH_4)_2SO_4$, 1.5 g/l; $MgSO_4.7H_2O$, 0.2 g/l; $CaCl_2$, 0.02 g/l; K_2HPO_4, 0.114 g/l; Fe-EDTA (77 mg $FeSO_4.7H_20$ + 103 mg NaEDTA in 50 ml distilled water), 1.0 ml/l; $Na_2MoO_4.2H_2O$, 1.0 μg/l; $MnCl_2.4H_2O$, 2.0 μg/l; $CoCl_2.6H_2O$, 2.0 μg/l; $CuSO_4.5H_2O$, 20.0, μg/l; $ZnSO_4.7H_2O$, 100 μg/l; $CaCO_3$, 2.0 g/l, and phenol red, 0.5 mg/l (Jones and Hood, 1980).

2.1.1.5. Medium of Alexander and Clark for cultivation of *Nitrobacter* (as a representative of nitrite oxidizing bacteria group), prepared in artificial seawater, with the pH adjusted to 7.3 by adding 0.1 M NaOH. The composition of medium were (g/l): KNO_2, 0.006; K_2HPO_4, 1.0; NaCl, 0.3; $MgSO_4.7H_2O$, 0.1; $FeSO_4.7H_2O$, 0.03; $CaCO_3$, 1.0 and $CaCl_2$, 0.3 (Alexander and Clark, 1965).

2.1.2. Reagent

2.1.2.1. Griess-Ilosvay reagent for checking NO_2-N contained the combination of 3 reagents (a, b and c) with the ratio of 1:1:1. All reagents were stored in a refrigerator at dark and mixed freshly before use (Alexander and Clark, 1965).

Reagent a: 0.6 g sulfanilic acid was added in 70 ml of hot distilled water, after cooled down, 20 ml of concentrate HCl was added, adjusted the volume up to 100 ml with distilled water.

Reagent b: 0.6 g alpha-naphthylamine was added into 10-20 ml of distilled water containing 1 ml concentrated HCl, adjusted to final volume of 100 ml with distilled water.

Reagent c: 16.4 g of $CH_3COONa.3H_2O$ was added to 10-20 ml of distilled water and then diluted to 100 ml final volume with distilled water.

2.1.2.2. NZY^+ broth for cultivation of transformant cells contained 10 g/l of NZ amine, 5g/l of yeast extract, 5g/l of NaCl, after autoclaving, added 12.5 ml of solution [1 M Mg_2Cl and 12.5 ml of 1 M Mg_2SO_4] and 10 ml of a 2 M filter sterile glucose solution.

2.2. Repetitive methods

2.2.1. MPN (most probable number) technique

Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in the SBRs were enumerated by culture dependent method using the most probable number (MPN) technique in microtiter plates which performed on the protocols described by Rowe *et al.*, 1977). A volume of 1 ml of sample was added to 9 ml of sterile artificial seawater (Stephen *et al.*, 1996) and

approximately 10 sterile glass beads (3 mm in diameter) and the mixture was vigorously shaken for 1 min. For each of AOB and NOB, 0.15 ml of the appropriate sterile medium (see below) was dispensed into each well of a 96-well microtiter plate, and eight replicate two-fold serial dilutions of 0.15 ml of sample were performed. After incubation in the dark for 21 days at room temperature, the presence of AOB or NOB was determined (see below) and the numbers were calculated using MPN tables-see Appendix 1.1 (Rowe *et al.*, 1977).

AOB were enumerated in modified Watson's medium (Jones and Hood, 1980) prepared in artificial seawater and the pH was adjusted to 7.5 using sterile 5% Na_2CO_3. Phenol red was added into the medium to indicate when the pH dropped below 7. Wells in the microtiter plate were recorded as positive for AOB by acid production and by detection of NO_2^- and NO_3^- through development of a blue colour after addition of 1 or 2 drops of 0.2% (w/v) diphenylamine in concentrated H_2SO_4 –see Appendix 1.2 (McCaig *et al.*, 1999).

NOB were enumerated in the medium of Alexander and Clark (1965) prepared in artificial seawater. Wells in the microtiter plate were recorded as positive for NOB when nitrite was absent, as indicated by the lack of colour change upon addition of Griess Ilosvay reagents- see Appendix 1.2 (Alexander and Clark, 1965).

2.2.2. FISH (fluorescence *in situ* hybridization) technique

Samples from the SBRs were analyzed by FISH using oligonucleotide probes detailed in Table 5 and methods described by Manz *et al.*, 1992). The paraformaldehyde-fixed samples were spotted on gelatin-coated slide glasses, dried and dehydrated in an ethanol series (50%, 80% and 98%), each for 3 min. Hybridization buffers were made from 0.9 M NaCl, 20 mM Tris/HCl, 0.01% sodium dodecyl sulfate (SDS) and formamide (see Table 5), and the pH was adjusted to 7.2. Fluorescent-labeled oligonucleotide probes were dissolved with the hybridization buffer, and hybridized with the sample for 2 h at 46°C. After hybridization, washing was done in a wash buffer

without formamide (20 mM Tris/HCl, 0.01% SDS and NaCl concentration depended on probe, pH 7.2) for 20 min at 48°C. Slides were rinsed with cold distilled water, air dried, mounted with mounting fluid for fluorescence microscopy (90 ml glycerol added to 10 ml of 0.5M carbonate buffer-Na_2CO_3 pH 9.0 containing 100 mg p-phenylenediamine) to avoid bleaching and observed using a confocal laser-scanning microscope (CLSM). Image Analysis (Crocetti *et al.*, 2002) was used to quantify the proportion of bacteria that were hybridized with interested probes.

Table 5. Oligonucleotide probes and detail were used in this study

Probe	%FA	Probe sequence (5'-3')	Target site	Specificity	Reference
EUB338 [a]	0-70	GCTGCCTCCCGTAGGAGT	16S rRNA (338-355)	most Bacteria	Amann *et al.* (1990)
EUB338-II	0-50	GCAGCCACCCGTAGGAGT	16S rRNA (338-355)	Bacterial domain (Planctomycetes)	Daims *et al.* (1999)
EUB338-III	0-50	GCTGCCACCCGTAGGAGT	16S rRNA (338-355)	Bacterial domain (Verrucomicrobia)	Daims *et al.* (1999)
NSO190	55	CGATCCCCTGCTTTTCTCC	16S rRNA (190-208)	ammonia oxidizing β-Proteobacteria	Mobarry *et al.* (1996)
NSO1225	35	CGCCATTGTATTACGTGTGA	16S rRNA (1224–1243)	ammonia oxidizing β-Proteobacteria	Mobarry *et al.* (1996)
NEU	40	CCCCTCTGCTGCACTCTA	16S rRNA (653-670)	halophilic and halotolerant members of the genus *Nitrosomonas*	Wagner *et al.* (1995)
Nsv443	30	CCGTGACCGTTTCGTTCCG	16S rRNA (444–462)	*Nitrosospira* spp.	Mobarry *et al.* (1996)
NmV	35	TCCTCAGAGACTACGCGG	16S rRNA (174–191)	*Nitrosococcus mobilis* ("*Nitrosomonas*") lineage	Juretschko *et al.* (1998)
NIT3	40	CCTGTGCTCCATGCTCCG	16S rRNA (1035-1048)	*Nitrobacter* spp.	Wagner *et al.* (1996)
NSR1156	30	CCCGTTCTCCTGGGCAGT	16S rRNA (1156-1173)	*Nitrospira moscoviensis*, freshwater *Nitrospira* spp.	Schramm *et al.* (1998)
Ntspa662	35	GGAATTCCGCGCTCCTCT	16S rRNA (662-679)	4 sublineages of *Nitrospira*	Daims *et al.* (2001)
NSR827	20	GTAAMCCGCCGACACTTA	16S rRNA (827-847)	*Nitrospira* genus	Schramm *et al.* (1998)
Ntspa714	55	CCTTCGCCACCGGCCT	16S rRNA (714-729)	phylum Nitrospira	Daims *et al.* (2001)
ALF1b	20	CGTTCGCTCTGAGCCAG	16S rRNA (19-35)	alpha subclass of Proteobacteria	Manz *et al.* (1992)
ALF969	20	TGGTAAGGTTCTGCGCGT	16S rRNA(969-986)	α-Proteobacteria and *Fibrobacter*	Neef (1997)
BET42a	35	GCCTTCCCACTTCGTTT	23S rRNA (1027-1043)	beta subclass of Proteobacteria	Manz *et al.* (1992)
GAM42a	35	GCCTTCCCACATCGTTT	23S rRNA (1027-1043)	gamma subclass of Proteobacteria	Manz *et al.* (1992)
CF319a	35	TGGTCCGTGTCTCAGTAC	16S rRNA (319-336)	*Cytophaga-Flavobacterium* cluster of CFB phylum	Manz *et al.* (1996)
HGC69a	25	TATAGTTACCACCGCCGT	23S rRNA (1901-1918)	gram-positive bacteria with high DNA G+C content	Roller *et al.* (1994)
LGC354a [b]	35	TGGAAGATTCCCTACTGC	16S rRNA (354-371)	part of Firmicutes	Meier *et al.* (1999)
LGC35b	35	CGGAAGATTCCCTACTGC	16S rRNA (354-371)	part of Firmicutes	Meier *et al.* (1999)
LGC354c	35	CCGAAGATTCCCTACTGC	16S rRNA (354-371)	part of Firmicutes	Meier *et al.* (1999)

[a] EUBMIX comprised equal amounts of EUB338, EUB338-II and EUB338-III. [b] LGC354 combined equal amounts of LGC354a, LGC354b and LGC354c, %FA = %Formamide

2.2.3. Clone library preparation and analysis

2.2.3.1. DNA extraction

The DNA was extracted following the protocol in Burrell *et al.* 1998. Biomass from the NFSBR (2 ml) was centrifuged at 12,000 x g for 5 min. The supernatant is discarded, and the pellet is re-suspended in 500 µl of saline-EDTA (150 mM NaCl, 100 mM EDTA [pH 8.0]). A volume of 20 µl of freshly prepared 100 mg/ml lysozyme was added to the mixture and incubated at 37 °C for 1.5 hours. The mixture is then subjected to four cycles of freezing and thawing at –70°C and 65°C, respectively, each step for 3 min. Following this, 100 µl of 25% (w/v) sodium dodecyl sulfate (SDS) and 50 µl of 2% (w/v) protenase K (Merck, Ltd.) were added to the mixture and the mixture was incubated at 60 °C for 1.5 hours. The DNA is recovered from the tube by phenol-chloroform extraction. The nucleic acid from the upper phase was precipitated by adding 0.12 ml of sterile 3 M sodium acetate and 1 ml absolute ice-cold ethanol and incubating for 1 hour at –70°C or -20°C overnight. The DNA pellet was recovered by centrifuging the solution at 12,000 x g for 20 minutes at 4 °C. The pellet was washed by adding 500 µl of 70% ice-cold ethanol then centrifugation at 12,000 *x* g for 10 minutes at 4 °C. The pellet was then air dried in speed vacuum for 10 min with medium heat, and the nucleic acids were dissolved in 30-100 µl of sterile milliQ-purified (MQ) water. Residual RNA was removed from the nucleic acid solution by adding 3 µl of 10-mg/ml RNase and incubating at 37 °C for 1 hour. The DNA extraction efficiency and DNA concentration was determined by running on a 1% Tris-acetate (TAE; 0.04 M Tris-acetate, 0.001 M EDTA) agarose gel containing ethidium bromide (1 µl of 10 mg/ml/50 ml of agarose).

2.2.3.2. Polymerase chain reaction of the 16S rDNA (16S rRNA genes)

Amplification of the near complete 16S rRNA genes from the extracted DNA was done by employing the conserved bacterial primers (Lane, 1991). The PCR reagents were composed of 10x *Tth* Plus reactions

buffer (Biotech International, Australia), 1.5 mM $MgCl_2$, 200 µM (each) deoxynucleotide phosphate (dNTPs), 0.5 U of *Tth* Plus DNA Polymerase (Biotech International, Australia), 1 µl of 200 ng/µl of each primer and 10-100 ng of DNA template. The sterile MQ water was added to make up a final volume of 100 µl.

Amplification reactions placed in a thermocycler (Perkin-Elmer DNA Thermal Cycler) with the cycling program of 30 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 2 min. This was followed by an extension cycle of 48°C for 2 min and 72°C for 5 min. The PCR products were detected by agarose gel electrophoresis on a 1% Tris-acetate TAE agarose gel containing ethidium bromide (1 µl of 10 mg/ml/50 ml of agarose) and viewed on a UV trans-illuminator.

2.2.3.3. Cloning of the 16S rDNAs

Amplicons were cloned immediately in a ligation reaction mixture using the TA Cloning kit (Invitrogen, CA, USA). Ligation reaction was composed of 1 µl of T_4 DNA ligase (1 U/ µl), 5 µl of 2 x ligation buffer, 1 µl of pGEM-T Easy vector (50 ng), 3 µl of amplicons (24 ng). Ligation occurred at 4 °C overnight. Ultracompetent cells were thawed on ice in preparation for the transformation step. A volume of 50 µl of thawed cells was gently placed in a chilled 50-ml Falcon tube, and 1 µl of β-mercaptoethanol was added. The mixture was incubated on ice for 10 minutes with gentle swirling every 2 minutes. Then, 5 µl of the ligation mixture was added to the cells, and incubated on ice for 30 minutes. A heat shock step was done by immersing the Falcon tube in a 42 °C water bath for exactly 30 seconds. Cells were then returned to ice for 2 minutes. A volume of 450 µl of preheated (42 °C) sterile NZY^+ broth was added to each tube of transformed cells and incubated at 37°C for 1 hour with shaking at 225-250 rpm.

A volume of 50-100 µl of transformed cells was spread inoculated onto Luria-Bertani (LB) agar plates containing ampicillin, X-Gal (5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside, and IPTG (isopropyl-β-D-thiogalacto

pyranoside (LB Ampicillin/ X-Gal/ IPTG), which were incubated at 37 °C for 12 to 16 hours and then at 4 °C for 1 hours to enhance the growth of blue colonies. Positive clones (those containing 16S rDNA PCR inserts) appeared white and negative clones (no inserts) were blue. Positive clones were picked and patched onto LB Ampicillin/ X-Gal/ IPTG agar plates to ensure that the first screening was correct. Positive clones were picked; homogenized into 100 µl of sterile 20% glycerol, and stored at–70 °C until required. These clones constituted the clone libraries.

2.2.3.4. Sequencing DNA fragments

16S rDNA inserts from individual clones were amplified and grouped according to restriction fragment length polymorphism (RFLP) analysis using methods previously described (Burrell *et al.*, 1998). Clones having the same bands pattern were grouped into the same operational taxonomic units (OTUs). Selected clones for sequence determinations were representatives of each OUT. All amplicons of clone inserts were partial sequenced using the ABI dideoxy sequencing kit (ABI Australia) according to manufacturer's instruction and using the universal primers 530f (position 515-530 of *E. coli* 16S rRNA; 5'GTGCCAGCMGCCGCGG 3'). Interested clone were sequenced using more 5 primers in order to obtained full size 16S rDNA (Table 6). Sequencing reactions were comprised of 1 µl of version 3 BDT (Big Dye Terminator, ready reaction mix), X µl of SP6-T7 PCR product (50-100 ng) and 1 µl of primer (25 ng/l) and made up to a final volume of 10 µl with sterile MQ water. The sequencing cycle on the thermocycler consisted of 1 cycle at 94 °C for 5 minutes and 30 cycles of 94°C for 10 seconds 50 °C for 5 seconds, and 60°C for 4 minutes.

Table 6. 16S rRNA sequencing primers

Name	*E. coli* rRNA position	Sequence 5'-3'
27f	8-27	AGAGTTTGATCCTGGCTCAG
519r	519-536	GWATTACCGCGGCKGCTG
530f	515-530	GTGCCAGCMGCCGCGG
907r	907-926	CCGTCAATTCMTTTRAGTTT
926f	907-926	AAACTYAAAKGAATTGACGG
1492r	1492-1513	TACGGYTACCTTGTTACGACTT

M = C:A, Y= C:T, K=G:T, W= A:T, R= A:G, all 1:1

Source: Lane (1991)

2.2.3.5. Analysis of sequence data

Partial 16S rDNA sequences were compiled and manually aligned using the software package SeqEd (Applied Biosystems, Australia) and analysed using the Basic Local Alignment Search Tool, BLAST, Altschul *et al.*, 1990) to determine their putative identities. The sequences were also manually aligned, considering secondary structural constraints, with sequences from members of the domain *Bacteria*. Phylogenetic analysis of aligned data sets was carried out by using ARB software package and database (http://www.arb-home.de/). Phylogenetic trees were constructed by carrying out evolutionary distance analyses on the 16S rDNA alignments, using the appropriate tool in the ARB database.