



Isolation and Characterization of a Putative Ammonia monooxygenase (*amo*) gene of *Morganella morganii* (Fulton, 1943) from Seafood Processing Waste Water

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Abstract

In the aquatic environment, microbial oxidation of ammonical nitrogen is an important biological process. The bacterial enzyme ammonia monooxygenase is the key enzyme involved in the oxidation of ammonia. In this study, the ammonia monooxygenase (*amo*) gene from *Morganella morganii* (Fulton, 1943) was isolated and characterized by full-length gene amplification, cloning, sequencing and *in-silico* protein structure and function predictions. The nucleotide sequence of ammonia monooxygenase gene was derived which showed 99% similarity with the homologous gene of *M. morganii* sub sp. *morganii*. *In-silico* structural and functional analyses of the ammonia monooxygenase gene revealed a class of transporter protein with a putative role in ammonia transportation. Further study is necessary to understand the role of this protein in ammonia oxidation by *M. morganii*.

Keywords: Ammonia oxidation, ammonia monooxygenase, *Morganella morganii*, nitrification, waste water

Introduction

The demand for seafood has been increasing owing to its superior nutrient content and excellent sensory features. As a consequence, the production of fish and shellfish from both capture and aquaculture activities has increased phenomenally in many Asian countries including India. Seafood processing activities generate a considerable amount of waste water with a high load of organic matter in soluble,

colloidal and particulate forms that get released into the natural environment causing pollution. The nature and the quantum of pollution depends on the type of seafood processing operation. High levels of phosphorous and nitrogen in the waste water discharged from the processing plants may cause eutrophication in the recipient water bodies leading to oxygen depletion affecting the aquatic flora and fauna (Singh et al., 2011). The emission of ammonia (NH₃) from waste water treatment also causes environmental pollution problems (Li et al., 2013). In addition, high levels of ammonia from protein degradation are toxic to aquatic wild life and therefore, waste water treatment and waste disposal are critical to the health of the aquatic ecosystem. The most important aspect in waste water treatment process is nitrogen removal. Nitrogen plays a vital role in eutrophication of recipient water bodies and to do so, biological nitrification is one of the most economical processes (Gupta & Gupta, 2001). Nitrification is part of the nitrogen cycle in which ammonium ions are biologically oxidized to nitrite and further to nitrate, which is the final product of nitrification beneficial to plants as a nutrient for growth (Yamamoto et al., 2011). Therefore, nitrification can be considered as the gatekeeper of nitrogen cycle. Ammonia oxidation process is achieved by two distinct groups of microorganisms, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). Ammonia oxidation was basically thought to be an obligatory aerobic, chemolithotrophic process performed by a few groups of β and γ -proteobacteria (Koops & Moller, 1992; Kowalchuk & Stephen, 2001). Ammonia oxidation is the first and critical step in nitrification in which ammonium ions are converted into hydroxylamine and further converted into nitrite and nitrate (Chain et al., 2003). Ammonia (NH₃) is oxidized to hydroxylamine (NH₂OH) due to the activity of a membrane-bound hetero-trimeric

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copper enzyme ammonia monooxygenase (AMO) (Richardson, 2000; Bergmann et al., 2005). Since *amoA* gene encodes α -subunit of the AMO enzyme, the *amoA* gene is used as a marker gene to study the nitrification process (Norton et al., 2002). Several past studies have extensively described the diversity and abundance of autotrophic ammonia oxidizing bacteria, the *amoA* gene sequences, enzyme structures and the primers for the amplification of the *amoA* gene (Rotthauwe et al., 1997; Yeager et al., 2005; Ball et al., 2010; Onodera et al., 2010; Szukics et al., 2010; Rasche et al., 2011; Zeglin et al., 2011; Hynes & Germida, 2012; Long et al., 2012; Petersen et al., 2012; Szukics et al., 2012), but only a few studies are available on the heterotrophic nitrification process in which heterotrophic nitrifying bacteria remove ammonia from water (Kim et al., 2005; Andersson et al., 2001; Zhang et al., 2011; Yang et al., 2011; Chen et al., 2012; Yao et al., 2013). *M. morgani* is a gram-negative bacterium commonly found in the aquatic environment and in association with marine fish and shellfish. *M. morgani* is also an important histamine former in fresh fish. Therefore, the primary aim of this work was to isolate and characterise the ammonia monooxygenase (*amo*) gene of *M. morgani* from seafood processing waste water.

Materials and Methods

Four isolates of *M. morgani* MM16, MM17, MM21 and MM22 were isolated from seafood processing waste water in the laboratory using standard techniques and the species identity of the isolates was confirmed by 16S rDNA amplification and sequencing (Bioserve Biotechnologies, Hyderabad). The isolates were stored in Luria Bertani (LB) broth containing 25% glycerol at -80°C till further use.

The total genomic DNA was extracted from the isolates using the cetyl trimethylammonium bromide (CTAB) method (Ausubel et al., 1995). Briefly, 3.0 ml of the overnight culture of *M. morgani* in LB broth was centrifuged at 14000 g for 5 min and the pellet was resuspended in 567 μl TE buffer followed by the addition of 30 μl of 10% SDS and 3 μl of 20 mg ml^{-1} proteinase K. The mixture was incubated at 37°C for 1 h. Following this, 100 μl of 5M NaCl and 80 μl of CTAB/NaCl were added, mixed thoroughly and incubated at 65°C for 10 min. The mixture was extracted with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 14000 g for 4-5 min. The aqueous

supernatant was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged again at 14000 g for 5 min. DNA was precipitated from the aqueous layer by slowly adding 0.6 volumes of isopropyl alcohol, washed with 70% ethanol and dried in a vacuum drier. DNA was dissolved in 100 μl of 1xTE buffer and stored at -20°C for further use. The concentration of DNA and the purity were determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

A total of three sets of primers were designed to amplify full-length sequence of *M. morgani* ammonia monooxygenase (*amo*) gene. Primers were designed from the complete genome sequence of *M. morgani* (GenBank accession number NC_020418.1) using primer BLAST (Ye et al., 2012) (Table1). PCR was performed in 25 μl volumes containing 2.5 μl of 10xPCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.1 and 0.1% Triton X-100) (MP Biomedicals, USA), 1.5 mM MgCl_2 , 200 mM of concentrations of each of the four dNTPs, 30 pico moles of forward and reverse primers each and 1U of *Taq* DNA polymerase (MP Biomedicals, USA). One-hundred nano grams of the template DNA was used in the amplification. The PCR products were resolved on ethidium bromide stained (0.5 $\mu\text{g ml}^{-1}$) agarose gels and photographed using a gel documentation system (BioRad, Hercules, CA, USA). The amplicons were purified from the gel using a gel extraction kit (Qiagen, Germany) and the concentrations were determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

The purified PCR products were cloned using the CloneJET PCR cloning kit following the manufacturer's instructions (Thermo Fisher Scientific, USA). Ligation with pJET 1.2/blunt vector was performed overnight at 4°C . Transformation was performed using competent cells of *Escherichia coli* (DH5 α) prepared using the TransformAid bacterial transformation kit according to the manufacturer's instructions (Thermo Scientific, USA). The transformants were selected by ampicillin resistance and screened by colony PCR using insert specific primers to identify the recombinant clones. Plasmids were extracted from the positive clones using Rapid Pure Plasmid extraction kit (MP Biomedicals, USA) and the inserts were sequenced in both the directions by primer walking (Xcleris Genomics Labs, Ahmedabad, India). The sequences were subjected to nucleotide BLAST analysis against homologous sequences in the GenBank (Altschul et

al., 1997). The multiple sequence alignment was performed using CLUSTAL-OMEGA program (Sievers et al., 2011). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Phylogenetic dendrogram was constructed using MEGA7 (Kumar et al., 2016).

Iterative Threading Assembly Refinement (I-TASSER) hierarchical method (Yang 2008; Ambrish et al., 2010) was used to predict protein structure and function. A multiple threading approach using local meta-threading-server (LOMETS) was followed to identify the structural templates from the Protein Data Bank (Wu & Zhang, 2007). Iterative template fragment assembly simulations were used for the construction of full-length atomic models. The 3D models were derived by threading through BioLip protein function database and the final functional features of the target were derived (Ambrish et al., 2012; Ambrish & Yang, 2012; Yang et al., 2013)

Results and Discussion

All the three primer sets designed in this study were (Table 1) successfully amplified *M. morganii* ammonia monooxygenase gene (*amo*) from all the four isolates used in this study. To determine the full-length nucleotide sequences, the amplicons were cloned and sequenced. The nucleotide-nucleotide BLAST analysis of the sequences showed 99% similarity with *M. morganii subsp. morganii* ammonia monooxygenase gene. The nucleotide sequences of *amo* gene were aligned with homologous sequences retrieved from the GenBank. Protein-protein BLAST analysis of the amino acid sequence of ammonia monooxygenase gene of *M. morganii* also showed 99% similarity to ammonia monooxygenase of *M. morganii* with putative conserved domains of *amoA*

super family of proteins (GenBank accession number WP_036417963.1W).

The phylogenetic relationship was inferred for *amoA* gene of both autotrophic as well as heterotrophic ammonia oxidizing bacteria using the Neighbour-Joining method (Saitou & Nei, 1987; Tamura et al., 2013). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985) (Fig. 1.) The isolated strains were all heterotrophic in nature. However, the *amo* gene nucleotide sequences of cluster-1 (heterotrophic) and the cluster-2 (autotrophic) ammonia oxidizing bacteria showed <50% similarity. The *amoA* gene is used as a marker to study the nitrification process and is responsible for encoding α -subunit of the ammonia monooxygenase enzyme (Norton et al., 2002). Many studies have been done on the structure and abundance of *amoA* gene (Quan et al., 2008; Pajares & Bohannan 2016, Wang et al., 2014). All these studies were based on the primer sets which were designed from reference species of autotrophic ammonia oxidizing bacteria *N. europaea* (L08050) *amoA* sequences. The *amoA* F/2 R primer set was extensively used to study the *amoA* gene (Rotthauwe et al., 1997; Onodera, 2010; Zeglin et al., 2011; Petersen et al., 2012). By using these primers, only partial sequence can be obtained which is insufficient to study the structure and function of ammonia monooxygenase gene. Therefore, full-length sequence of ammonia monooxygenase gene of *M. morganii* was determined in this study and it was observed that *M. morganii* isolates of this study harbored ammonia monooxygenase gene. A membrane-associated ammonia monooxygenase was identified in the whole-genome sequence of *M. morganii* KT (Yu-Tin et al., 2012).

The nucleotide sequence of *amoA* gene of autotrophic ammonia oxidizing bacteria *N. europaea* (GenBank accession number NC_004757.1) and nucleotide sequence of *amo* gene of heterotrophic bacterium *M. morganii* derived in this study were used to predict the 3-dimensional structure of the ammonia monooxygenase protein. High confidence score was obtained for the secondary structures of ammonia monooxygenases of both autotrophic and heterotrophic bacteria. Higher confidence scores are indicative of better prediction of secondary structures of proteins. Normalized B-factor plot for both autotrophic and heterotrophic bacteria suggested that the amino acid residues were more stable in the

Table. 1. Details of primers used for the amplification of *M. morganii amo* gene in this study

Sl. No.	Primer	Sequence (5' - 3')	Product size (bp)
1	MM1F	tgcgcaccgtttcaatgttt	640
2	MM1R	gtcaccatattgcgcgagcag	
3	MM2F	ttccgtgaccacactgatt	690
4	MM2R	cgctttgccggacaataacc	
5	MM3F	ctgtttccgctgaggtcact	556
6	MM3R	gattgaaatcaccggcagc	

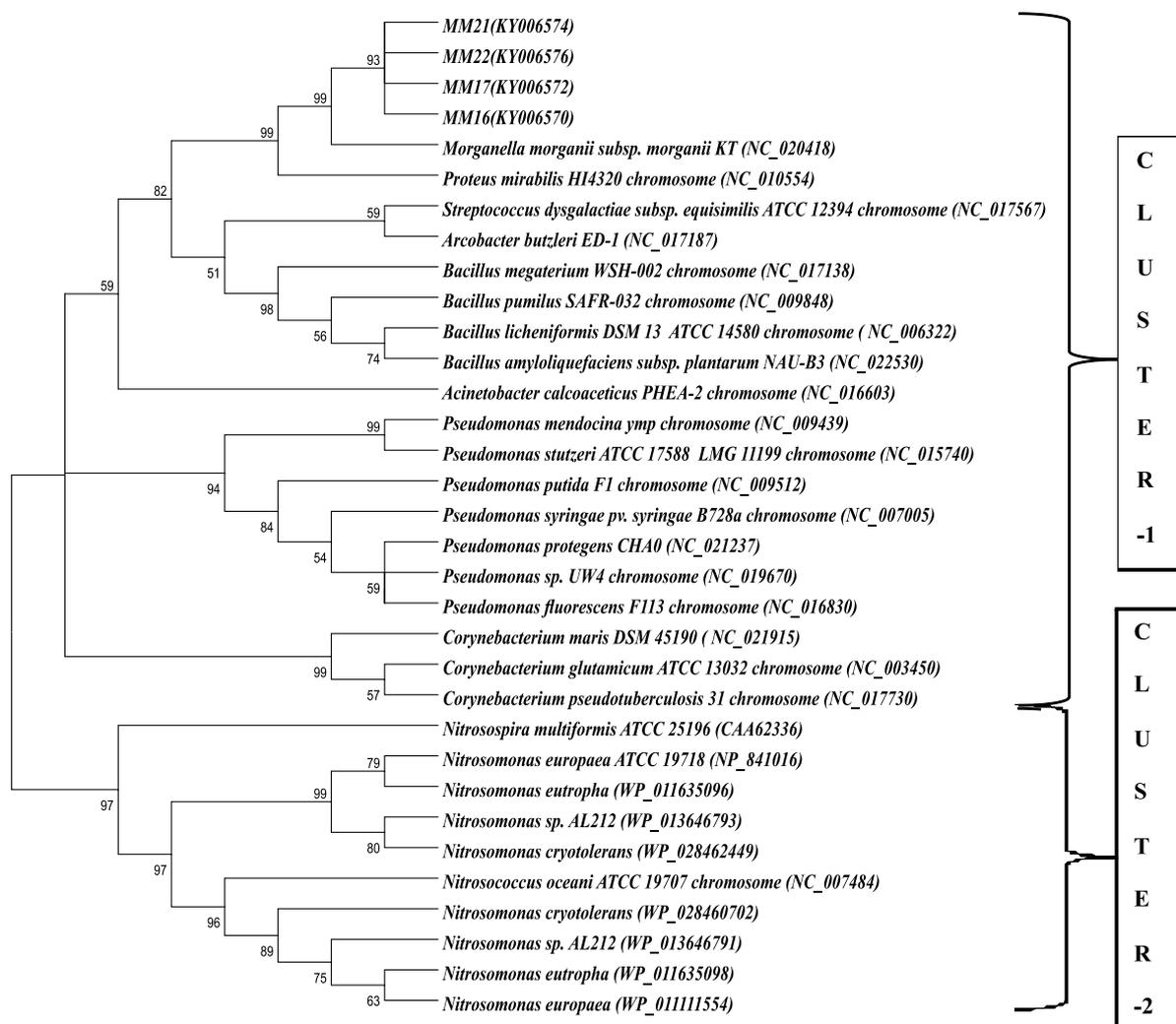


Fig. 1. Dendrogram showing the evolutionary relationships of ammonia monoxygenase gene (*amo*) of *M. morganiii* isolates MM16, MM17, MM21 and MM22 with the homologous genes of both heterotrophic and autotrophic ammonia oxidizing bacteria by the neighbour-Joining method. The accession numbers of the sequences are indicated in parentheses.

structure (data not shown). The top ten threading alignments for protein sequences of ammonia monoxygenase of both *N. europaea* and *M. morganiii* generated by LOMETS were from the top threading templates based on conserved regions to obtain higher structural accuracy. The statistical significance of the best threading alignment was judged based on the Z-Score and a Z-Score of >1 obtained in this study indicates good alignments (data not shown). Top five 3D Models were predicted for both the bacteria and the final 3D models were selected based on the C-Score. The C-Scores (Fig. 2 A & B) for ammonia monoxygenase of *N. europaea* and

M. morganiii were -0.48 and -0.69 respectively which indicated that the models generated were accurate. Proteins structurally close to the predicted structures in this study were identified in the Protein Data Bank (Fig. 3 A & B). The ammonia monoxygenase of autotrophic ammonia oxidizing bacteria *N. europaea* showed similarity with a class of oxidoreductase, methane monoxygenase (PDB ID 4PHZ) (Fig. 3B), which is a membrane-bound metalloenzyme that oxidizes methane to methanol in methanotrophic bacteria. The predicted structure of the ammonia monoxygenase of *M. morganiii* showed similarity to a class of membrane

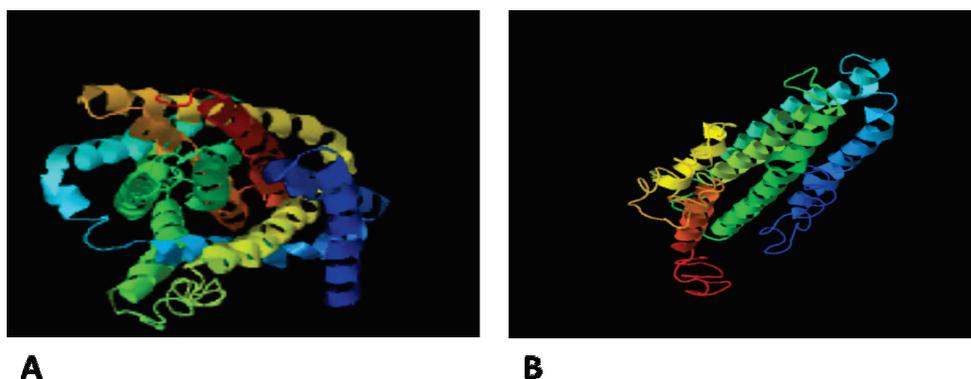


Fig. 2. Predicted 3-dimensional structure of ammonia monooxygenase of *M. morganii* using the sequence derived in this study (A), and the ammonia monooxygenase of *N. europaea* (B) (GenBank accession number NC_004757.1)

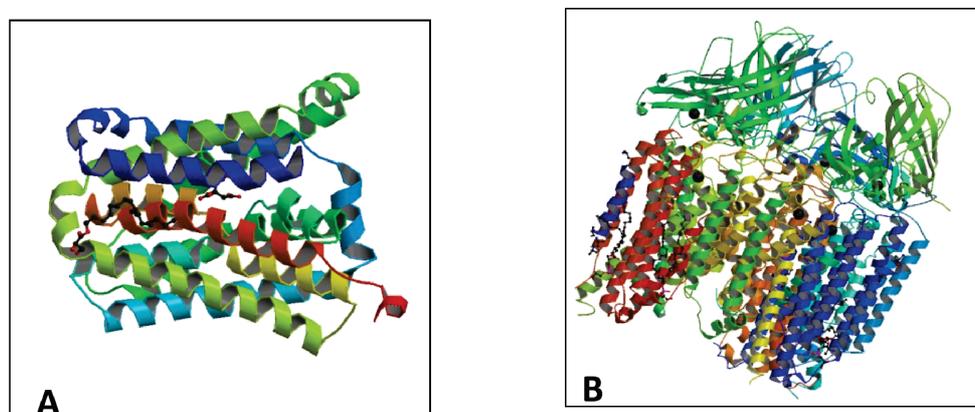


Fig. 3. Structural homologues of the putative proteins of *M. morganii* derived in this study and the ammonia monooxygenase of *N. europaea* in Protein Data Bank (PDB). The putative protein, the gene sequence of which was derived in this study, was structurally similar to a sodium-bile symporter protein (PDB structure ID 4N7W) (A), and the ammonia monooxygenase of *N. europaea* was structurally similar to methane monooxygenase (PDB ID 4PHZ) (B).

transporter protein, the sodium bile acid symporter (PDB ID 4N7W) (Fig. 3A). The amino acid sequence similarity and the predicted tertiary structure of the gene sequence from *M. morganii* derived in this study presumably has a putative role in ammonia transportation. Further studies are necessary to identify the complete genetic set up involved in ammonia oxidation in *M. morganii* which can be a potential biotechnological tool for bioremediation of ammonia-rich waste water generated from fish processing industries.

The *amo* gene sequences of *M. morganii* generated in this study have been deposited in GenBank under accession numbers (KY006570, KY006572, KY006574 and KY006576).

The biological process of oxidation of ammonia is accomplished by the bacterial enzyme ammonia monooxygenase (AMO). In this study, we amplified and sequenced a putative *amoA* gene from *Morganella morganii*. The 3-dimensional structure predicted using bioinformatics tools revealed that the gene identified in this study encodes a membrane protein homologous to sodium bile acid symporter with a putative ammonium transportation activity. Further studies are necessary to establish the role of the transporter protein identified in this study in ammonia utilization as well as other proteins involved in the process of ammonia oxidation by *M. morganii*.

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