

Characterization and Phylogenetic Studies of Marine Bacteria Isolated from Sundarbans, Bangladesh

ABSTRACT

Aims: Marine environment is a vast but represents a largely untapped source for isolation of new microorganism having ability to produce novel bioactive compound(s). This present study was designed with an aim to discover and identify a new marine bacteria strain producing antibacterial metabolites from marine soil samples of Sundarbans, Bangladesh.

Study design: Serial dilution technique, streak plate technique, cover slip culture method and 16S rDNA sequencing were used for this study.

Place and Duration of Study: Pharmaceutical Microbiology Research Laboratory, Department of Pharmacy, University of Rajshahi, Rajshahi-6205, Bangladesh between August 2010 and July 2011.

Methodology: Totally thirty nine bacterial colonies were isolated from the marine soils collected from the different location of Sundarbans, a mangrove forest of Bangladesh. Among them ANAM-39 were selected for morphological, cultural, physiological, biochemical and taxonomic characterization as it was exhibited highest antibacterial activity against a series of test organisms.

Results: The 16s rDNA sequence of the strain ANAM-39 showed close similarity with *Smithella propionica* (95.99%), *Syntrophus aciditrophicus* (93.63%) and *Syntrophus gentianae* (93.17%). But the cultural, morphological, physiological and biochemical characteristics of the strain and its closely associated strains were completely different. So, it is not logical to assign the strain ANAM-39 to the genus *Smithella*.

Conclusion: To confirm the taxonomic position of the strain and to have a correct solution from this taxonomic problem, further studies is needed in respect to the DNA relatedness studies, DNA-DNA hybridization, small subunit (SSU) sequences, cell wall composition and other characteristics of the strain.

Keywords: Characterization, Phylogenetic Studies, Marine Bacteria, Sundarbans.

1. INTRODUCTION

Marine microbial biodiversity has opened up unexpected new horizons for finding novel organism for trapping their potential resources. More than 70% of our planet's surface is covered by oceans and life on Earth originated from the sea. Marine sediment is an inexhaustible resource that has not been properly exploited. It is estimated that less than 1% of potentially useful chemicals from marine environment has been screened so far, with microbial products representing approximately 1% of the total number [1]. Marine ecosystems and coastal regions are particularly promising, because of the rightly adopted species found in this harsh environment. Studies have shown that microorganism isolated from the marine environment are metabolically active and have adapted to life in the sea [2]. So, the exploration of microbial secondary metabolites from marine environment has led to the discovery of hundreds of biologically active compounds.

Marine environments are a rich source of both biological and chemical diversity. This diversity has been a potential source for unique microorganism producing different types of bioactive compounds [3]. Marine microorganisms have become important in the study of novel microbial products exhibiting antibacterial, antiviral, antitumor as well as anticoagulant and cardioactive properties [4-5]. Recently, the marine derived microorganisms have become recognized as a source of novel antibiotic and anticancer agent with unusual structure and properties [6].

Antibiotic resistance arising mainly through antibiotic misuse is now recognized as a global health problem. The situation is exacerbated by the fact that no novel antibiotics have been discovered for last 20 years. Although many pre-existing antibiotics have been modified to yield new derivatives, bacteria have the potential to mutate known resistance mechanisms to combat these [7-8]. The need for new, safe and more effective antibiotic is a major challenge to the pharmaceutical industry today, especially with the increase in antibiotic resistance. For this constant increasing of bacterial resistance, it is now desperate need to search for new sources of antibiotics.

As the frequency of novel bioactive compounds discovered from terrestrial microorganism decreases with time, much attention has been focused on screening of microorganism from diverse environments. Marine environments are largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites. As marine environmental conditions are extremely different from terrestrial ones, it is summarized that marine microorganism have different characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds [9]. Marine derived antibiotics are more efficient at fighting microbial infections than the terrestrial bacteria have not developed any resistant against them. Among marine microorganisms, actinomycetes particularly the genus *Streptomyces* are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities [10].

Actinomycetes populations are relatively lower than other soil microbes and contain a predominance of *Streptomyces* that are tolerant to acid conditions [11]. *Streptomyces* is the largest genus of the Actinobacteria and are Gram positive bacterium with high GC content [12]. *Streptomyces* includes filamentous bacteria which produce well developed vegetative hyphae (between 0.5-2.0 μm in diameter) with branches. Members of *Streptomyces* are found almost everywhere, and the largest reservoir of these microorganisms is soil. *Streptomyces* covers around 80% of total antibiotic product, with other genera trailing. *Streptomyces* are widely recognized as sources of antibiotics and other important novel metabolites, including antifungal agents [13], antitumor agents [14], antihelminthic agents [15] and herbicides [16]. Although thousands of antibiotics have been isolated from *Streptomyces*, only small fraction of the repertoire of bioactive compounds produced [17-18].

In this study, we explored the presence of antibiotic producing microorganism in soils of Sundarbans, the largest tidal halophytic mangrove forest in the world. Marine bacteria producing antibacterial compounds were isolated from Sundarbans, Bangladesh and were characterized. This study demonstrated that the diversity of the Sundarbans as a rich source of bioactive molecules producing new and potential marine bacteria species.

2. MATERIAL AND METHODS

2.1 Isolation and Selection of Organism

A total of nine marine soil samples were collected carefully from the various depth of the earth, ranging from layers just beneath the upper surface to 1.5 meter depth from different location of Sundarbans, Bangladesh (08-16 August, 2010). The isolation of the marine

bacteria from these soil samples were carried out by spread plate technique [19]. A total of 39 strains [ANAM-1 to ANAM-48] were isolated as pure culture as their colonies showed *Streptomyces* like appearance under light microscope from these soil samples. The purified isolates were preserved on yeast-extract-glucose-agar slants at 4°C. Then all the pure isolates were preliminary screened for antibacterial activity through the use of streak plating technique [20] on yeast-extract-glucose-agar medium. Due to the highest antibacterial activity in preliminary antibacterial screening, visibility of the metabolite as well as excellent growth properties, the isolates ANAM-39, a brownish yellow colored microorganism was chosen for further characterization.

2.2 Morphological Characterization

Morphology had played a major role in distinguishing bacteria and in the characterization of bacteria species. The morphological characteristics of the strain ANAM-39 was determined in accordance with the method described by Shirling and Gottlieb [21]. Microscopic characterization was done by cover slip culture method. In this method, four sterile cover slips were inserted aseptically into the sterile solidified agar medium in a petri dish at an angle of 45° to 60° by means of a sterile forceps and the strain was inoculated by streaking method, at the free space of the plate. The hyphae of the strain grew and spread on the cover slip and produced spores during incubation period (at 37.5°C for 10 days). After this period, the cover slips were then taken out smoothly by using sterile forceps, mounted on slides and observed under microscope, using lactophenol cotton blue [22]. Colonies was identified on the basis of their colony morphology and color [21]. The characteristics of the spore-bearing hyphae and spore chains were determined by direct microscopic examination of the culture surface on opened dishes. To establish the presence or absence of chains of spores 100x - 700 x magnifications were used.

2.3 Cultural Characterization

The cultural characteristic of the strain was also determined according to the method described by Shirling and Gottlieb [21]. Cultural characteristics are best made on a variety of standard cultivation media such as International *Streptomyces* Project (ISP) 2 (yeast-extract-malt extract agar); ISP 3 (oatmeal agar); ISP 4 (inorganic salts-starch agar); and ISP 5 (glycerol-asparagine agar), ISP 1 (Trypton –yeast extract agar), ISP 7 (Tyrosine agar) and yeast-extract – glucose agar (YEGA). A National Bureau of Standards Color Chart was used to determine the color of the substrate mycelia, aerial mycelia, and spore mass and pigment production [23].

2.4 Biochemical and Physiological Characterization

The biochemical and physiological characteristics of the strain such as production of melanin, optimization of pH and temperature, utilization of carbon and nitrogen sources etc. were determined as described by Shirling and Gottlieb [21]. The growth of the strain ANAM-39 was determined on different carbon sources like D-glucose, sucrose, D-fructose, L-rhamnose, D-galactose, xylose, rhamnose, maltose, lactose, starch and mannitol. Here, basal mineral salts agar (ISP medium 9) and different carbon sources were used for the medium of the test. The optimization of nitrogen sources such as yeast extract, peptone, ammonium chloride, ammonium sulphate, NaNO₃, casein, beef extract, KNO₃, sodium nitrate and L-asparagine was carried out by adding nitrogen source (0.2%) to the basal medium containing glucose (3%). The pH of the medium was adjusted using hydrochloric acid (1M) and sodium hydroxide (1M). For the optimization of temperature, after sterilizing and inoculating with spores the flasks were incubated at different temperatures (25-47°C).

2.5 Phylogenetic Characterization

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods [24]. So, significance of

phylogenetic studies based on 16S rDNA sequences is increasing in the systematics of bacteria and marine microorganism. In 16S rDNA sequencing, for the preparation of genomic DNA the isolated colony from the agar plate was dispersed in 500µl of saline-EDTA buffer (NaCl 150 mM; EDTA 10 mM; pH 8.2) and incubated for 1hr at 37°C. Then, 10 µl of lysozyme solution (5mg/ml), 5 µl of proteinase K solution (15mg/ml) and 10 µl SDS solution (25%) were added and incubated for 30 minutes at 55°C. Lysate was extracted, purified and dissolved in nano pure sterile water (60 µl). The 16S rDNA was amplified by the PCR in reaction mixture containing KOD FX buffer with 200 µM dNTP, 100 ng genomic DNA and 0.5 µg forward (5'-AGAGTTTGATCCTGGCTCAG -3') and 0.5 µg reverse (5'-GGTACCTTGTACGACTT -3') primer. The resultant PCR solution was used in sequencing reaction with Pre-Mix and primer. The reaction mixture was applied to thermal cycles and DNA was purified by using Prep-A-Gene Kit (Bio-Rad). The liquid has evaporated and the dried DNA was dissolved in 20µl of polyacrylamide sequencing gel and sequenced by Applied Biosystems automated DNA sequence. The resulting sequences were analyzed with DDBJ/EMBL/GenBank database using analysis softwares- BLAST and BIBI (Bioinformatics Bacterial Identification Tool) [24]. BIBI software is available online (<http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibiexecx.cgi>). The phylogenetic trees were constructed by using BIBI. The 16s rDNA sequences of the isolates ANAM-39 was submitted to the GenBank.

3. RESULTS AND DISCUSSION

This present study was designed with an aim to discover a new marine bacteria strain producing antibacterial metabolites. With this view point, the isolates ANAM-39, a brownish yellow colored microorganism was chosen for further characterization due to the highest preliminary antibacterial activity, visibility of the metabolite as well as excellent growth properties of the strain.

The isolate ANAM-39 grows very slowly at first and gives white colonies after 24 hrs (Fig. 1.) After 48 hrs, it gradually turns into velvety cream colonies.



Fig. 1. Aerial and substrate mycelial view of ANAM-39 on 21st day of incubation.

Then it grows very fast and after 72 hrs ANAM-39 starts to release a soluble brown pigment through the medium. It became light brown after 96 hrs and give brownish yellow colonies after 120 hrs. As the time passes, the aerial mycelium became more powdery as well as deep brown due to sporulation of the microorganism. Under microscope, the marine bacteria ANAM-39 produce well developed substrate mycelium and aerial hyphae which are differentiated into long and extended filamentous chains with cylindrical, smooth spores occurring in chains of more than 7. Spores are observed after five days. The isolates ANAM-39 exhibited morphology of typical *Streptomyces*; the colonies were slow growing, aerobic, glabrous or chalky, folded, and with aerial and substrate mycelia [24]. In addition, this isolate possessed an earthy odor

175 and fitted to the description of genus *Streptomyces* in Bergey's Manual of Systemic
176 Bacteriology.

177

178 Cultural characteristics of the strain ANAM-39 in different cultural media are presented in
179 Table 1, showing morphology and properties of typical *Streptomyces*. The growth and
180 pigmentation of the strain was very low in ISP 5 medium compared with both ISP 2 and ISP
181 4 medium where the growth of the strain was very high, the spore mass was profuse in
182 these medium and pigmentation was very high. In ISP 1, ISP 3, ISP 7 and YEGA medium
183 the growth of the strain was moderate. The color of the substrate mycelium was tended to
184 either yellow, pinkish yellow, dark white or white. Whereas, aerial mycelium found to be
185 either brownish yellow, deep brown, light brown, yellowish brown, or light yellow. The strain
186 produces yellowish brown to brown pigment or a distinct brown pigment modified by other
187 color on ISP 1 and ISP 3 medium.

188

189 **Table 1. Cultural characteristics of strain ANAM-39. The sign ‘++’ indicates strongly**
190 **positive utilization, ‘+’ sign positive utilization, ‘±’ sign utilization doubtful and ‘-’**
191 **indicate utilization negative.**

Medium	Growth	Spore mass	Aerial mycelium	Reverse side substrate mycelium	Pigmentation
Trypton –yeast extract agar (ISP 1)	++	Very low	Light brown	Dark white	+
Yeast-extract-malt extract agar (ISP 2)	+++	Abundant	Brownish yellow	Pinkish yellow	++
Oatmeal agar (ISP 3)	++	Moderate	Brownish yellow	Yellow	++
Inorganic salt-starch agar (ISP 4)	+++	Abundant	Deep brown	Yellow	+++
Glycerol-asparagine agar (ISP 5)	+	Very low	Light yellow	Yellow	-
Tyrosine agar (ISP 7)	++	Moderate	Yellowish brown	Dusky yellow	+
Yeast-extract glucose agar (YEGA)	++	Low	Brownish yellow	White	+

192

193 Utilization of carbon and nitrogen sources by the strain ANAM-39 is presented in Table 2.
194 The production of antibiotic by various *Streptomyces* species was greatly influenced by
195 suitable carbon and nitrogen sources [25]. The growth and production of yellow pigment was
196 good by the strain in the presence of glucose, lactose and sucrose as carbon sources in the
197 medium. In the presence of D-xylose as a sole carbon source in the medium, no growth was
198 observed. Utilization and metabolite production by the strain in the presence of inositol and
199 mannitol was doubtful. In the absence of any carbon source (Negative control) the strain
200 could not grow. Therefore, it may be concluded that, proper carbon source is needed for
201 optimum growth of the strain and for optimum production of pigment. In this present study,
202 the strain was found to produce high amount of metabolites in the medium supplemented
203 with glucose, lactose and sucrose as sole carbon source. In case of *Streptomyces* species,
204 with regards to carbon sources species specific variation may occur for cell growth and
205 secondary metabolites production [26].The strain used different inorganic and organic
206 nitrogen sources differently and exhibited differences in metabolites production. The growth
207 of the strain as well as metabolite was very good in the medium supplemented with yeast
208 extract and casein as nitrogen sources. In the presence of peptone, ammonium chloride,
209 ammonium sulphate, NaNO₃ and beef extract as nitrogen sources the growth and metabolites

production of the strain was moderate. The growth and pigmentation was doubtful for the strain in the medium containing KNO₃ and sodium nitrate. The growth of the strain was doubtful and metabolite production was negative in the medium containing L-asparagine. Different types of carbon and nitrogen sources were found to have significant effect on growth and secondary metabolite production by various *Streptomyces* species [27]. On this respect, the strain ANAM-39 showed similar characteristics with *Streptomyces* species. The growth of the strain was very high and production of high levels of antibiotic when culture medium incubated at 30 °C and pH 6.0. Extreme pH and temperature were unfavorable for antibiotic production. So, the morphological, cultural, biochemical and physiological properties of the strain ANAM-39 were consistent with its assignment to the genus *Streptomyces* sp.

Table 2. Utilization of carbon and nitrogen sources by the strain ANAM-39. The sign ‘++’ indicates strongly positive utilization, ‘+’ sign positive utilization, ‘±’ sign utilization doubtful and ‘-’ indicate utilization negative.

Carbon source	Utilization	Pigmentation	Nitrogen source	Utilization	Pigmentation
D-Glucose (Positive control)	++	++	Yeast extract	++	++
No carbon (Negative control)	-	-	Peptone	+	+
L-Rhamnose	+	+	Ammonium chloride	+	+
Starch	+	+	Ammonium sulphate	+	+
D-fructose	+	+	NaNO ₃	+	+
D-Xylose	-	-	Casein	++	++
Lactose	++	++	Beef extract	+	+
Mannitol	+	±	KNO ₃	±	±
Inositol	±	±	Sodium nitrate	±	±
Sucrose	++	++	L-asparagine	±	-

On identifying isolates at the species level, incongruities were observed. The 16s rDNA sequence of strain ANAM-39 was determined and compared to those deposited in GenBank using the BLAST and BIBI and Figure 2 shows a phylogenetic tree derived from these sequences. It was observed that the sequence was actually similar to those sequences of more than one species (Table 3). Species identification was based on maximum score, identity and coverage values. The genetically related species were *Smithella propionica*, *Syntrophus aciditrophicus*, *Syntrophus gentianae*, *Syntrophus buswellii*, *Pelobacter propionicus*, *Algidimarina propionica* and *Algorimarina butyrica*. But, this strain showed is most closely related (95.99 % sequence similarity) to strain *Smithella propionica*, an anaerobic, syntrophic, propionate-oxidizing bacteria whose sequence was determined in the laboratory of David R. Boone [28]. This strain also closely related to *Syntrophus aciditrophicus* (93.93% sequence similarity) and *Syntrophus buswellii* (92.52%), but these similarities were not sufficient to indicate that strain ANAM-39 should be classified within the genus *Syntrophous*. According to Drancourt et al [29], >99% and >97% sequence similarities

were used as the cut-offs for species and genus identification, respectively, depending on the bacterial genus under investigation. On the other hand, Janda and Abbott in their recommended guidelines suggested that a minimum of >99%, and ideally >99.5%, sequence similarity be used as the criteria for species identification [30]. They also proposed that other properties, e.g. phenotype, should be considered in final species identification. Although different studies have identified groups of bacteria for which 16S rDNA sequences are not sufficiently discriminative and for which other gene targets have to be used.

Table 3. Sequences producing significant alignments with the 16S rDNA sequence of ANAM-39.

Accession No.	Species/Strain	Total score	Query coverage (%)	E value	Max identity (%)
AF126282	<i>Smithella propionica</i>	2286	100%	0.0	95.99%
CP000252	<i>Syntrophus aciditrophicus</i>	2242	100%	0.0	93.63%
X85132	<i>Syntrophus gentianae</i>	2198	100%	0.0	93.17%
GU993263	<i>Syntrophus aciditrophicus</i>	2185	100%	0.0	93.52%
X85131	<i>Syntrophus buswellii</i>	2113	100%	0.0	92.52%
U86447	<i>Syntrophus aciditrophicus</i>	1836	100%	0.0	93.93%
X70954	<i>Pelobacter propionicus</i>	1455	100%	0.0	90.59%
CP000482	<i>Pelobacter propionicus</i>	1455	100%	0.0	90.59%
AY85129	<i>Algidimarina propionica</i>	1447	100%	0.0	90.17%
AY851292	<i>Algorimarina butyrlica</i>	1435	100%	0.0	90.64%

For comparison we examined *Smithella propionica* and found important differences. *Smithella propionica* is strictly anaerobic, gram-negative, syntrophic, rod shaped, 0-5 µm in diameter, contain granules of poly-P-hydroxybutyrate, weakly motile bacteria [28] but the strain ANAM-39 was aerobic, gram-positive, produce well developed substrate mycelium and aerial hyphae which are differentiated into long and extended filamentous chains with cylindrical, smooth spores occurring in chains of more than 7, colonies glabrous or chalky, folded, and with aerial and substrate mycelia and possessed an earthy odor. *Smithella propionica* grows fast with pH near neutral, could not grow at pH values of 6.3 or below and could not grow well above or below 37°C temperature [28] whereas strain ANAM-39 showed excellent growth at 30°C and pH 6.0. *Smithella propionica* grows axenically on crotonate, producing acetate and butyrate but does not grow axenically on propionate whereas strain ANAM-39 utilizes mostly D-Glucose, Lactose, Starch and D-fructose as energy source.

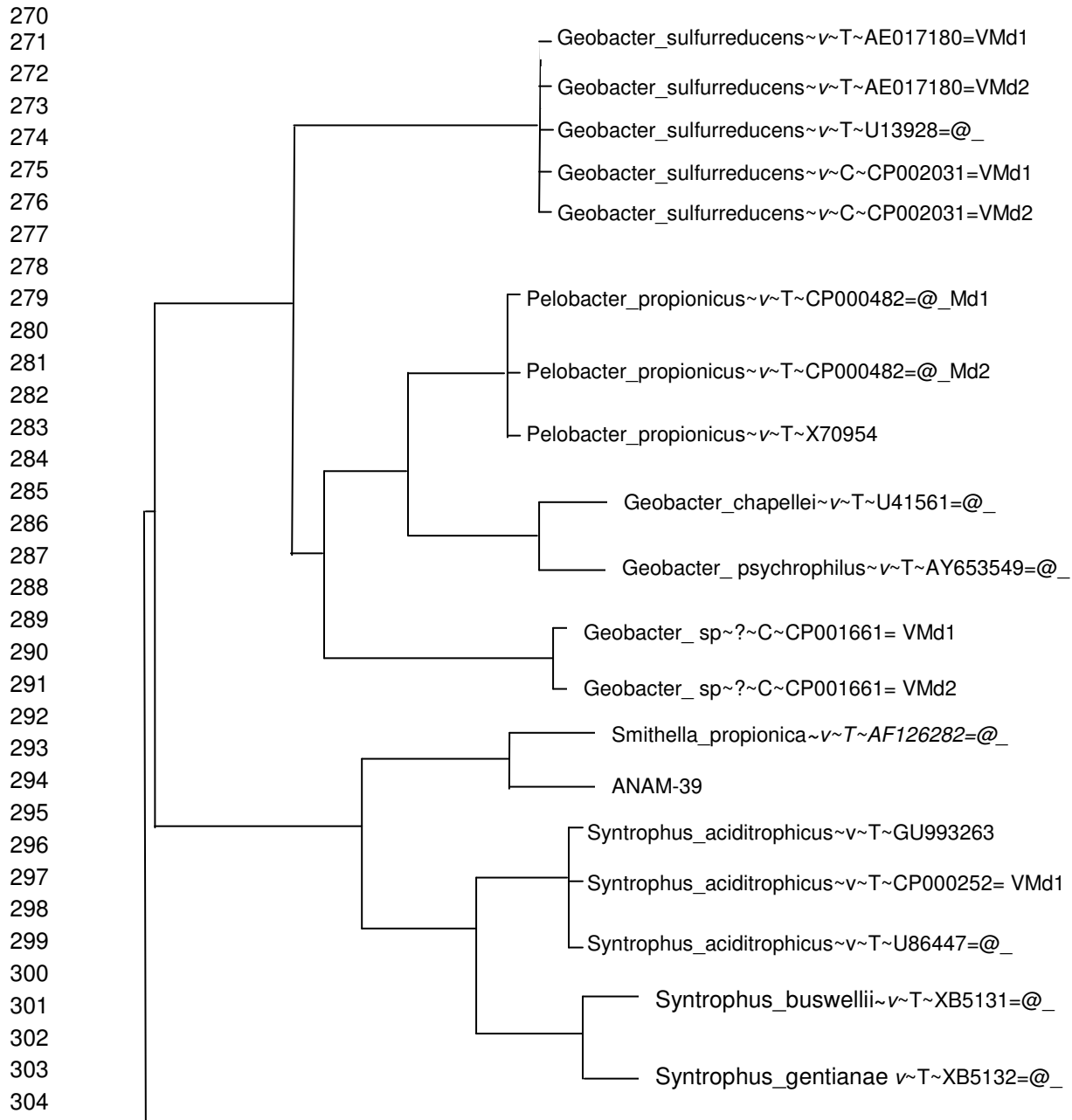


Fig. 2. Phylogenetic tree of the strain ANAM-39 based on partial 16S rDNA gene sequences (using sequence database Bacteria_TS_SSU-rDNA-16s-stringent).

Although 16S rDNA sequencing is being used increasingly for bacterial identification in clinical microbiology laboratories but there is some limitations of this technique like the ever-expanding sequence databases and taxonomic complexity, and the inaccuracies in some databases [28-29]. The use of 16S rDNA sequencing for bacterial identification depends on significant interspecies differences and small intraspecies differences in 16S rDNA sequences. Therefore, one of the major limitations is that when two different bacterial species share almost the same 16S rDNA sequence, this technique would not be useful for distinguishing between them [31]. However, there are 'blind spots' within some major

genera, in which 16S rDNA sequences are not sufficiently discriminative for the identification of certain species [32-33]. 16S rRNA gene sequencing is highly useful in regards to bacterial classification; it has low phylogenetic power at the species level and poor discriminatory power for some genera [34-35]. Considering these limitations, in order to increase the accuracy of 16S rDNA sequencing for identification of bacteria, it would be necessary to interpret the results of 16S rDNA sequencing with preliminary morphological, cultural, biochemical and physiological test results.

The results obtained on analyzing various characteristics and phylogenetic studies based on partial sequences of the 16S rDNA region encoding gene, demonstrate that though the strain ANAM-39 is most closely related to the organism *Smithella propionica* in phylogenetic development as well as sequence similarity but the various phenotypic characteristics of ANAM-39 like morphological, cultural, physiological and biochemical were completely different from *Smithella propionica*. So, the consignment of the strain ANAM-39 to the genus *Smithella* is not logical. Thus, further studies should be carried out in respect of the DNA relatedness studies, DNA-DNA hybridization, small subunit (SSU) sequences, cell wall composition and other characteristics to confirm the taxonomic position of the strain ANAM-39.

4. CONCLUSION

Our findings indicate that the strain ANAM-39 differs morphologically, culturally, physiologically, biochemically and phylogenetically from *Smithella propionica*. Strain ANAM-39 is phylogenetically most similar to the genus *Smithella*, but the phylogenetic relationship is not sufficiently close to permit classification of strain ANAM-39 in this genus. So, the strain ANAM-39 cannot be assigned to the genus *Smithella*. Thus to confirm the taxonomic position and to provide absolute resolution to these taxonomic problem of the strain ANAM-39, more studies should be carried out in respect of the DNA relatedness studies, DNA-DNA hybridization, small subunit (SSU) sequences, cell wall composition and other characteristics.

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