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Studies of the Rate Homology between 16S rRNA Gene in theUncultured*Paenibacillus*Species Clone: T-168 and Other 16S Rrna Gene in the*Paenibacillus*Species.

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ABSTRACT

Theuncultured*Paenibacillus*speciesclone: T-168 were isolated from muscle tissue of *salmotruttacaspius*(fish species) for studies of their 16S rRNA gene. A one pair of 16S rRNA gene were designed by BLAST and DNAMAN computer program. After amplifying of RNA gene, were sequenced and deposited GenBank, accession number, AB828175.1. 16S rRNA gene of uncultured*Paenibacillus* clone: T-168 were aligned between some species of bacteria specially *Paenibacilus*. The results showed that there was homology between 16S rRNA gene of uncultured*Paenibacillus* clone: T-168 and 16S rRNA gene in other species of the bacteria.

Keywords: 16S rRNA gene of uncultured*Paenibacillus* clone: T-168**,** Bacteria, sequencing.

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INTRODUCTION

Paenibacilli have been isolated from a variety of sources including soil, water, the plant rhizosphere, food, and diseased insect larvae, but not from clinical specimens (Daane*et al*. 2002). This species were applied for industrial and agricultural development. Some *paenibacilus* species were used for nitrogen fixation in which atmospheric nitrogen including 45 recognized *Paenibacillus* species (NCBI homepage, November 2003). Also their ability to produce phytohormones, suppress phytopathogens through antagonistic functions and solubilize organic phosphate (Mavingui and Heulin, 1994; Lebuhn*et al.* 1997; Pires and Seldin, 1997), they can also be of industrial importance due to the production of antibiotics, chitinases, amylases and proteases (Mavingui and Heulin, 1994; von der Weid*et al.* 2003). Were studied on the phylogenetic of *Paenibacillus* species for finding rate of homology between species and subspecies of the *Paenibacillus*. As following that, studied on the 16S rRNA sequences, were used studies on phylogenetic of the new genus of *Paenibacilus* species. (Ash *et al*. 1993; Heyndrickx*et al.* 1996; Coelho *et al.* 2003; Silva *et al.* 2003; Daane*et al*. 2002; Chung *et al.* 2000; Stackebrandt*et al.* 1997). For *Paenibacilus* species were found 10 copies of the 16S rRNA gene in their genomics (Heyndrickx*et al*. 1996; Berge *et al.* 2002). 16S rRNA gene also were analyzed in Bacterial genomics (Woese*et al*., 1990) and analysis of bacterial diversity(Bosshard*et al.* 2003; Fox *et al.*1980). the similarity of some members of the genus *Paenibacillus* was observed <92%, this similarity is very important between and whiten species of *Paenibacillus.* There are some reports about uncultured*Paenibacillus*species T-160in GenBank (accession number, HQ616297.1),*Paenibacillus* sp. C-168 16S rRNA gene, accession number, Y16129.1, *Paenibacillus* sp. T-168 16S rRNA gene, accession number, Y16128. They found that a 1400 bp to 1500 full length sequencing that revealed a high similarity with uncultured *Paenibacillus*speciesand also 16S rRNA gene inbacterial. In this study, we sequenced and deposited a fragment 677 bp. of 16S rRNA gene in GenBank, accession number; AB828175.1. Therefore, we aimed analyses of the rate relationship between and within uncultured *Paenibacillus*species, because there was some publications deal with intraspecificpolymorphisms in 16S rRNA genes (Bricker, 2000;Cilia *et al*. 1996; Lieftin*get al*. 1996; Martolnez-Murcia *et al*. 1999; Ninet*et al*. 1996; Nubel*et al*. 1996; Petterssone*t al*. 1998; Reischl*et al*. 1998). Hence, we decided toinvestigate this strain by molecular techniques.

MATERIALS AND METHODS

Bacterial isolation and phenotypic characterization

Uncultured *Paenibacillus*were isolated from muscle tissue of *Salmotruttacaspius*(fish species). The morphology of the bacteria was examined using visual investigation and a light microscope. Motility tests were performed using the hanging-drop technique and motility medium plates (1% nutrient broth, 5.3 gelatin, 0.3% agar, 0.1% KNO3, pH 7.2) that were incubated overnight at 30ºC. Anaerobic growth was determined by incubating LA plates overnight at 30ºC in Incubator. Optimum growth temperatureswere determined in LB by shaking at 160 rpm and spectrophotometric reading.

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DNA isolation

Total cellular DNA was isolated from bacterial colony following described by Sambrook et al., (1998). Briefly, samples was digested in a digestion buffer, containing; (100 mMNaCl, 10 mM Tris pH 8.0, 25 rnM EDTA pH 8.0, 0.5% sodium dodecyl sulphate (SDS), 0.1 mg/ml proteinase K). Samples were then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (24:25:1).Vortexed 10s then centrifuged at 3000×g for 5 min at room temperature. DNA was precipitated overnight at 4°C with 1/2 Vol 7.5 M ammonium acetate and 2 vol 100% ethanol. Then DNA solved in TE buffer (10 rnM Tris-Cl, 1mM EDTA) pH 8.0. Samples were stored at -20°C until analysis. Then the DNA genomics amplified 16S rRNA gene gene was separated by 1.5 % agarose gel electrophoresis. After electrophoresis, the DNA full length was visualized ethidium bromide and then was taken photos by gel DOC Bio RAD Company.

Designing of primers

Primers designed to specifically amplify the 16s rRNAgene based on conserved sequences from regions identified by the alignment of all the available sequences data from several the *Paenibacillus* species. These primers can amplified from first to end of 16s rRNAgene. These primers including:

Forward Primer 5' CACCTCCCTTACACCGAGAA 3'
Reverse Primer 5' GCCGAGTTCCTTCTCTTCCT 3'

The PCR programs

The PCR reaction used 10 microgram PCR reactions contained: 2µl template DNA, 2 µl forward primer (100 ng/µl), 2 µl reverse primer (100 ng/µl), 2 µl dNTP mix (2.5mM each), 5 µl 10X ChromTaq Assay buffer, 0.5 µl ChromTaq enzyme (3U/µl), Water 37.5 µl, in a total volume, 50 µl. 95° of 10 min, 30 cycles of 95°C 60 Sec., 52-60°C 55 Sec., and 72°C 1.5 min. Two to ten µl of each PCR reaction were run on 1.5% agarose gels in TAE buffer containing ethidium bromide. One µl 500bp, DNA ladder (Gibco-BRL) was used as a size standard. Then the PCR products after purification by the Chromous kit purification were sent to the ChromousGeni Company-India for doing sequence.

Sequencing of 16S rRNA gene in the Uncultured *Paenibacillus* species clone T-168

For sequencing of 16S rRNA gene we designed one set of primer that process of the sequencing including:

PCR Purification

Amplified PCR product was purified using QIAquick PCR Purification Kit Protocol:

A. Added 5 volumes of Buffer PB to one volume of the PCR sample and mixed. **B**. Placed a QIAquick spin column in a provided 2 ml collection tube. **C**. Centrifuged at 8000 rpm for 30–

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60 s. **D**. Discarded flow-through. Placed the QIAquick column back into the same tube. **E**. Washed with 0.75 ml Buffer PE to the QIAquick column and centrifuged for 30–60 s. **F**. Discarded the flow-through and placed the QIAquick column back in the same tube. **G**. Centrifuged the column for an additional 1 min at maximum speed. **H**. Placed QIAquick column in a clean 1.5 ml micro centrifuge tube. **K**. To elute PCR product, added 40 µl of B H2O to the center of the QIAquick membrane and centrifuged the column for 1 min.

Sequencing of Amplified 16S rRNA gene

Sequencing was performed along with the Forward and reverse primers in ABI 3730XL high throughput sequencer machine. Forward and reverse sequences were assembled and edited.

Sequence data analyzes

Sequence data were analyzed mostly by BLAST (NCBI Network system) and DNAMAN computer program genetics.

RESULTS

DNA extraction

In this study, after acquired the colony of bacteria, we extracted total DNA genomic from uncultured*Paenibacillus* T-168 (Figure 1). For amplification of 16S rRNA gene were designed one pair of primer from some species of *Paenibacillus* that reported in GenBank. We analysed by NCBI Network system and DNAMAN program computer. We selected one pair universal primer for amplification part of the 16S rRNA gene. These primers produced around 670 bp. PCR product that shown in Figure 2.

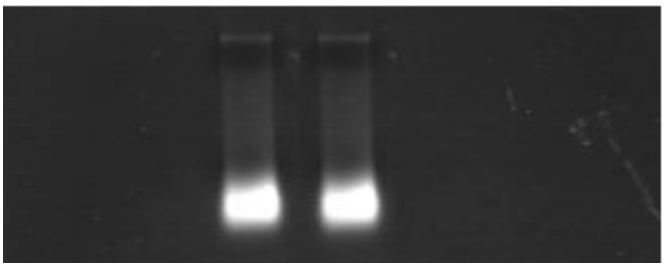

Figure 1: Total genomic DNA of Uncultured *Paenibacillus* sp. clone: T-168.

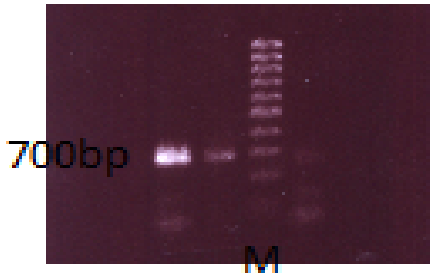

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Figure 2: PCR product ofpartial sequence of 16S rRNA gene in Uncultured *Paenibacillus*sp. clone: T-168. M: Size marker 1000bp.

Identification and characterization of 16S ribosomal RNAgene

The PCR amplification generated approximately a 700bp. The nucleotide sequences as well as deposited in (GenBank, accession number, AB828175.1. Sequence of 16S rRNA gene in Uncultured *Paenibacillus*sp. clone: T-168 were aligned with 16S rRNA genes in *Paenibacillus*species T-168 16S, accession no. Y16128.1. in BLAST. The homology between both sequences was high however this homology was partial sequences of them (Figure 3and4). Sequence of acquired were analysed with some 16S rRNAgenes including:*Paenibacillus*sp. T-168, accession no. Y16128.1. *Paenibacilluslentimorbus* strain ATCC 14707, accession no. NR_040889.1. Uncultured*Paenibacillus* sp. clone T- T0275, accession no. HQ616297.1. *Paenibacilluslentimorbus*, accession no. AB110988. *Paenibacillus*sp. C-168, accession no.Y16129.1. *Paenibacillus*sp. accession no. EU876668.1. Uncultured *Paenibacillus* sp. clone P4S-226, accession no. GQ329306.1. *Paenibacillus* sp. accession no. Y11583. Uncultured *paenibacillus*sp. clone P8S-359, accession no. GQ329445 (Figure 5) and these sequences again were analyzed with DNAMAN computer program (Figure 6). The results showed that, there was homology between sequences, approximately 82.1%.

Y16128.1

GGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTACTACAAT 1213

AB828175.1

GGTGGGGAAGACCTCAAATCAAAATACCCCTTTTGACTTGGGCTGCACACTTCTTACAGT 452

Y16128.1

GGCTGGTACAACGGGAAGCGAAGCCGCGAGGTGGAGCGAATCCTAAAAAGCCAGTCTCAG 1273

AB828175.1

GGATAGGCCGACGTCAAGCGAAGCCGAGAGGCGGGGTGAATCCATAAAAATGAGTGTCAG 512

Y16128.1

TTCGGAATGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAAT 1324

AB828175.1

CCGGCGGCGCATAATGCAACTCGCCTGGAAGAAGTCGAAATTGTTTTTAAT 563

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Figure 3: The sequences of 16S rRNA gene in Uncultured *Paenibacillus* species clone: T-168.Accession no. AB828175.1and, *Paenibacillus*species T-168 16S rRNA gene, accession no. Y16128.1, were analysed in Blast NCBI Network system. The results showed that there were homology between them.

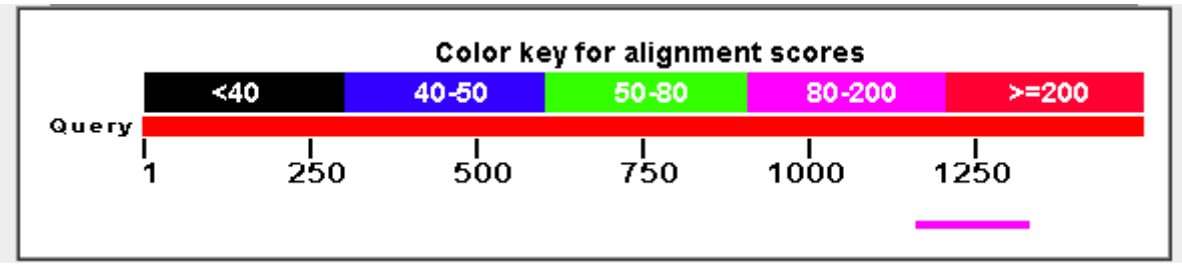

Figure 4: Structure of graphical 16S rRNA gene in Uncultured *Paenibacillus* sp. clone: T-168.AB828175.1(down line color) and, *Paenibacillus*species. T-168 16S rRNA gene, accession no. Y16128.1(above line color). Positions of colors indicated that there were homology only space of 80 to 200 nucleotides between them.

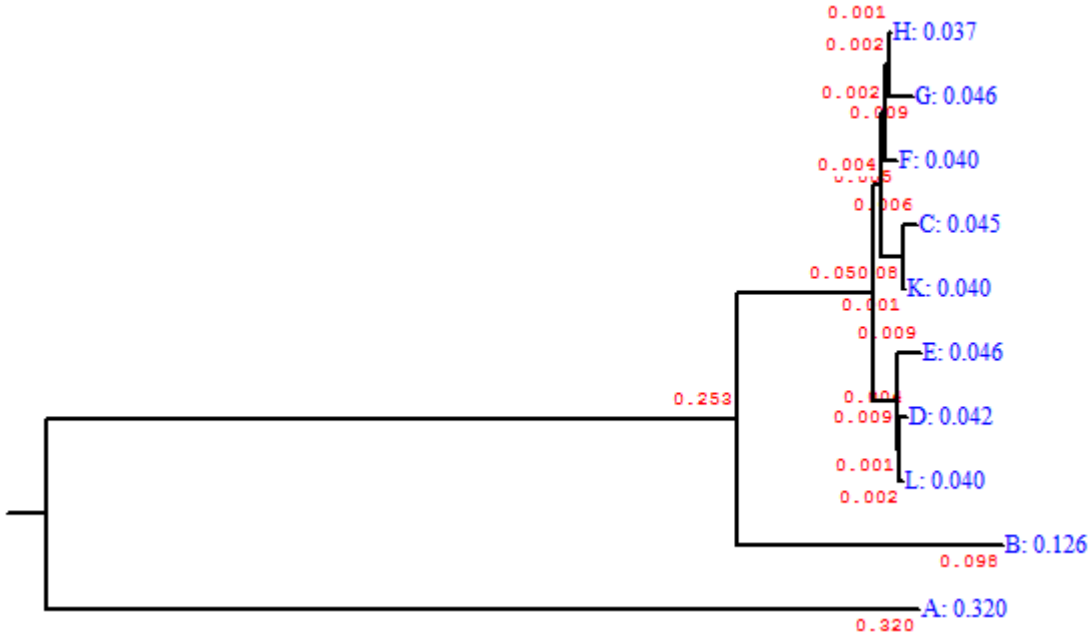

Figure 5:Dendrogram analysis of 16S rRNA gene in Uncultured *Paenibacillus* sp. clone: T-168.AB828175.1(A). *Paenibacillus*sp. T-168, accession no. Y16128.1(B).*Paenibacilluslentimorbus* strain ATCC 14707, accession no. NR_040889.1(C). Uncultured*Paenibacillus* sp. clone T- T0275, accession no. HQ616297.1(D). *Paenibacilluslentimorbus*, accession no. AB110988(E). *Paenibacillus*sp. C-168, accession no.Y16129.1 (F). *Paenibacillus*sp. accession no. EU876668.1(G), and, Uncultured *Paenibacillus* sp. clone P4s-226, accession no. GQ329306.1 (H). *Paenibacillus* sp. accession no. Y11583(K). Uncultured *paenibacillus*sp. clone P8s-359, accession no. GQ329445 (L). Dendrogram indicated that there were homology between them but it is low.

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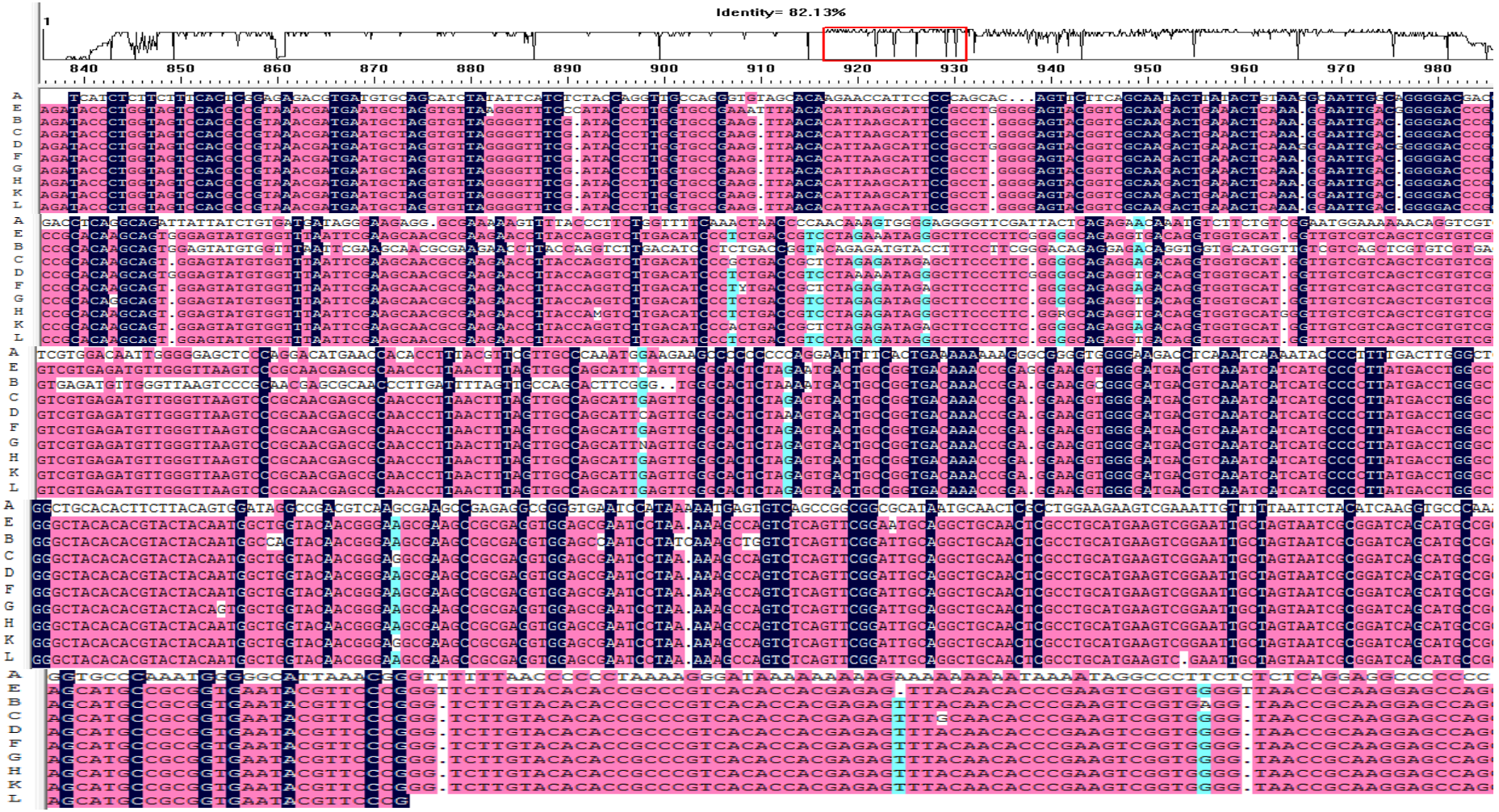

Figure 5: The sequences of 16S rRNA gene in Uncultured *Paenibacillus* sp. clone: T-168.AB828175.1(A), 16S rRNA gene in Uncultured Paenibacillus sp. clone: T-168. AB828175.1(A). *Paenibacillus* sp. T-168, accession no. Y16128.1(B). *Paenibacilluslentimorbus* strain ATCC 14707, accession no. NR_040889.1(C). Uncultured Paenibacillus sp. clone T- T0275, accession no. HQ616297.1(D). Paenibacilluslentimorbus, accession no. AB110988 (E). *Paenibacillus* sp. C-168, accession no.Y16129.1 (F). *Paenibacillus* sp. accession no. EU876668.1(G), and, Uncultured *Paenibacillus* sp. clone P4s-226, accession no. GQ329306.1 (H).*Paenibacillus* sp. accession no. Y11583(K). Uncultured *paenibacillus*sp. clone P8s-359 16S rRNA gene, accession no. GQ329445 (L). Positions of colors indicated that there were homology only space of 80 to 200 nucleotides between them.

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DISCUSSION

This study we isolated uncultured *Paenibacillus* species from muscle of fishes. Uncultured *Paenibacillus*live in environment such as, soil, water, vegetable matter, forage and insect larvae, human, as well as clinical samples (Lal and Tabacchioni, 2009; McSpadden Gardener, 2004; Montes*et al.* 2004; Ouyang, 2008). Still, today, there is not any report that fish species is host of uncultured *Paenibacillus*species. Therefore,this is a novel discovery regarding different bacteria species. We cultured *Paenibacillus* in muscle fishes after screening bacteria; we isolated DNA genomic and finally had done sequencing of 16S rRNA gene. There were many studies about functional of 16S rRNA gene in uncultured *Paenibacillus*in phylum of Bacteria (Cloud *et al.* 2002; Tortoli*et al.* 2001; Hall *et al.* 2001; Stackebrandt*et al.* 2002).

They also found a good homology between bacterial 16S rRNA gene and *Paenibaclus* species (reflected to NCBI Network system, accession number of 16 SrRNA gene in bacterial and specially *Paenibacilus* species) but our results showed that the rate of homology was medium, these results suggests that three theory including:1) may be this species (Uncultured *Paenibacillus*sp, clone T-168) a novel bacteria, that can be existence by genetic mutation, 2) still we didn't sequence full length of 16S rRNA gene, therefore, after getting full length may be reduce the genetic variation and has been high homology between uncultured *Paenibacillus*sp, clone T-168 and other same species. 3) may be only variation was there on the 16 S rRNA gene and the rate of homology after getting sequence from other nuclear genomics may be increased. However, the third theory is impossible because there is more research denote that 16S rRNA gene usually between organisms has been high homology, therefore, in the bacterial genomics also, 16S rRNA gene has high homology between species, and there are more reports about phylum of bacterial that used 16S rRNA gene. Bosshard*et al.* (2003), who reported that 14% of all aerobic gram-positive rods isolated in their clinical microbiology laboratory would require 16S rRNA gene sequence-based identification (3). These data indicate that molecular identification of unidentifiable bacterial isolates offers an opportunity for the description of new bacterial species encountered in clinical microbiology laboratories.

The fact that the commercially available 16S rRNA gene sequence-based identification kits have been favourably evaluated (Coelho, 2003; Tang, 2000; Woese*et al.* 1990) indicates that an exponential increase in the use of the technique can be expected within the next few years. Indeed, 16S rRNA gene sequencing offers an unprecedented tool for the description of new bacterial species. In figure 3-6, 16S rRNA gene in uncultured *Paenibacillus* species, clone T-168 with 16S rRNA genein *Paenibacillus sp*. T-168. were analysed by Blast, NCBI Network system. Results showed that the homology between both were low, 16S rRNA genein *Paenibacillus sp*. T-168 has around 1250 bp. that approximately had homology 200 bp of 16S rRNA gene in uncultured *Paenibacillus*species clone T-168 with 16S rRNA genein*Paenibacillus*species T-168, this annotated only the part of end of both sequences had high homology and first to medium of sequences there was not any homology. However in figure 5, 16S rRNA geneinuncultured *Paenibacillus* species clone T-168 with 16S rRNA geneof bacterial and uncultured *Paenibacillus*species by DNAMAN computer program were aligned, the results showed that there were homology between sequences of 16S rRNA gene in

between and within of species bacteria(approximately 82%). As above results, we concluded that should be sequence full length of 16S rRNA geneanduse more genes for studies of phylogenetic in bacteria.

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