Synthesis of SCL-LCL-PHA Co-Polymer from Starch by *Pseudomonas Aeruginosa* OU67 Isolated from Polluted Water

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Summary

Pseudomonas aeruginosa OU67 was isolated from polluted water and found to produce short-chain-length-long-chain-length polyhydroxyalkanoate (SCL-LCL-PHA) co-polymer and characterized. Strain OU67 grew well on starch at temperatures 30^0 C, pH7 and accumulated 57.77 PHA%(wt) at 30 h of incubation. The purified polymer sample from cells was determined as SCL-MCL-PHA co-polymer by gas chromatographic and nuclear magnetic resonance analysis of polymers. The polymer extracted from *P. aeruginosa* OU67 exhibited comparable material properties with the commercial polymer. As compared with heterotrophic bacteria, *P. aeruginosa* OU67 was able to utilize a wide range of carbon sources with good enzymatic potentials, for PHA production is thus, low-cost PHA production can be achieved. The 16S rDNA gene sequences of *P. aeruginosa* OU67 have been deposited in the EMBL data library under accession number FN663622.The type strain was submitted in the (JCM 17284^T = CCTCC AB 2010470^T)

Keywords: *Pseudomonas aeruginosa* OU67;starch;co-polymer;NMR.

Introduction

Polyhydroxyalkanoates (PHA) are a family of bio polyesters synthesized by many types of bacteria as carbon and energy reserve materials[1].Due to increasing industrial interest exists in the biotechnological production of Polyhydroxyalkanoates (PHA) from renewable resources to arrive at bio-based and biodegradable polymeric materials that can act as alternatives for common plastics derived from petrol [2,3].For these reasons, researchers have focused their endeavours on the development of biomaterials (natural products that are synthesized and catabolized by different organisms and that have broad biotechnological applications) to generate fully biodegradable compounds with potential industrial applicability[4]. PHA can be divided into three classes depending on the number of carbon atoms in their monomer units; shortchain-length (SCL), medium-chain-length (MCL) and long-chain-length polyhydroxyalkanoates (LCL-PHAs), composed by hydroxyacids with 3–5, 6–14 or more than 14 carbon atoms respectively. About more than 100 different monomer units reported so far, none of them contain more than 14 carbon atoms as constituents of PHA [5].

Anderson and collaborators referred to the capability for PHA biosynthesis from unrelated substrates [1,6] this is very important as it allows the synthesis of PHAs from simple substrates rather than fromexpensive or relatively toxic substrates [6]*Pseudomonas aeruginosa* and other fluorescent pseudomonads including *P. putida*, *P. mendocina* and *P. stutzeri*, *p. fluorescens* were reported to produce mcl PHAs from the unrelated carbon source gluconate or glucose [7,8,9].

In the present study we have investigated the influence of cultural conditions by using starch as carbon source, on the synthesis and accumulation of PHA by *Pseudomonas aeruginosa* OU67.The potential of the isolate to accumulate copolyesters of short-chain-length-long-chain-length polyhydroxyalkanoate(SCL-LCL-PHA) using starch as carbon substrate has also been evaluated.

Materials and methods

Microorganism and cultural conditions

Polyhydroxyalkanoate accumulating strain *P. aeruginosa sp.* OU67 which was isolated from the polluted water was used in this study. E2 medium [10] was used for PHA production. Aerobic conditions were maintained by shaking the inoculated Erlenmeyer flasks at 30^0 C for 28 h. Pure cultures were isolated on agar containing Nile blue .Cultures were directly monitored for the fluorescent colonies by exposing to ultraviolet light to detect accumulation of lipid storage compounds including PHA.

Estimation of viable cell count

To estimate viable cell count, the samples were serially diluted with sterile saline solution (1%% w/v NaCl). The diluted samples (0.1 ml) were plated in triplicate, on nutrient agar plates and incubated at 30 °C for 24 h to form fully developed colonies. The results were expressed as colony forming units per ml (cfu/ml).

Characterization of *P. aeruginosa* OU67

Morphological, growth and biochemical studies were performed using standard methods [11, 12]. Nutrient agar was used for growth, maintenance of the strain and the determination of the phenotypic characteristics. The isolate OU67 was characterized by its growth at various temperatures (5, 30, 45 and 60°C), tolerance at different salt levels (2, 4 and 10 g NaCl/100 ml) and reduction of nitrate. In addition, lecithinase, lipase, gelatinase , protease production and starch hydrolysis were examined, and anaerobic growth of the isolate was also performed. Biochemical characteristics were also checked with the Hi25 Enterobacteriaceae identification kit (KB003) and Hi Carbohydrate kit parts A, B and C (KB009) (both from HiMedia) according to the manufacturer's protocol. The antibiotic sensitivity of the isolate OU67was tested against different antibiotics. The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC[13].

For phylogenetic characterization, the 16S rRNA gene was amplified [14], and the PCR product was purifieded using the QIA quick PCR purification kit (Qiagen). Sequencing was performed by using ABI PRISM model 3700 automatic DNA sequencer and the Big Dye Terminator cycle sequencing kit (both from Applied Biosystems). The 16S rRNA gene sequence (1670 nucleotides) was submitted to the RDP website, aligned and used to build a phylogenetic tree of the isolate OU67 by neighbor-joining method using the tree builder tool. [15].

Scanning Electron Microscopy
For microscopic studies sample was transferred to vials and fixed in 2.5% Glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 hr at 4^0C and post fixed 2% aqueous Osmium Tetroxide in the same buffer for 2 h. After the post fixation samples were dehydrated in series of graded alcohol and dried to critical point drying with Electron Microscopy Science CPD unit. Then dried samples were mounted over the stubs with double sided conductivity tape. Finally, applied a thin layer of gold metal over the sample using an automated sputter coater (JEOL JFC-1600) for about 3 min. Then scanned the samples in scanning electron microscope (Model: JOEL-JSM 5600, JAPAN).

Nucleotide sequence accession number. The 16S rDNA gene sequences of *P. aeruginosa* OU67 have been deposited in the EMBL data library under accession number FN663622.The type strain was submitted in the (JCM 17284^T = CCTCC AB 2010470^T).

Estimation of biomass
The microorganism was grown aerobically in 250 and 500 ml Erlenmeyer flasks with 50 ml of the culture medium and incubated in a rotary shaker at 150 rev/min and 30 °C during 48 h.

Pha extraction and purification
For the extraction of PHA, 300mL of the cells were harvested by centrifugation at 5000×*g* and then lyophilized. The following methods were then employed. The PHA extracted from the cell pellet by the hypochlorite method[16],was washed with methanol and acetone consecutively and centrifuged at 8000 rpm for 20 min. The polymers were then dissolved in hot chloroform (60°C) and the solution poured onto glass trays. The chloroform was allowed to evaporate slowly by placing the trays in the cold room at 4°C. The film of PHA so obtained was used for further analysis.

Effect of different carbon sources
To increase the yields of polymer PHA, various carbon sources (2% w/v) such as sucrose, fructose and lactose and starch were added to the nitrogen free medium with the inoculum of 2% (v/v). Finally starch was selected with E_2 mineral medium to optimize the studies.

Analytical methods
Microbial growth was monitored by measuring the cell density of the culture at 600 nm after suitable dilution with distilled water. Organic nitrogen in the samples was estimated following its mineralization with hot sulphuric acid. PHA quantification was quantitated according to the method of Law and Slepecky (1961), whereby the dried pellets containing intracellular PHA were hydrolysed using concentrated sulfuric acid for 1 h to obtain crotonic acid, which was quantified by measuring absorbance at 235 nm. Analysis was performed in triplicates for shake flask samples. Cell dry weight (cdw) was measured by lyophilizing harvested cells from 3 ml culture broth. PHA content and its composition were determined by gas chromatography using PHA standards. Cell concentration was defined as cell dry weight per litre of the culture medium. The PHA content was defined as the ratio of PHA concentration to cell concentration given as percentage [17].

Gas chromatography (GC)

PHA was quantified using a slight modification of the gas chromatographic method of Riis and Mai (1988).Instead of whole cells, pure, extracted PHA was used. The precipated polymer was weighed in tightly sealable vials (volume 10 ml). Two ml of 1,2-Dichloroethane (DCE), 2 ml n-Propanol containing hydrochloric acid (HCl) (1 volume concentrated HCl + 4 volume n-Propanol) and 200 µl internal standard (2.0 g benzoic acid in 50 ml n-Propanol) were added. The mix was incubated for 4 h in a water bath at 85^0C. The mixture was shaken intermittently. After cooling to room temperature, 4 ml water were added and the mixture shaken for 20 – 30 s. The heavier DCE-Propanol phase was collected and injected directly into the gas chromatograph. Quantitative evaluation was affected by means of the peak areas of standard polymer and benzoic acid. [18].

IR

For FT-IR analysis, the PHA was precipitated from the chloroform using cold ethanol. The precipitated polymer was used to prepare KBr discs (sample: KBr, 1:100). An FT-IR spectrum 1720X spectrometer (Perkin Elmer, USA) was used under the following conditions: spectral range, 4,000–400 cm^{-1}; window material, CsI; 16 scans; resolution 4 cm^{-1}; the detector was a temperature-stabilized, coated FR-DTGS detector.

NMR

The ^{1}H NMR analysis of the polyester samples was carried out on Varian-300 spectrometer (USA). The 300 MHz ^{1}H NMR spectra were recorded at 24°C in $CDCl_3$ solution of polyester (50 mg/ml) with a acquisition time of 2.0480 seconds, sweep width of 4000 Hz. Tetra methyl saline was used as an internal chemical shift standard. The spectra was recorded for commercial PHA (Sigma-Aldrich, USA) and for the polymer extracted from test strains. All experiments were performed in triplicate to check the reproducibility.

Results and discussion

Characterization of *P. aeruginosa* OU6

Gram –ve, motile, short rods(figure 1a), (1–1.5 µm×2.5–4.0 µm) occurring singly, 3 mm diameter colonies on nutrient agar medium, greenish yellow pigmented colonies, diffusible pigment, turns to dark green on aging and able to emit fluorescence under the UV light (figure1b). Non spore are formers, colonies are circular glistening, growth good at 20 to 30^{o}c and pH 7.Catalase +ve, oxidase+, able to degrade caseine, esculin, melanin and starch. HCN +ve, able to solubilise phosphate, cellulose and lipids. Utilizes L-arabinose, xylose, adonitol, melibiose, glucose, lactose and citrate. Reduces nitrate to nitrite. Able o accumulate mcl-scl PHA. Sensitive to Penicillin, novobiocin, nalidixicacid, nitrofurazone and kanamycin. The G+C content of strain OU67 was 54 mol. %.The 16S rDNA gene sequence is deposited in gene bank with accession number **FN663622.** A phylogenetic tree (Figure 5) demonstrated that the isolated strain was a memberof the genus *Pseudomonas* , and it formed a monophyletic lineage. Sequence similarity calculations after a neighbor-joining analysis indicated that the closest relatives of strain OU67 was *Pseudomonas aeruginosa* LMG 1242^T (Z76651) with 99.5% similarity. According to the phylogenetic tree (Figure 5), based on 16S rDNA sequences, the new strain OU67was found to be affiliated to the genus *Pseudomonas*.

Biosynthesis of poly-β-hydroroxyalkanoates

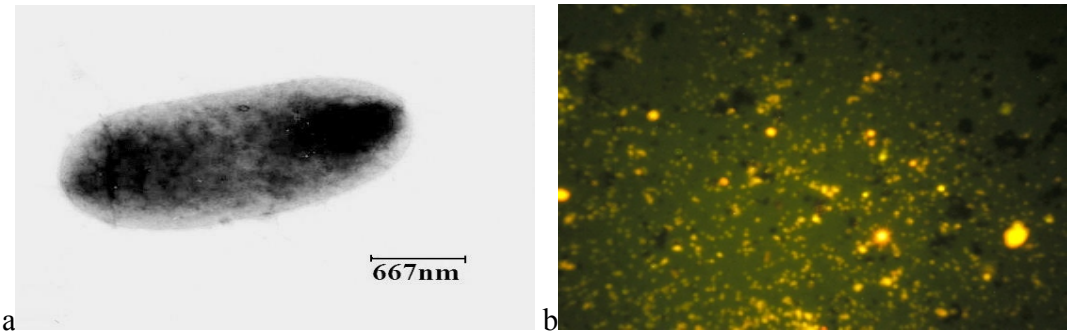
In shaken flask cultures, the cell mass increased steadily, leading to a maximum cell density within 30 h of cultivation after which there was a gradual decrease (Figure 4). Cell density of the culture at 600 nm (OD600) was attained to maximum value of 0.573 after dilutions.. The pH of the culture medium decreased during the growth, from its initial value of 7to a minimum of 5 .5. PHA conc g/L was up to 1.755.Cessation of logarithmic growth coincided with the approach of the pH minimum and rapid consumption of starch. PHA accumulated rapidly during the stationary phase and reached a maximum concentration of 57.77% of dry cell weight (dcw) at 30 h of growth. It is well known that the ratio of carbon source to nitrogen source (C/N) affects PHA accumulation; this was also known to be true for strain OU67 grown in glucose media. However, the production of PHA obtained maximum at 30^0C, assumed that the temperature was one of the significant factors to the PHA production for strain OU67. Previously the strain of *Haloferax Mediterranean* and *Azotobacter chroococcum* were employed to produce PHA in a starch medium [19,20] where hydrolysis of starch was carried out separately. Ramsay *et al* reported the accumulation of only 27% medium-chain-length PHA (MCL-PHA)by using nonanoic acid.[21]

Polymer analysis

IR spectra were recorded for the polymers dissolved in chloroform. Spectra showed two intense absorption bands at 1,730.22 and 1,280.66 cm¡1, corresponding to C = O and C–O stretching groups, respectively. Other absorption bands at 1,379, 1,456, 2,924 and 3,437 cm^{-1} corresponding to -CH3, -CH2, -CH and O–H groups are shown in Figure 2. The polymer analysis revealed the presence of hetero polymer. Its composition was confirmed by ^{1}HNMR (Figuer 3). The resonance, as observed at0.880, 1.199, 1.622, 2.518 and 5.251 δ by 1H-NMR analysis were, respectively, for CH3 (3HV, 3HHD and 3HOD side group), CH3 (3HB side group), CH2 (3HV, 3HHD and 3HOD side group), CH2 (3HV, 3HB, 3HHD and 3HOD bulk structures), CH (3HV, 3HB,3HHD and HHD bulk structures) of theCDCl3 -soluble fraction of the polymer confirmed the presence of the copolymer consisting of 3HB, 3HV, 3HHD and 3HOD units in *P. aeruginosa* OU67(figure 3). In GC analysis, three major ester peaks were found for the PHA isolated from starch (data not shown).Strain proved good capability to synthesize SCL-LCL-PHA co-polymer from unrelated carbon sources. The reason for this may be α-amylase production of the strain and the versatility of this bacterium in the selection of the carbon source may provide an attractive alternative for the utilization of starch-derived raw materials.

Conclusion

The results shown above demonstrated that the bacterium, which was isolated from polluted water, identified as *Pseudomonas aeruginosa* OU67. It was able to produce good amount of PHA (up to 57.77% of dry cell weight) in the presence of excesss starch as carbon source in the E_2 mineral medium (Fig. 4), is capable of accumulating LCL 3-hydroxyhexadecanoate and 3-hydroxyoctadecanoate units with SCL 3-hydroxybutyrate and 3-hydroxyvalerate as constituents of PHA. Use of inexpensive substrates such as starch could contribute to reducing the PHA production cost.



a b

Figure 1: a Scanning electron micrograph showing morphology of *P. aeruginosa* OU67
b. Flourescence micrograph of *P. aeruginosa* OU67

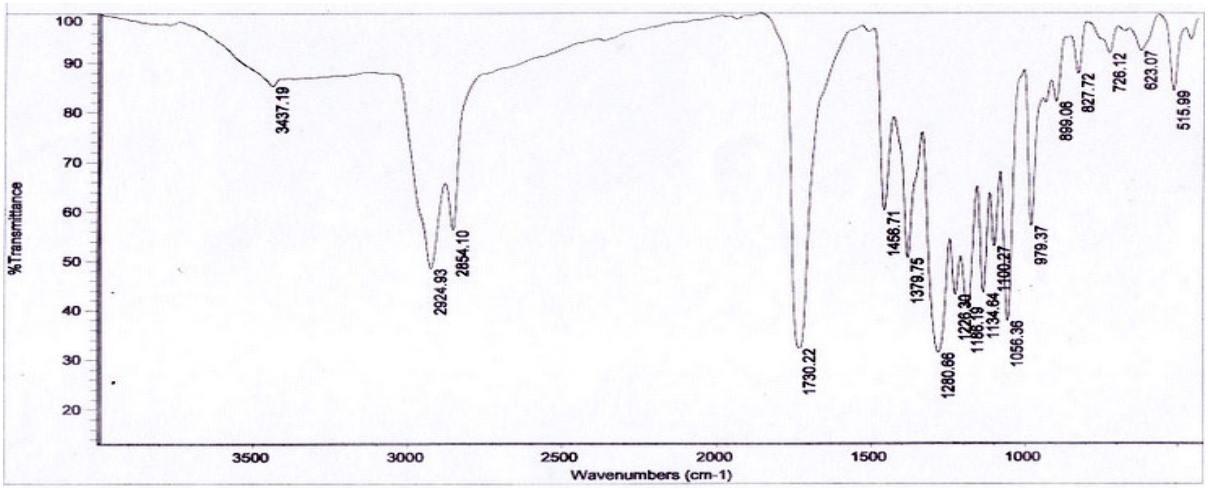


Figure 2: IR spectra of polymer from isolate OU67, grown on starch

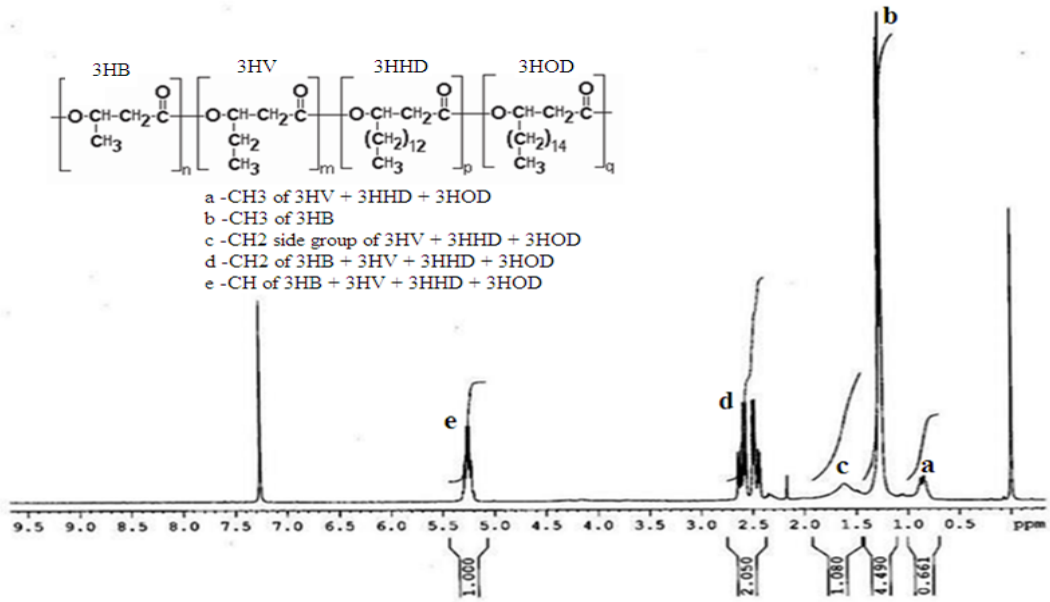


Figure 3: H^1 NMR of polymer from isolate OU67 grown on starch.

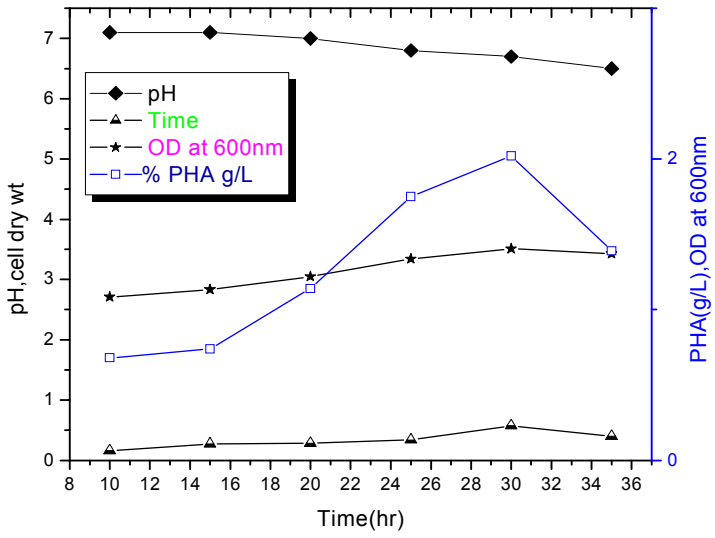


Figure 4: Cell growth and PHA accumulation by *Pseudomonas aeruginosa* OU6 grown on the starch containing medium. Each point represented an average of three test tubes.

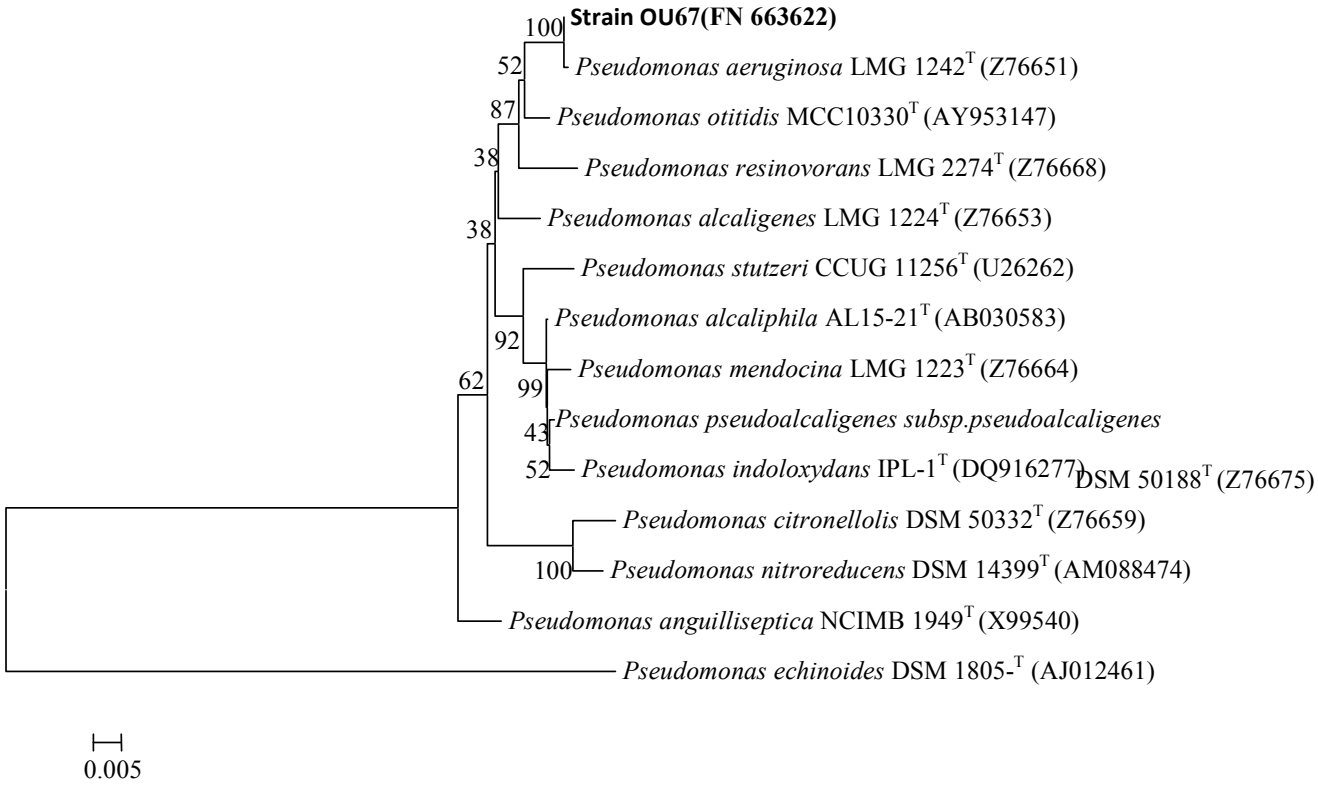


Figure 5: Phylogenetic position of the bacterium OU67 with the genus *Pseudomonas* with EMBL/GENE bank **accession No FN663622**

The evolutionary history was inferred using the Neighbor-Joining method [15] . The bootstrap consensus tree inferred from 1000 replicates [22](Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4[23] (Tamura et al., 2007). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method [24](Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site.

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