16S rDNA analysis for characterization of Pseudomonas sp. strain MF30 isolated from *Rumex acetocella* roots in northern Sweden

(Received: 15.09 .2006; Accepted: 28.09.2006)

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ABSTRACT

A bacterial strain obtained from the northern part of Sweden previously classified as Pseudomonas veronii based on biochemical and physioloical tests. In this study, phylogenetic tree was constructed using a nearly complete sequence within the 16S rDNA gene. The strain of Pseudomonas sp. subdivided into two rather distinctly related groups, neither of which is very close to the group within the Pseudomonas fluorescens cluster. Although the phylogenetic analysis is not conclusive, it is consistent with other observations, especially the capacities of the this strain as a biocontrol agent. Taken all together, the results suggest that the MF30 strain should be classifed as another Pseudomonas species, either Pseudomonas antarctica or P. meridina.

***Key words**: Phylogenetic analysis, 16S rDNA gene, Pseudomonas species, P. veronii, P. antarctica, P. meridina.*

INTRODUCTION

Bacteria belonging to the fluorescent pseudomonads, known for the diversity of their metabolites *(Leisinger and Margaff, 1979)*, contain species that are recognized as human and as animal pathogens (Nakazawa and Abe, 1996) and some are important phytopathogens (Jaunet *et al.,* 1995). Most species are however, saprophytic, and as a group, they are ubiquitous in water (Verhille *et al.,* 1997), soil (Lemanceau *et al.,* 1995), plant spermosphere (Fukui *et al.,* 1994), phyllosphere (Rainey *et al.,* 1994) and rhizosphere (Lambert *et al.,* 1990). In recent years certain species, for example *Pseudomonas chloroaphis*, *P. fluorescens*, *P. veronii*, *P. putida*, have received attention even though they possess abilities to influence plant growth and development through different mechanisms (Weller, 1988; O'Sullivan and O'Gara, 1992). They are now recognised as being antagonistic to several opportunistic soil-borne fungi (Weller, 1988; Keel *et al.,* 1996) and to seed-borne fungi (Hökeberg *et al.,* 1997). Also, some strains are responsible for significant plant promoting effects (DeFreitas and Germida, 1991; Kropp *et al.,* 1996).

Taxonomically, this flourecent *Pseudomonas* spp. belong to the rRNA group I of the gamma subclass of *Proteobacteria* (Palleroni, 1993; Kersters *et al.,* 1996). The species within this group have been differentiated on the basis of their DNA/Dna homologies (Johnson and Palleroni, 1989; Gardan *et al.,* 1992). This method of differentiating bacterial species has for several years been the most reliable, since traditional classification of bacteria based on phenotypic features does not always correlate with molecular taxonomy (Woese, 1987). Other methods based on cataloguing 16S rRNA have been shown to be reliable and effective (Woese *et al.,* 1984; Woese, 1987). Indeed, using the 16-rDNA approach, Woese *et al.* (1990) were able to show definitively that life can be divided into three major lines of descent presently named Archea, Bacteria and Eukarya.

For bacteria belonging to the genus *Pseudomonas*, data collected on 16S rDNA genes make taxonomical studies possible, including those in the rhizosphere. As a result of such studies, many organisms classified as species of this genus have been reclassified (Kersters *et al.,* 1996). However, classification of the fluorescent pseudomonads has yet to be clarified completely, in part due to the many new species being decribed (Elomari *et al.,* 1996). This paper a case in point. In earlier study, Attitalla *et al.* (2001) identified a bacterial strain (MF30) based on the characteristics revealed by API NE 20 test (BioMerieux, Marcy l'Etoile, Marcy, France), the bacterial strain MF30 was assigned to the genus *Pseudomonas*, also partial sequences of 16S rDNA showed 99.6% similarity to *Pseudomonas veronii* and high similarity to other members of RNA group I of the *Pseudomonas*. Strain testing was done in Deutsche Sammlung von Mikro-organismen aus Zelkulturen GmbH, Braunschweig, Germany (DSMZ), which also found that the profile of the cellular fattty acids was typical for that RNA-group, and although physiological tests were unable to distinguish between the species in this group, yet they were all closely related to biovars of *Pseudomonas fluorescens*. This strain has been shown to produce biologically active metabolities namely massetolides type A, a low molecular weight lipopeptide (Gerard *et al.,* 1997). This compound, as well as several derivatives (massetolides B-H), were recently isolated from two *Pseudomonas* spp. isolates of a marine algae and a marine tube worm origin (Gerard, 1997). To date, there only two reports of *in vitro* anti-tuberculosis activity expressed by massetolides (El-Sayed *et al.,* 2000).

In the present paper, we successfuly applied restriction fragment length polymorphism and sequencing of the 16S rDNA for clarifying the classification of the strain MF30. This study indicates that it differs from the *P. veronii*. However, since 16S rDNA data precluded the prospect of conclusive results from phylogenetic analysis, one can rule out the possibility that the strain can represent a specific group or biotype of *P. veronii*. Using sequencing data from nearly complete sequence of the 16S rDNA gene together with their biological characters, we describe the strain MF30. We proposed that this bacterium should be reclassified as a new *Pseudomonas* species, either *P. antarctica* or *P. meridina*.

MATERIALS AND METHODS

Bacterial strain and cultivation

Using the procedure described by Hökeberg *et al.,* (1997), the bacterial strain MF30 was isolated from root of a wild plant (*Rumex acetocella*) in the mountain area of northern Sweden: The strain MF30 from the dicotyledonous plant. The strain was maintained as freeze-dried or as deep-frozen (-70°C) stock culture. The bacterium was grown in 50% strength Tryptic Soy Broth (15g/k, TSB, Difco) it shaking (120-rev min^{-1}) at 20°C for 48 hours.

Secretion of extracellular proteases

Proteolytic activity was estimated in 48 hours old cell free supernatant from bactrial culture grown in 50% normal strength TSB (15g/l) (Tryptic Soy Broth, Difco). Bacterial culture was incubated on rotary shaker (120-rev min^{-1}). Cells were removed by centriifugation for 20 min at 16.000 g and supernatant was then filter sterilized. The radial diffusion assay (Dow *et al.,* 1990) in milk agar plates was used. Fifty microliters of supernatant was applied to a well cut (6 mm in diameter) in the middle of skim milk agar plates, which were then incubated at 37°C. Clear zones (mm) around the well were measured after 24 hours. The test was repeated twice with two Petri-dishes per treatment.

Phenotypic characterization

The strain was also characterized phenotypically with regard to production of fluorescent colonies on King'B medium, and for motalility in a swarming assay (Bondesson and Haas, 2002; Thorsson, 2002).

Fluorescence

Siderophore production was detected as fluoresence under UV light (wave length = 320 nm) when cells were spread on KB medium (King *et al.,* 1954). Plates were incubated at 25°C for 2 days prior to visual examination of fluorescence on UV a transilminator.

Swarming

To test swarming ability, 1 µl of bacterial suspension (a small loop of fresh bacterial cells suspended in 100 µl high purity water) of the strain was inoculated onto liquid medium (LM plates containing 10g/l Bacto-trypton, 6g/l Bacto-yeast extract, .5 g/l K_2HPO_4, 0.5 g/l NaCl, 0.4g/l $MgSO_4{*}7H_2O$).

DNA isolation and PCR amplification

The bacterium was grown in 50% normal strength TSB on a rotary shaker (120-rev min^{-1}) at 20°C for 48 hours. The RapidPrep^{TM} genomic DNA Isolation Kit for Cells and Tissues (Pharmacia Biotech) was used to isolate DNA as advised by the manufacturer. DNA amplification was conducted on pure 2 to 3 µl DNA sample with about 150 ng of DNA per 1 µl of sample in a perkin Elmer (Nowalk, CT) thermocycler. The fD1 and rD1 primers amplifying the 16S rDNA gene in most of bacteria was used for DNA amplification (Weisburg *et al.,* 1991). PCR reactions were conducted in a final volume of 100 µl with 0.2 µM of each primer, 2.0 mM $Mgcl_2$, 200µM dNTPs and 2.5 units of AmpliTag DNA polymearse mixed in the PCR buffer (Perkin Elmer). DNA was amplified over 35 cycles of denaturation for 1 min at 94°C annealing at 55°C for 1.5 min and extension at 72°C for 2 min. After the last cycle, DNA was extended at 72°C for 10 min. Amplification was confirmed by analyzing 5µl of PCR reaction mixture on 1% agarose gel (Promega).

DNA sequencing

The PCR-product was purified using QIAquik PCR Purification Kit (Qiagen), and sequenced using automatic ABI 310 DNA Sequencer, Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer. The sequencing was performed in both direction using previously described primers (Lane *et al.,* 1985; Lane, 1991). Sequencing data was analzed by two different computer alignment programs, DNAStar (DNASTAR, Inc., USA) and Sequence Navigator (Perkin, Corp., USA).

Determination of phylogenetic relationships

The BLAST database (Altschul *et al.,* 1997) of National Center for Biotechnology

Information was used to compare resolved sequence of the MF30 strain with known 16S rDNA sequences. Determination of phylogenetic realtionships was analyzed by the program Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b1 for Macintosh (Swofford, 1993). The robustness of the internal branches of the trees was estimated by bootstrap analyses using 1000 replications in a heuristic search with random stepwise addition (3 replications) (Vinnere *et al.,* 2002). Bootstrap majority-rule (> 50%) consensus trees were obtained.

RESULTS AND DISCUSSION

Restriction fragment analysis of PCR-amplified 16S rDNA gene was used to classify an important bacterial strain. Attitalla *et al.,* (2001) attempted to identify the MF30 strain on the basis of morphological and biochemical characters; the strain was close to *P. veronii*. Considering the fact that the estimated number of culturable bacteria is probably only 0.1 to 1% of those present in nature (Bintrim *et al.,* 1997), many totally new bacterial strains are still being recovered (Borowicz, 1998). The traditional taxonomic methods based on morphological physiological, and biochemical characters are now accompained by DNA-based methods like DNA-DNA hybridization, RFLP and sequencing of 16S rRNA genes (Grimont *et al.,* 1996; Hartung, 1998).

Table.(1). The phenotype characterization of the strain MF30.

Bacterium	Fluorescence	Swarming	Protease
MF30 strain	+	+	++

The available gene databases on 16S rRNA genes presently contain a number of sequences, which allow reconstruction of phylogenetic relationships and precise positioning of even unknown species. In case of genus *Pseudomonas* (sensu stricto), nearly-complete sequences have been determined for the PCR amplified 16S rRNA genes of as many as 21 species (Moore *et al.,* 1996). In this paper, we have further investigated the taxonomic position of the bacterial strain (MF30), which we propose here represent a new spesies either *Pseudomonas antarctica* or *P. meridin*, upon sequencing most of the 16S rDNA gene, we discovered that the strain MF30 shares the same identical sequence, which is nearly the complete 16S rRNA gene. The identity of this sequence to the closest pseudomonads strains is 98-99%. Therfore, the previous identification of the strain MF30 by API system (Attitalla *et al.,* 2001), and the identification of this strain by fatty acid analysis as well as species of *P. veronii* was not confirmed.

It is interesting to note that in the phylogenetic tree constructed in this study, the strain MF30 is subdivided into 2 rather distantly related groups, neither of which is very close to the group within which the strains *P. antarctica* or *P. meridin* cluster (Fig. 1). The strain MF30 is clearly shown to be an extracellular protease producer (Table 1). The proteolytic activity on skim milk agar medium was visulay determined, and by measuring the proteolytic activity zone of clearing in mm, the zone was 15.0 mm.

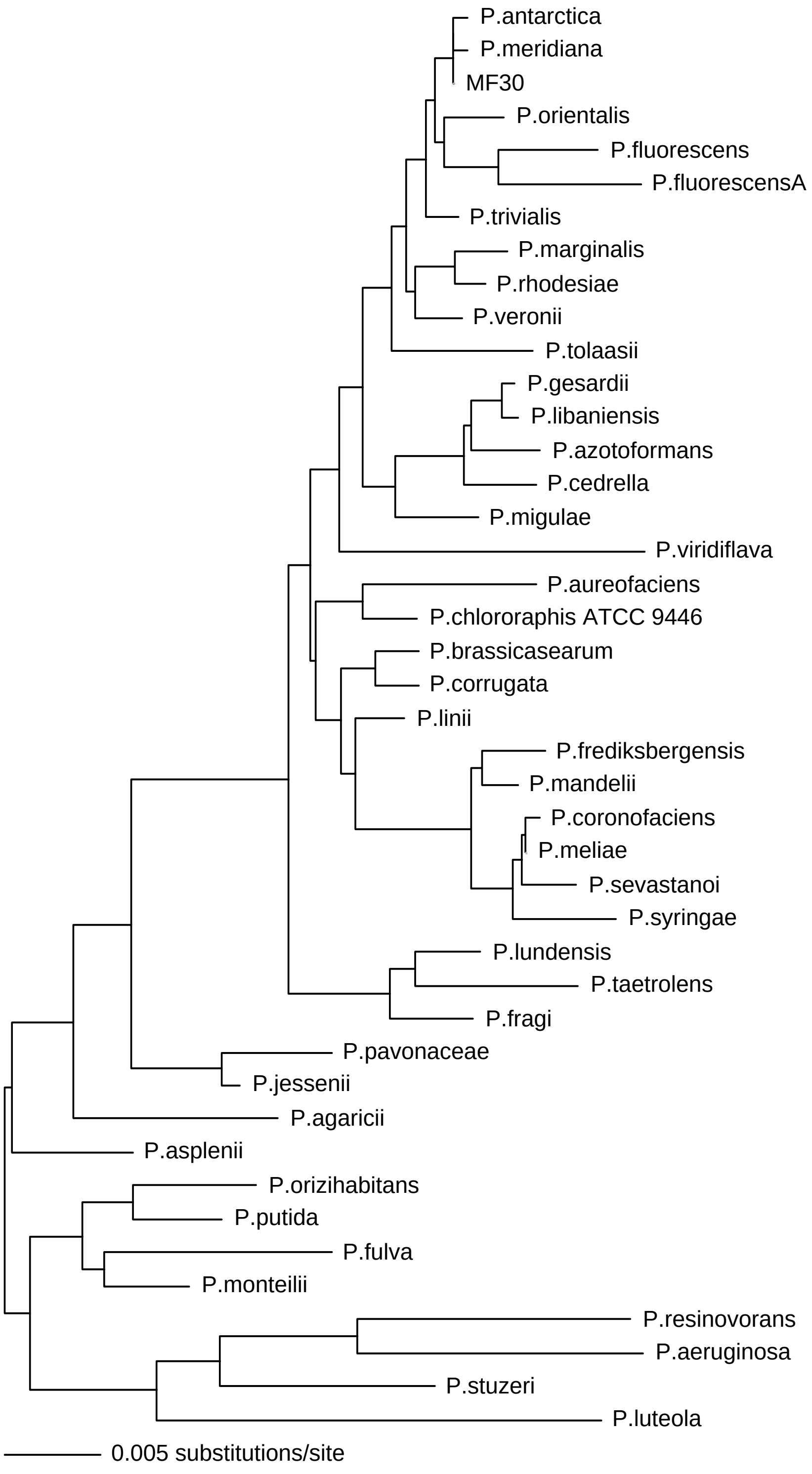

Fig. (1): The BLAST database (Altschul et al., 1997) of National Center for Biotechnology Information (NCBI) was used to compare resolved sequence of the MF30 strain with known 16S rDNA sequences. The Phylogenetic tree was reconstructed for the strain MF30.

The finding that protease production by the strain MF30 was inversely related to its growth rate in the tested medium (data not shown) further confirms this hypothesis. The protease-producing bacterium was also capable of secreting protease(s) when grown in non-protein medium such as mineral medium (MMB) supplemented with glucose as a single carbon source. This suggests that at least some protease(s) are constitutively produced (Whooley *et al.,* 1983).

Based on phylogenetic study, the analysis of the 16S rDNA gene sequences data for the strain MF30, *P. antarctica*, and *P. meridina* share the same sequence, but differ from *P. veronii*. The strain studied was probably too closley related to support a meaningful parsiomony analysis and construction of a phylogenetic tree. The genetic relationships between the strain MF30 and known members of other species of *Pseudomonas* genus were estimated by parsimony analysis (Swofford, 1993) using haustoric search with TBR branch swapping (100 replicates). The bootstrap analyses were run with TBR MULPARS and 1000 replicates. Nine equally parsimonious trees, which showed few differences in topology analysis is shown in Fig. (1). These findings support further taxonomic analysis of the isolates by sequencing of the full 16S rRNA gene by DNA/DNA hybridization and/or by PCR analysis of other genes, preferably from non-coding DNA region.

ACKNOWLEDGEMENTS

We gratefully acknowledge Professor Siv Andersson and Dr. Paul Johnson (Uppsala University, Sweden) for assistance in interpreting the data and for critical reading and refining of the manuscript.

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الملخص العربي

تحليل الـr DNA 16S لتوصيف السلالة Mf 30 لبكتريا السيدوموناس المعزولة من جذور نبات Rumex acetocella فى شمال السويد

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السلالة البكتيرية MF30 والمعزولة من شمال المملكة السويدية والتي في السابق صنفت كسلالة *Pseudomonas veronii*، وذلك إستناداً إلى الإختبارات البيوكيميائية والفسيولوجية. في الدراسة الحالية، شجرة التطور الوراثية والتي تم إستخلاصها بإستخدام تتابع (قريب من التتابع الكامل) لجين 16S rDNA. وهذه السلالة *Pseudomonas* قسمت إلى مجموعتين محددتين، أي من كليهما قريب من مجموع *Pseudomonas fluorescenes*، وبالرغم من أن تحليل التطور الوراثي ليس شاملاً، لكنه أظهر بأن يتفق مع أو يدعم المشاهدات الأخرى، خصوصاً لسعة هذه السلالة كعامل في المكافحة الإحيائية. بأخذ كل هذه الإعتبارات جملةً، أظهرت الاستنتاجات أو توصلت إلى أن سلالة MF30 يجب أن تصنف كنوع أخر من جنس *Pseudomonas*، إما أن يكون *Pseudomonas antarctica* أو *Pseudomonas merdina*.