J. BIOL. ENVIRON. SCI.,
2012, 6(16), 15-23

Characterization of *Pseudomonas* spp. from seawater of the southwest coast of Turkey

Aysel Uğur[1*], Özgür Ceylan[2] and Belma Aslım[3]
[1] Department of Biology, Faculty of Science, Mugla University, Mugla-48187, Turkey
[2] Apiculture Program, Ula Ali Kocman Vocational School, Mugla University, Ula, Mugla-48640, Turkey
[3] Department of Biology, Faculty of Arts and Sciences, Gazi University, Ankara-06500, Turkey

ABSTRACT

In this work, 46 gram negative bacteria isolated from the seawater on the southwest coast of Turkey. These isolates were identified as 20 *Pseudomonas spp*., 15 *Aeromonas spp*., 8 *Chryseomonas spp*., 2 *Burkholderia spp*. and 1 *Vibrio spp*. by biochemical and conventional methods. And then study was continued for the 20 *Pseudomonas* isolates. Species identification of these isolates were made by means of API 20 NE. Enzymatic activities of these strains were determined by API ZYM. The strains were further characterized by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins and 16S ribosomal DNA sequencing. According to the results obtained by phenotypic methods; the 20 isolates were identified as 6 *Pseudomonas aeruginosa*, 7 *P.fluorescens*, 3 *P.putida*, 2 *P.stutzeri*, 1 *P.mendocina* and 1 *P.alcaligenes.* The results of protein profiles and 16S rDNA sequence correlated well with phenotypic classification for *P.aeruginosa, P.putida, P.stutzeri* and *P.mendocina* strains. Good overall agreement between phenotypic and genotypic identification procedures was found for the 17 *Pseudomonas spp.* But discordance of identification between phenotypic methods and 16S rDNA sequence analysis was observed for two *P. fluorescens* (P 5-4 and P 9-2). Indeed these isolates were genotypically identified as *P.putida*. One strain (P 4-3), identified as, *P.alcaligenes* based on phenotypic characters, were identified as *P.pseudoalcaligenes* by 16S rDNA sequencing. These results indicate that the procedures for the identification of *Pseudomonas* spp. based on phenotypic characteristics should be additionally verified by the molecular methods to obtain results that are meaningful as well as accurate.

Key Words *Pseudomonas* ; characterization; marine; SDS-PAGE; 16S rDNA

INTRODUCTION

The genus *Pseudomonas* is the most heterogenous and ecologically significant group of known bacteria, and includes Gram-negative motile aerobic rods that are wide-spread throughout nature and characterized by elevated metabolic versatility, thanks to presence of a complex enzymatic system. The nutritional requirements of *Pseudomonas* spp. are very simple, and the genus is found in natural habitats like soil, fresh water, marine environments etc., but it has also been isolated from clinical instruments, aseptic solutions, cosmetics and medical products (Franzetti and Scarpellini, 2007). Certain members of the genus *Pseudomonas* are considered to be important phyto-pathogens and agents or carriers of human infections, whereas its other strains and species exhibit activities of bioremediation and biocontrol (Tripathy et al. 2006). Pseudomonads are well adapted to survival in whirlpools, hot tubs and indoor pools because of the warm water temperatures. These waters are especially prone to contamination during periods of high use when it is difficult to maintain adequate disinfection levels (Tirodimos et al.2010). *Pseudomonas aeruginosa* belongs to a vast of obligate aerobic, non-fermenting, saprophytic, Gram negative bacilli widespread in nature, particularly in moist environments such as water, sewage, soil, plants and animals (Goldberg, 2000). The organism is able to grow and multipl in variety of water sources including river water, seawater, wastewater and bottled mineral water (Kimata et al. 2004; Hunter 1993). *Pseudomonas aeruginosa* is an important agent of opportunistic infection in patients, particularly in those with respiratory complications and burns. *Pseudomonas* spp. was one of the most frequently identified agents associated with waterborne outbreaks of dermatitis (rash or folliculitis), as well as conjunctivitis, otitis externa and other symptoms, in recreational water in the United States of America(14%) (Craun et al. 2005).

Thus, given the great interest in the genus *Pseudomonas,* and its primary role in the use as biocontrol agents in seawater, the aim of this work was to to establish a taxonomic, molecular and phenotypic framework to enable identification and hence tracking of *Pseudomonas* species from marine environments in the touristic coast of Bodrum, Mugla in Turkey. The isolates were initially classified according phenotypic characteristics and further identified by molecular methods such as SDS-PAGE and 16S rRNA sequencing. Results from both identification methods were analyzed and compared.

MATERIALS AND METHODS

Location and Sample collection

Bacteria studied in sea water were isolated from 9 various locations along the Bodrum Coast of Mugla province, Turkey. The study area is 7 km region that includes 6 beaches, 2 jetties and a harbor. Sea water samples for

[*] Corresponding author: ayselugur@hotmail.com

bacteriological analysis were collected aseptically and transported to the laboratory, using 500 ml sterile bottles where they were analysed within 8 h of collection (APHA 1995), following which they were processed for isolation bacteria.

Isolation procedure and maintenance

Isolates were prepared from the samples collected by using membran filter method together with a selective growth medium. *Pseudomonas* spp. were isolated, using a qualitative method, after concentrating 100 ml of sea water through 0.45µm membranes which were then preenriched in NKS Cetrimide plates (Sartorius AG, Germany) at 30°C for 24 h. Enrichment followed in Pseudomonas Selective Medium (Oxoid, CFC-SR103) at 30°C for 24 h. Positive cultures were subcultured on Nutrient Agar (NA) (Difco) for the isolation of a pure, single colony for identification. Species identification of isolates were made by means of API 20 NE, API ZYM test kits and some biochemical tests. All cultures were maintained at −20°C in 15% (w/v) glycerol.

Morpholocigal, biochemical and physiological characterization

Colony characteristics, including pigment production were determined on Pseudomonas P Agar (Acumedia) and NA (Difco). All strains were characterized by the following classical tests according to Bergey's Manual of Systematic Bacteriology (Palleroni, 1984): Gram staining, cytochrome oxidase production, catalase production and growth on McConkey Agar and 42°C. Isolated strains were biochemically identified by conventional tests followed by use of API 20 NE identification system (API 20 NE, Biomerieux). To examine the enzyme profiles of our isolates the API ZYM (Biomerieux) system was used, essentially as recommended by the supplier. The results were obtained in duplicate and analyzed employing the Apilab Plus Software (Biomerieux).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The extraction of total cell protein content of the strains studied was determined according to Pot et al.(1994) and separation of proteins based on their molecular weight was achieved through SDS-PAGE technique (Laemmli 1970). The cultures were used as an inoculum (1% v/v) in 10 ml NB, and incubated at 37 ˚C for 24 h. The pellet washed two or three times with sodium phosphate buffer (0.01M) containing 0.8 % NaCl (pH 7.3; sodium phosphate-buffered saline). The extracts were sonicated on ice during 45 seconds. After lysis, the sonicated extracts were centrifuged at 5000xg for 10 min. The pellet was discarded and the supernatant was supplemented with sample buffer (0.062 M Tris- HCl, 0.75 g; 2-merkapto etanol, 5ml; glycerol, 10ml; bromophenol blue, 1mg) and added 20% SDS, 0.1ml, heated for 10 min at 95˚C. Protein extracts underwent SDS-PAGE on vertical slab gels in an electrophoresis apparatus. Electrophoresis was performed using a power supply operated at a constant current of 20 mA through the stacking gel and 30 mA through the resolving gel. A standard protein solution (MBI Fermentas, SM 0439), containing 7 proteins ranging in size from 14.4 to 116 kDa, was used as a molecular weight marker and for the normalization and interpolation of the protein patterns. The proteins were run on 10% SDS-PAGE and gels were stained with Coomassie blue R250. Afterwards, the gels were distained until protein bands become clearly visible.

DNA extraction and 16S rRNA sequence determination

Genomic DNA isolated according to the methods of Sambrook et al. (1989). Design of specific primer and sequencing were performed by Thermo Electron GmbH (Ulm, Germany). Polymerase chain reactions were performed using the Hybaid termocycler. PCR reaction mixture consisted of; 10 ng template DNA, 1 unit Taq DNA polymerase (MBI Fermentas), 1x reaction buffer, 50 p mol of each primer, 2.5 – 3 mM $MgCl_2$, 0.2 mM dNTPs and dd H_2O to a final volume of 50 µl. The amplification conditions were as follows: initial denaturation for 2 min at 94^{o}C was followed by 35 cycles of 1 min denaturation at 92 ^{o}C, 30 s at the anneling temprature of the primers and 30 s an extention time at 72 ^{o}C, a final extention at 72 ^{o}C for 10 min. The presence of PCR products was controlled on 1% agarose gel electrophoresis. The sequencing process was performed by the METIS-Biotecnology (Ankara, Turkey). The sequence of the PCR product was compared with known 16S rRNA gene sequences in the GenBank using the blast function.

RESULTS

A total of 46 putative *Pseudomonas* isolates was prepared from the samples collected from the Bodrum coast of Mugla Province (Fig 1.) using membran filter method together with a selective growth medium. For conducting investigations in the present study, these isolates were obtained from seawater and the frequency of occurrence of the isolates is shown in Table 1. In the present study, the evaluated samples were found to contain 5 bacterial

species. However, among the isolated strains, the *Pseudomonas spp.* was the most frequently detected microorganism, revealing 20 strains. These strains were shown to be gram-negative, oxidase and catalase positive rods. Species identification of these isolates were made by means of API 20 NE test kits and some biochemical tests. The enzyme activities of the strains were determined by API ZYM enzyme kits were used in order to compare the results of conventional tests and API 20 NE test. The enzymatic capabilities assayed with the API ZYM system showed that all strains possess similar profiles with strong esterase (C4), esterase lipase (C8), and leucine-arylamidase activities. In the experimental conditions used none of the examined strains presented activity on substrates for the cystine arylamidase, α-chymotrypsine, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosidase, α-mannosidase and α-fucosidase. Isolates and their biochemical characteristics are shown in Table 2. According to the results of the identifications, 20 strains belong to the various species of *Pseudomonas* genus. And then the study was continued for the 20 strains. The isolated strains included 6 of *P. aeruginosa*, 7 of *P. fluorescens*, 3 of *P. putida*, 2 of *P. stutzeri*, and 1 each of *P. mendocina*, and *P. alcaligenes* (Table 2).

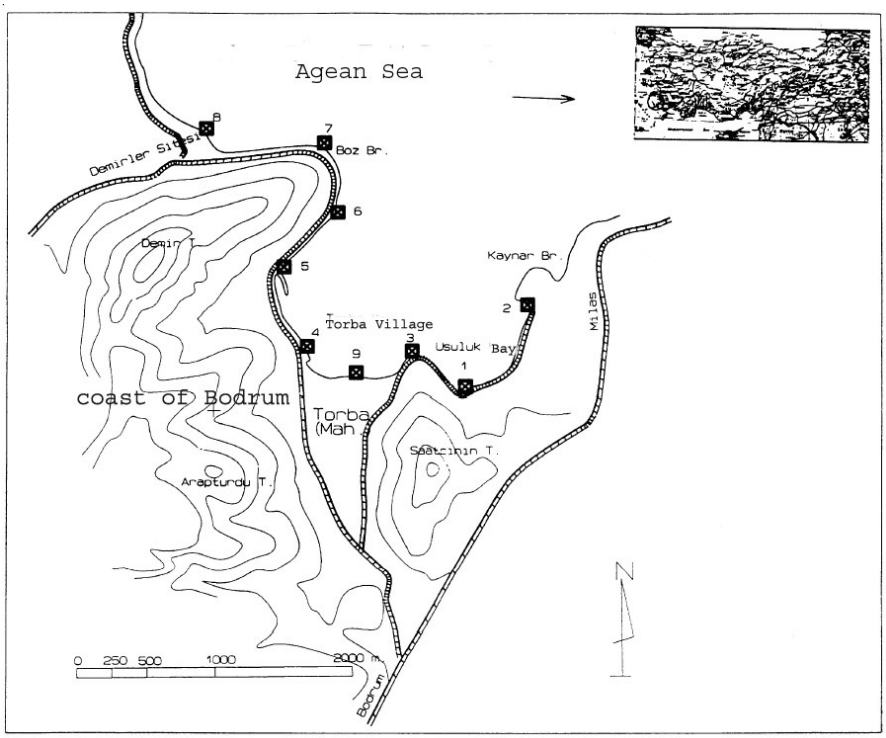

Figure 1. Map of investigated area and sampling stations.

Table 1. Number of species of Gram negative bacteria isolated from Torba Bay on the southwest coast of Turkey.

Genus	Number of isolates	%
Pseudomonas spp.	20	43.47
Aeromonas spp.	15	32.60
Chryseomonas spp.	8	17.39
Burkholderia spp.	2	4.34
Vibrio spp.	1	2.17
Total	**46**	**100**

Table 2. Identifications and characteristics that differentiate groups of *Pseudomonas* strains.

Characteristics	Reaction[a] of the following *Pseudomonas* strains					
	P.aeruginosa(6)	*P.fluorescens*(7)	*P.putida*(3)	*P.stutzeri*(2)	*P.mendocina*(1)	*P.alcaligenes*(1)
Classical tests						
Oxidase reaction	+	+	+	+	+	+
Catalase reaction	+	+	+	+	+	+
Growth at 42°C	+	-	-	+	+	+
Production of fluorescent pigment	+	+	-	-	-	-
API 20 NE						
Indol production on tryptophan	-	-	-	-	-	-
Glucose acidification	-	-	-	-	-	-
Arginine dihydrolase	+	+	+	d	+	+
Urease	+	-	-	-	-	-
Esculin hydrolisis	-	-	-	-	-	-
Gelatin hydrolisis	+	-	-	-	-	-
β – Galactosidase	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	-
L-Arabinose	-	-	-	-	-	-
D-Mannose	-	+	-	-	-	-
D-Mannitol	+	+	-	d	-	+
N-Acetyl-D-glucosamine	+	-	-	-	-	-
Maltose	-	-	-	+	-	-
Gluconate	+	+	+	+	+	+
Caprate	+	+	+	+	+	+
Adipate	+	-	-	-	-	-
L-Malate	+	+	+	+	+	+
Citrate	+	+	+	-	+	+
Phenylacetate	-	-	-	d	-	-
API ZYM						
Alkaline phosphatase	d	+	+	+	+	+
Esterase(C4)	+	+	+	+	+	+
Esterase Lipase(C8)	+	+	+	+	+	+
Lipase (C14)	+	-	d	-	+	+
Leucine arylamidase	+	+	+	+	+	+
Valine arylamidase	d	-	d	+	+	-
Cystine arylamidase	-	-	-	-	-	-

Trypsin	-	+	+	+	+	+
α-chmotrypsin	-	-	-	-	-	-
Acid phosphotase	+	+	+	-	+	+
Naphtol-AS-phosphohydrolase	d	+	+	+	+	-
α-galactosidase	-	-	-	-	-	-
β-galactosidase	-	-	-	-	-	-
β-glucuronidase	-	-	-	-	-	-
α-glucosidase	-	-	-	-	+	-
β-glucosidase	-	-	-	-	-	-
N-acetyl- β glucosidase	-	-	-	-	-	-
α-mannosidase	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-

[a] +, 65% or more the total; -, 35% or less the total; d, 36 to 64% the total.

Partial DNA sequences of 16S rRNA genes were obtained for 11 *Pseudomonas* isolates. Isolates were selected for sequencing to provide least one representative from each of the six groups. These 16S rRNA sequences were used for phylogenetic analyses in combination with the previously described 16S rRNA sequences for the *Pseudomonas*. These results showed that out of 11 isolates, 4 were *P.putida*, 3 were *P.aeruginosa*, 2 were *P.stutzeri*, 1 was *P.mendocina* and 1 was *P.pseudoalcaligenes*. Our sequences of *Pseudomonas* spp. showed 100-95% similarities to the reference sequences obtained from the GenBank. These results were consistent with the results obtained from the API 20 NE kits (Table 3). In many cases, comparison of the sequence results to known 16S rRNA gene sequences deposited in GenBank revealed that species identifications based on phenotypic characteristics were in agreement with the molecular data. Nevertheless, 16S rRNA sequences of two isolates, P9-2 and P5-4, identified as *P. fluorescens* based on phenotypic characters, revealed that they were *P. putida* (96%) and 16S rRNA sequences of one isolate, P4-3, phenotypically identified as *P. alcaligenes* revealed that it was *P. pseudoalcaligenes* (97%). Table 3 summarizes the results of the SDS-PAGE protein pattern analysis and compare them with the results of the phenotypic and molecular study.

The electrophoretic patterns of whole cell soluble proteins of the isolates were visualized on SDS gels. It was found that there were patterns of homogenity between the strains of same species. The *P.aeruginosa* strains identified by the phenotypic tests (Table 2) were also assayed by the SDS-PAGE technique. The results indicated that from the two approaches yielded the same species identification (Fig. 3). In addition, comparison of SDS-PAGE patterns of *P.fluorescens* strains were similar, except for P9-2 and P5-4 strains. The patterns of proteins from P9-2 and P5-4 were different from the patterns of proteins prepared from the other *P.fluorescens* strains (Fig. 2). Indeed, both strains (P9-2 and P5-4) were later identified as *P.putida* followed by 16S rRNA analysis. Then, the protein profiles of P9-2 and P5-4 strains were compared with that of *P.putida* strains and a similarities between the two strains (data not shown).

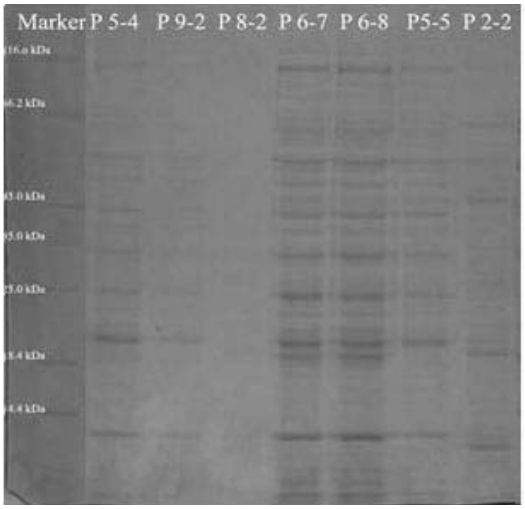

Figure 2. SDS-PAGE of whole-cell proteins of *P.fluorescens* identified by phenotypic methods. Lane 1: protein molecular weight markers (Fermentas #SM0431), from the top of the bottom, β galactosidase (116.0 kDa); bovine serum albumin (66.2 kDa); ovalbumin (45.0 kDa); lactate dehydrogenase (35.0 kDa); restriction endonuclease *BSP*981 (25 kDa); β lactoglobulin (18.4 kDa); lysozyme (14.4 kDa). Lane 2-3: 16S rRNA sequences of two isolates, P5-4 and P9-2, identified as *P.fluorescens* based on phenotypic characters, revealed that they were *P.putida* Lane 4-8: *P.fluorescens* P8-2, P6-7, P6-8, P5-5, P2-2.

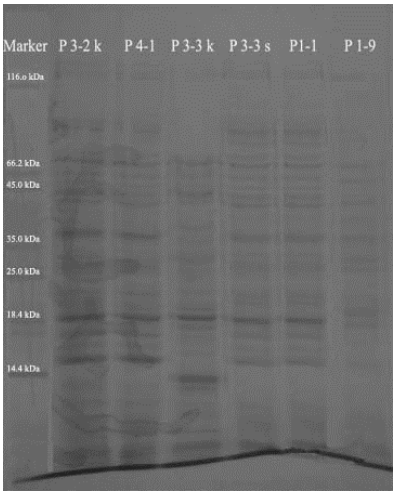

Figure 3. SDS-PAGE of whole-cell proteins of *P.aeruginosa*.Lane 1: protein molecular weight markers (Fermentas #SM0431), from the top of the bottom, β galactosidase (116.0 kDa); bovine serum albumin (66.2 kDa); ovalbumin (45.0 kDa); lactate dehydrogenase (35.0 kDa); restriction endonuclease *BSP*981 (25 kDa); β lactoglobulin (18.4 kDa); lysozyme (14.4 kDa). Lane 2-6: *P.aeruginosa* P3-2k, P4-1, P3-3k, P3-3s, P1-1, P1-9.

Table 3. Comparison of biochemical test, API 20 NE kit, API ZYM enzyme kit, 16S rRNA analysis and SDS-PAGE whole cell proteins identifications of *Pseudomonas* spp. strains.

Strain No	[a] Biochemical test	[b] API 20 NE	API ZYM	[c] SDS-PAGE	[d] 16S rRNA analysis	GenBank access number	Species[e]
P 3-3-k	+	+	+	+	+	AB037553	*P.aeruginosa*
P 4-1	+	+	+	+	+	AB037553	*P.aeruginosa*
P 3-2-k	+	+	+	+	+	AB037553	*P.aeruginosa*
P 9-2	-	+	+	+	+	AY785244	*P.putida*
P 5-4	-	+	+	+	+	PPU249825	*P.putida*
P 6-5	+	+	+	+	+	AY785244	*P.putida*
P 6-2	+	+	+	+	+	DQ192173	*P.putida*
P 5-7	+	+	+	-	+	PST288149	*P.stutzeri*
P 3-4	+	+	+	+	+	PST288149	*P.stutzeri*
P 6-6	+	+	+	+	+	AF094734	*P.mendocina*
P 4-3	-	+	+	-	+	AY880303	*P.pseudoalcaligenes*

[a]According to the traditional biochemical tests, the isolates which were same with *Pseudomonas spp.* were marked by "+".
[b]The isolates which were identified as *Pseudomonas spp.* by API 20 NE were marked by "+".
[c]According to the identification of SDS-PAGE, the isolates which were have common protein bands with *Pseudomonas spp.* were marked by "+".
[d]According to the 16S rRNA analysis, the isolates which were same with *Pseudomonas spp.* were marked with "+".
[e]Species identification by 16S rRNA analysis.

DISCUSSION

In this study, three broadly used typing methods were employed in order to assess and compare the identification and classification of environmental *Pseudomonas* strains. Twenty *Pseudomonas* isolates were characterized by a rapid identification system that is commercially available system (API 20NE). Also, the strains were analyzed by the API ZYM system, an enzyme activity profiling kit. The isolates were subjected to whole-cell protein electrophoresis (SDS-PAGE) and 16S rRNA sequence analysis.

Based on phenotypic characterization by the API 20 NE system, which evaluates oxidation patterns of 20 different substrates by a given isolate, and characterization by the API ZYM, our isolates were classified into six different species. The results show that all of the 20 *Pseudomonas* isolated from marine environment can be divided into six different species. These strains included six *Pseudomonas aeruginosa*, seven *P.fluorescens*, three *P.putida*, two *P.stutzeri*, one *P.mendocina* and one *P.alcaligenes* (Table 2).

Reliable identification of bacteria remains to be an important task in food and environmental microbiology. Molecular procedures are the most efficient but phenotypic approaches using either conventional or commercial systems are still used in the majority of laboratories. Phenotypic identification of isolates from natural sources has limitations related to the influence of cultural conditions on morphological and biochemical characteristics and to the effect of environmental pressure on gene expression (Soutourina et al. 2001; Maruyama et al.2000; Boivin-Jahns et al.,1995). Another problem is that databases of commercially available identification systems, have a considerable degree of accuracy for common species resulting in difficulties in identifying atypical strains and infrequently isolated species (Janda and Abbott 2002; Abbott et al., 1998). Hence, genotyping studies should be carried out to clarify the taxonomic status of the considered isolates (Alatossava and Alatossava 2006).

Molecular methods are necessary for the identification of environmental isolates and species with an incomplete phenotypic description (Garcı´a-Lo´pez et al. 2004). 16S rRNA data are widely used not only to define taxa but also to identify organisms present in clinical, veterinary and environmental samples (Drancourt et al. 2000; Maruyama et al.2000; Tang et al. 1998; Boivin-Jahns et al.,1995). We have shown that 16S rRNA sequence analysis improves the identification of environmental *Pseudomonas spp.* compared to conventional phenotypic methods.

Isolates well identified at the species level by conventional phenotypic methods served as a control for both conventional and molecular identification procedures. In general, the results obtained were in agreement. For this collection of isolates (n = 17), the two methods showed good overall agreement. In rare instances, (n = 3), 16S rRNA sequencing and conventional identification gave different results. 16S rRNA sequences of two

isolates, P9-2 and P5-4, identified as *P. fluorescens* based on phenotypic characters, revealed that they were *P. putida* (96%). In one case, a conflicting result was obtained (i.e., conventional identification resulted in *P.alcaligenes*, whereas sequence analysis resulted in *P. pseudoalcaligenes*). As seen from the results, the procedures of identification with phenotypic methods should be verified by molecular methods in order to come to more meaningful conclusions. In the other study, phenotypic characterization of the isolates was mainly performed using the API 20NE and BIOLOG GN2 identification systems. It was found that the identity of many isolates remained doubtful or controversial. The reasons for the limitations in the identification may be numerous: the determination of the sole phenotypic features (which constitutes only part of the polyphasic taxonomic approach recommended to identify micro-organisms), the Gram-negative orientated analyses (API 20NE and BIOLOG GN2 systems) that hindered the identification of some isolates, the number of tests in the strips, and the limits of the databases content (Alatossava and Alatossava 2006).

According to biochemical test results the strains of P5-4 and P9-2 identified as *P.fluorescens* according to 16S rRNA sequence results have turned out to be *P.putida*. The protein profiles of these two strains different from the protein profiles other *P.fluorescens* strains (Fig. 3). There are similarities between the protein profiles of these two strains and protein profiles of *P.putida* strains (Data not shown). The protein profiles of *P.aeruginosa* strains have high level of similarities when compared with each other (Fig. 2).

To date, various studies have suggested that the phenotypic classification techniques are not solely adequate for identification of *Pseudomonas spp*, which is problematic in classification. Hence, confirmatory identification of especially potentially risky pseudomonads by genotypic identification systems are required (Alatossava and Alatossava 2006).

It is important to monitor the presence of environmental *Pseudomonas* species due to their potential ecological significance. In order to make conclusions about their role in the ecosystem and human health, one must acquire further information regarding *Pseudomonas* species, using the phenotypic and genotypic methods discussed in this investigate.

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