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Characterisation of *Pseudomonas* spp. isolated from foods

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Abstract - Putative *Pseudomonas* spp. (102 isolates) from different foods were first characterised by API 20NE and then tested for some enzymatic activities (lipase and lecithinase production, starch hydrolysis and proteolytic activity). However subsequent molecular tests did not always confirm the results obtained, thus highlighting the limits of API 20NE. Instead RFLP ITS1 and the sequencing of 16S rRNA gene grouped the isolates into 6 clusters: *Pseudomonas fluorescens* (cluster I), *Pseudomonas fragi* (cluster II and V) *Pseudomonas migulae* (cluster III), *Pseudomonas aeruginosa* (cluster IV) and *Pseudomonas chicorii* (cluster VI). The pectinolytic activity was typical of species isolated from vegetable products, especially *Pseudomonas fluorescens*. Instead *Pseudomonas fragi*, predominantly isolated from meat was characterised by proteolytic and lipolytic activities.

Key words*: Pseudomonas fluorescens*, enzymatic activity, ITS1.

INTRODUCTION

The genus *Pseudomonas* is the most heterogeneous and ecologically significant group of known bacteria, and includes Gram-negative motile aerobic rods that are widespread throughout nature and characterised by elevated metabolic versatility, thanks to the 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.

Some species are important medically because they are considered opportunistic pathogens for humans and animals while others, like phytopathogens, are very important in the agricultural sector (Ridgway and Safarik, 1990; Palleroni, 1991a, 1993). The identification of the species of this genus was first described by Migula in 1894 (Palleroni, 1984); however, for a long time there were many gaps. Palleroni *et al*. (1973) began a complex study on the genus and recognized 5 rRNA similarity groups. Today, only rRNA similarity group 1 is considered the "true" *Pseudomonas* or *Pseudomonas* "*sensu stricto*", (Moore *et al.,* 1996) while different designations have been proposed for the members of other groups (Stanier *et al.,* 1966; Johnson and Palleroni, 1989; Palleroni, 1991b; Kozo, 1995).

Most members of group 1 are psychrotrophic, can be fluorescent or non-fluorescent, and have long been known to be responsible for chilled food spoilage. *Pseudomonas fluorescens, Pseudomonas putida*, and *Pseudomonas fragi* are the most frequently found species, however the species distribution within the food ecosystem remains relatively unknown (Arnaut-Rollier *et al.,* 1999). The principal microbial population of many vegetables in the field consists of species of the genus *Pseudomonas,* especially the fluorescent forms. During storage and processing their numbers increase, and in Minimally Processed Vegetables (MPV) they play an important role in the phenomenon of browning because of their pectinolytic activity (Ngyen-The and Carlin, 1994; Riva *et al.*, 2001). The most representative species are *Pseudomonas fluorescens, Pseudomonas putida*, *Pseudomonas chicorii* and *Pseudomonas maltophilia*.

Fish products serve as a good growth substrate for the *Pseudomonas* genus, especially under aerobic iced storage. Indeed, the genus is considered a major producer of the volatile compounds responsible for off-flavour compounds (aldehydes, ketones and esters) (Gram and Huss, 1996; Franzetti *et al.,* 2001) and *Pseudomonas fragi* appears the most to blame for fructy-odor production, a consequence of amino acid degradation (Molin and Ternstrom, 1986; Miller *et al.,* 1993; Tryfinopoulou *et al.*, 2002; Garcia-Lopez *et al.,* 2004).

Pseudomonas spp. also plays an important role in milk spoilage. During the storage of raw milk they produce many thermo-tolerant lipolytic and proteolytic enzymes that reduce both the quality and shelf life of processed milk (Wiedmann *et al.,* 2000; Dogan and Boor, 2003).

Thus, given the great interest in the genus *Pseudomonas,* and its primary role in the processes of foodstuff spoilage, the aim of this work was to classify, and to evaluate, the enzymatic activities of a large number of isolates, previously assigned to the *Pseudomonas* genus, coming from different foods.

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MATERIALS AND METHODS

Bacterial strains. A total of 102 isolates of putative *Pseudomonas* spp. were examined for phenotypic and genotypic characteristics. The strains, isolated since 1996 to 2005 and coming from different foods, were taken from the collection of freeze-dried cultures kept in the food-microbiology laboratory of DISTAM, Milan. The origin of the isolated strains was: 22 from minimally processed vegetables, 5 from pasteurised milk, 2 from fresh cheese, 16 from ground raw beef, 11 from uncooked chicken hamburger and 46 from sushi packed under modified atmosphere. The isolates were maintained on TSA slopes at 5 °C and sub-cultured every month. All isolates are listed in Table 1.

References strains. The study used the following reference strains as controls: *Pseudomonas fluorescens* biotype A ATCC 17555, *Pseudomonas fluorescens* biotype B ATCC 17482, *Pseudomonas fluorescens* biotype C ATCC 17559, *Pseudomonas fluorescens* biovar 3 DSMZ 50124, *Pseudomonas fluorescens* biotype G ATCC 17518, *Pseudomonas aeruginosa* DSMZ 50071^T, *Pseudomonas agarici* DSMZ 11810^T, *Pseudomonas alcaligenes* DSMZ 50342^T, *Pseudomonas asplenii* DSMZ 50254, *Pseudomonas chichorii* DSM 50259^T, *Pseudomonas chlororaphis* DSMZ 50083^T, *Pseudomonas fragi* DSMZ 3456^T, *Pseudomonas mendocina* DSMZ 50017^T, *Pseudomonas putida* DSM 291^T, *Pseudomonas syringae* DSMZ 10604^T, *Pseudomonas stutzeri* DSM 51909^T, *Pseudomonas viridiflava* DSMZ 11124.^{T} The strains were maintained on TSA slopes at 5 °C and sub-cultured every month.

Morphological, physiological and biochemical tests. Each isolate was tested for morphology, motility and Gram stain by phase contrast microscopy. The following physiological tests were performed: fluorescent and phenazine pigment production (King *et al.*, 1954), accumulation of poly-b-hydroxybutyrate (Palleroni and Doudoroff, 1972) levans production (Lelliot *et al.,* 1966), growth at 4, 10 and 41 °C and oxidative or fermentative acid production (Hugh and Leifson, 1953), API 20NE strips (Bio Mérieux, Marcy l'Etoile, France) were used for a preliminary biochemical characterisation of the strains and the species were identified using the API database. The enzymatic activities tested were: lipase production (Sierra, 1957), pectinolytic activity (Sand *et al.,* 1972), starch hydrolysis (Stanier *et al.,* 1966), lecithinase production (Nutrient Agar plates plus 5% v/v egg-yolk emulsion) and proteolytic enzyme production (Nutrient Agar plates containing 10% skim milk powder).

DNA extraction and PCR amplification. Template DNA was prepared by boiling 200 μl of bacterial suspension in MilliQ (OD_{600} = 0.6) in safe-lock Eppendorf tubes for 10 min. The tubes were immediately cooled on ice and centrifuged (20000 *x g* for 10 min, 5 °C); the supernatants were subsequently kept on ice or at –20 °C. One micro litre of template DNA suspension was used for each reaction (Johnsen *et al.,* 1996). All PCR reactions were performed in a volume of 50 μl containing approximately 50-100 ng of bacterial genomic DNA solution, 5 μl of 10X PCR reaction buffer, 200 μM of each dNTP, 2 mM of $MgCl_2$, 0.5 μM of each primer and 0.5 U of Taq Polymerase (Amersham-Pharmacia). DNA 16S region amplification was performed using the primer set 16SF-16SR (Alm *et al.,* 1996) (16SF 5'-AGAGTTTGATCCTGGCTCAG-3'; 16SR 5'-CTACGGCTACCTTGTTACGA-3') and the following thermal profile: 2 min at 94 °C; 5 cycles consisting of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min; 35 cycles consisting of 92 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min; final extension of 72 °C for 2 min; and final cooling at 4 °C. Amplification specific for *Pseudomonas* DNA was performed using the primer set 16SF-PSM_G (5'-CCTTCCTCCCAACTT- 3') (Johnsen *et al.,* 1999) and the following thermal profile: 6 min at 94 °C; 35 cycles consisting of 92 °C for 30s, 52 of 5 °C for 30 s, each cycle being followed by 1 min at 68 °C; on completion of all the cycles a further 6 min at 68 °C, and final cooling at 4 °C. DNA 16S-23S intergenic spacer (ITS1) region amplification was performed using the primer set 16F945 and 23R458 (Lane *et al*., 1985) (16F945 5'-GGGCCCGCACAAGCGGTGG-3'; 23R458 5'-CTTTCCCTCACGGTAC-3') and the following thermal profile: 5 min at 94 °C; 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; final extension of 72 °C for 2 min.

16S rRNA gene partial region (482-521 bp; 1311-1351 bp) specific for *Pseudomonas fluorescens* amplification was performed using the primer set 16SPSEfluF and 16SPSER (16SPSEfluF 5'-TGCATTCAAAACTGACTG-3'; 16SPSER 5'AATCACACCGTGGTAACCG-3') (Scarpellini *et al.,* 2004). Following amplification, 7 μl of each amplificate was analysed by electrophoresis at 100 Volt (1% agarose gel, 0.2 μg of ethidium bromide ml^{-1}) in TAE buffer. The amplification was performed in a DNA thermal cycle (Biometra T gradient, Germany).

Restriction analysis of the 16S rRNA gene and spacer (ITS1). Restriction digestion of each amplified 16S rRNA gene was carried out for 16 h at 30 °C in 25 ml reaction mixture containing 15 μl of template DNA, 2.5 μl of 10X PCR reaction buffer, 18.75 U of one restriction enzyme, either *Hae*III or *Vsp*I (Amersham Pharmacia Biotech). Restriction digestion of each amplified ITS1 was carried out for 16 h at 65 °C in 25 μl reaction mixture containing 15 ml of template DNA, 2.5 μl of 10X PCR reaction buffer, 18.75 U of *Taq* I (Amersham Pharmacia Biotech) (Guasp *et al.,* 2000). The restriction digestions were then analysed by agarose electrophoresis (3% w/v), containing 0.2 μg of ethidium bromide ml^{-1}, TAE buffer (Guasp *et al.,* 2000).

RFLP cluster analysis of ITS1. A computer similarities analysis was estimated by means of the jaccard coefficient (Sneath and Sokal, 1973) and clustering of strains was based on unweighted pair group method with arithmetic average (UPGMA). The NTSYS-PC computer program (version 1.30) (Rohlf, 1987) was used in the data analysis.

Total sequencing of the16S rRNA gene. After amplification of the 16S rRNA gene from extracted DNA, the PCR product was purified according to the instructions of a commercial kit (Qiaquick, Qiagen), and the strains were sequenced with the 16S forward primer and 16S reverse primer in a model 310 automatic DNA sequencer (Applied biosystem, Foster City, CA). The sequences obtained were elaborated by the software Chromas 2.13 (Technelysium Pty Ltd. Helensvale, Queensland, Australia) and the results were compared with sequences found in the gene bank www.ncib.nlm.nih.gov.

TABLE 1 - *Pseudomonas* species and their origin

Isolates	Origins	Phenotypical and biochemical identification (API 20NE)	Molecular identification	
			RFLP ITS1	16S rRNA gene sequencing
L1	Pasteurised milk	*P. fluorescens*	Profile A	*P. jessenii* (accession number gi. 3212001)
I14	Ready-to-use salad	*P. fluorescens*	Profile B	*P. orientalis* (accession number gi. 3142686)
Cluster I				
I3	Ready-to-use salad	*P. fluorescens*	P. fluorescens	
I15	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
L2	Pasteurised milk	*P. fluorescens*	*P. fluorescens*	
C15	Ground meat	*P. fluorescens*	*P. fluorescens*	
P10	Chicken hamburger	*P. fluorescens*	*P. fluorescens*	
V46	Sushi	*P. fluorescens*	*P. fluorescens*	
I7	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I8	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I11	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I16	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I18	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I19	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I21	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I22	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
V36	Sushi	*P. fluorescens*	*P. fluorescens*	
I20	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I13	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I5	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I10	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I23	Ready-to-use salad	*P. fluorescens*	*P. fluorescens*	
I12	Ready-to-use salad	*P. fluorescens*	P. fluorescens	
Cluster II				
P2	Chicken hamburger	*P. putida*	Profile C	*P. fragi (*accession number gi.10567504)
C2	Ground meat	*P. putida*	Profile C	*P. fragi (*accession number gi.10567504)
I2	Ready-to-use salad	*P. putida*	Profile C	*P. fragi (*accession number gi.10567504)
C3	Ground meat	*P. putida*	Profile C	
C4	Ground meat	*P. putida*	Profile C	
C16	Ground meat	*P. putida*	Profile C	
C20	Ground meat	*P. putida*	Profile C	
C11	Ground meat	*P. putida*	Profile C	
P1	Chicken hamburger	*P. putida*	Profile C	
V31	Sushi	*P. putida*	Profile C	*P. fragi (*accession number gi.10567504)
V13	Sushi	*P. fluorescens*	Profile C	
V33	Sushi	*P. putida*	Profile C	
V41	Sushi	*P. putida*	Profile C	
V29	Sushi	*P. putida*	Profile C	
V25	Sushi	*P. putida*	Profile C	
V21	Sushi	*P. putida*	Profile C	
V19	Sushi	*P. putida*	Profile C	
C14	Ground meat	*P. putida*	Profile C	
V32	Sushi	*P. putida*	Profile C	
V43	Sushi	*P. putida*	Profile C	
V30	Sushi	*P. putida*	Profile C	
V28	Sushi	*P. putida*	Profile C	
V22	Sushi	*P. putida*	Profile C	
C12	Ground meat	*P. putida*	Profile C	
P14	Chicken hamburger	*P. putida*	Profile C	
P8	Chicken hamburger	*P. putida*	Profile C	
P17	Chicken hamburger	*P. putida*	Profile C	
P13	Chicken hamburger	*P. putida*	Profile C	
V20	Sushi	*P. putida*	Profile C	
V35	Sushi	*P. putida*	Profile C	
V17	Sushi	*P. putida*	Profile C	
P7	Chicken hamburger	*P. putida*	Profile C	

(follow)

TABLE 1 - *Pseudomonas* species and their origin *(Continued)*

Isolates	Origins	Phenotypical and biochemical identification (API 20NE)	Molecular identification	
			RFLP ITS1	16S rRNA gene sequencing
Cluster III				
V6	Sushi	*P. putida*	Profile D	*P. migulae (*accession number gi 3309634)
C5	Ground meat	*P. putida*	Profile D	*P. migulae (*accession number gi 3309634)
V11	Sushi	*P. putida*	Profile D	
C13	Ground meat	*P. putida*	Profile D	
V14	Sushi	*P. putida*	Profile D	
V8	Sushi	*P. putida*	Profile D	
Cluster IV				
I25	Ready to use salad	*P. aeruginosa*	P. aeruginosa	*P. aeruginosa* (accesion number gi 17932687)
Cluster V				
L5	Pasteurised milk	*P. putida*	*P. fragi*	*P. fragi* (accession number gi. 4165419)
I6	Ready to use salad	*P. putida*	*P. fragi*	*P. fragi* (accession number gi. 4165419)
C7	Ground meat	*P. putida*	*P. fragi*	*P. fragi* (accession number gi. 4165419)
C1	Ground meat	*P. putida*	*P. fragi*	
V24	Sushi	*P. putida*	*P. fragi*	*P. fragi* (accession number gi. 4165419)
F4	Fresh cheese	*Pseudomonas* spp.	*P. fragi*	*P. fragi* (accession number gi. 4165419)
C6	Ground meat	*Pseudomonas* spp.	*P. fragi*	
V1	Sushi	*P. putida*	*P. fragi*	
V2	Sushi	*P. fluorescens*	*P. fragi*	
V44	Sushi	*P. putida*	*P. fragi*	
S3	Ground meat	*P. putida*	*P. fragi*	
I17	Ready to use salad	*P. fluorescens*	*P. fragi*	
L4	Pasteurised milk	*P. putida*	*P. fragi*	
P5	Chicken hamburger	*P. putida*	*P. fragi*	*P. fragi* (accession number gi. 4165419)
C8	Ground meat	*P. putida*	*P. fragi*	
V5	Sushi	*P. putida*	*P. fragi*	
V45	Sushi	*P. putida*	*P. fragi*	
V3	Sushi	*P. putida*	*P. fragi*	
V4	Sushi	*P. putida*	*P. fragi*	
V40	Sushi	*P. putida*	*P. fragi*	
V27	Sushi	*P. putida*	*P. fragi*	
V18	Sushi	*P. putida*	*P. fragi*	
V15	Sushi	*P. putida*	*P. fragi*	
V9	Sushi	*P. putida*	*P. fragi*	
V38	Sushi	*P. putida*	*P. fragi*	
V39	Sushi	*P. putida*	*P. fragi*	
V42	Sushi	*P. putida*	*P. fragi*	
V7	Sushi	*P. putida*	*P. fragi*	
V12	Sushi	*P. putida*	*P. fragi*	
V16	Sushi	*P. putida*	*P. fragi*	
V23	Sushi	*P. putida*	*P. fragi*	
Cluster VI				
I24	Ready to use salad	*P. fluorescens*	P. chicorii	*P. chicorii* (accession number gi 4165404)
L3	Ready to use salad	*P. putida*	*P. chicorii*	*P. chicorii* (accession number gi 4165404)
V37	Sushi	*P. putida*	*P. chicorii*	*P. chicorii* (accession number gi 4165404)
P15	Ready to use salad	*P. putida*	*P. chicorii*	*P. chicorii* (accession number gi 4165404)
F1	Ready to use salad	*P. putida*	*P. chicorii*	*P. chicorii* (accession number gi 4165404)
V10	Sushi	*P. putida*	*P. chicorii*	
V34	Sushi	*P. putida*	*P. chicorii*	
V26	Sushi	*P. putida*	*P. chicorii*	
P3	Ready to use salad	*P. putida*	*P. chicorii*	

RESULTS

Morphological, physiological and biochemical identification

The results of the phenotypic characterisation, based on physiological, morphological and biochemical tests, allowed all the strains to be assigned to group I, the one that, according to the most recent taxonomic revisions, is now called *Pseudomonas sensu stricto*. All the strains were Gram-negative, aerobic, catalase and oxidase positive rods, showed oxidative metabolism on Hugh Leifson medium and grew at 4 °C; only one strain (I25) grew at 41 °C, none were pigmented, nor did they accumulate poly ß-hydroxybutyrate granules. Pigment fluorescent production is a characteristic frequently found in the species of group I: 30% of the strains had this ability, and most of these were isolated from vegetables. Diffusible pigment production, favoured by low temperatures, was observed in 20% of the strains. Enzymatic activity varied from strain to strain.

The results of the biochemical characterisation, determined by means of API 20NE strips (Table 1), permitted the identification of 27 strains as *P. fluorescens,* 72 as *P putida,* 2 as *Pseudomonas* spp. and 1 as *P. aeruginosa.*

TABLE 2 - Biochemical characteristics of strains of six significant clusters

Characteristics	Custers (no. strains)								
	I (21) *P. fluorescens*				II (32) *P. fragi*	III (6) *P. migulae*	IV (1) *P. aeruginosa*	V (31) *P. fragi*	VI (9) *P. chicorii*
	B (6)	C (7)	3 (4)	G (4)					
API 20NE									
Denitrification	–	+	+	+	–	–	+	–	–
Indol	–	–	–	–	–	–	–	–	–
Glucose	–	–	–	–	–	–	–	–	–
Ammonia from arginine	+	+	+	+	+	+	–	+ (2)*	+
Urease	–	–	–	–	–	–	–	+ (3)	–
Esculin	–	–	–	–	–	–	–	–	–
Gelatine	+	+	+	+	+	–	+	–	–
p-Nitro-phenyl-β-D–galactopyranoside	–	–	–	–	–	–	–	–	–
Assimilation of:									
Glucose	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	–	+ (3)	–	+(3)	– (1)
Mannose	+	+	+	+	+	+ (3)	–	– (5)	– (1)
Mannitol	+	+	+	+	+	+ (3)	–	– (2)	–
N-acetyl-D-glucosamine	–	–	–	–	–	–	+	–	–
Maltose	–	–	–	–	–	–	–	–	–
Gluconate	+	+	+	+	+	+	+	+ (2)	+
Caprate	+	+	+	+	– (4)	–	+	– (2)	– (1)
Adipate	–	–	–	–	–	–	+	– (2)	–
Malate	V	V	V	V	+	+	+	+ (2)	+
Citrate	–	–	–	–	+	+	+	+	+
Phenyl-acetate	–	–	V	–	–	–	–	– (4)	–
Other tests									
Ossidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Oxidative in OF medium	+	+	+	+	+	+	+	+	+
Fluorescent pigment	+	+	+	+	–	+	+	–	+
Pyocyanin production	–	–	–	–	–	–	+	–	–
Growth at 4°C	+	+	+	+	+	+	+	+	+
Growth at 41°C	–	–	–	–	–	–	+	+	–
Lipase activity (tween 80)	–	–	–	W	+ (15)	W (3)	+	+	–
Egg yolk reaction	+	+	+	+	– (10)	–	–	+	–
Pectinolytic activity	+	+	+	+	–	+	–	–	+ (1)
Amidon hydrolysis	+	–	–	–	– (7)	–	–	+(8)	+ (1)
Proteolytic activity	+	w	–	–	+	W (2)	–	+ (3)	–
Levan production	+	+	–	+	–	+	–	–	–

(+): test positive; (–): test negative; (W): weak reaction; (V): variable reaction. * The number in parentheses represents the strains deviating from the most common result.

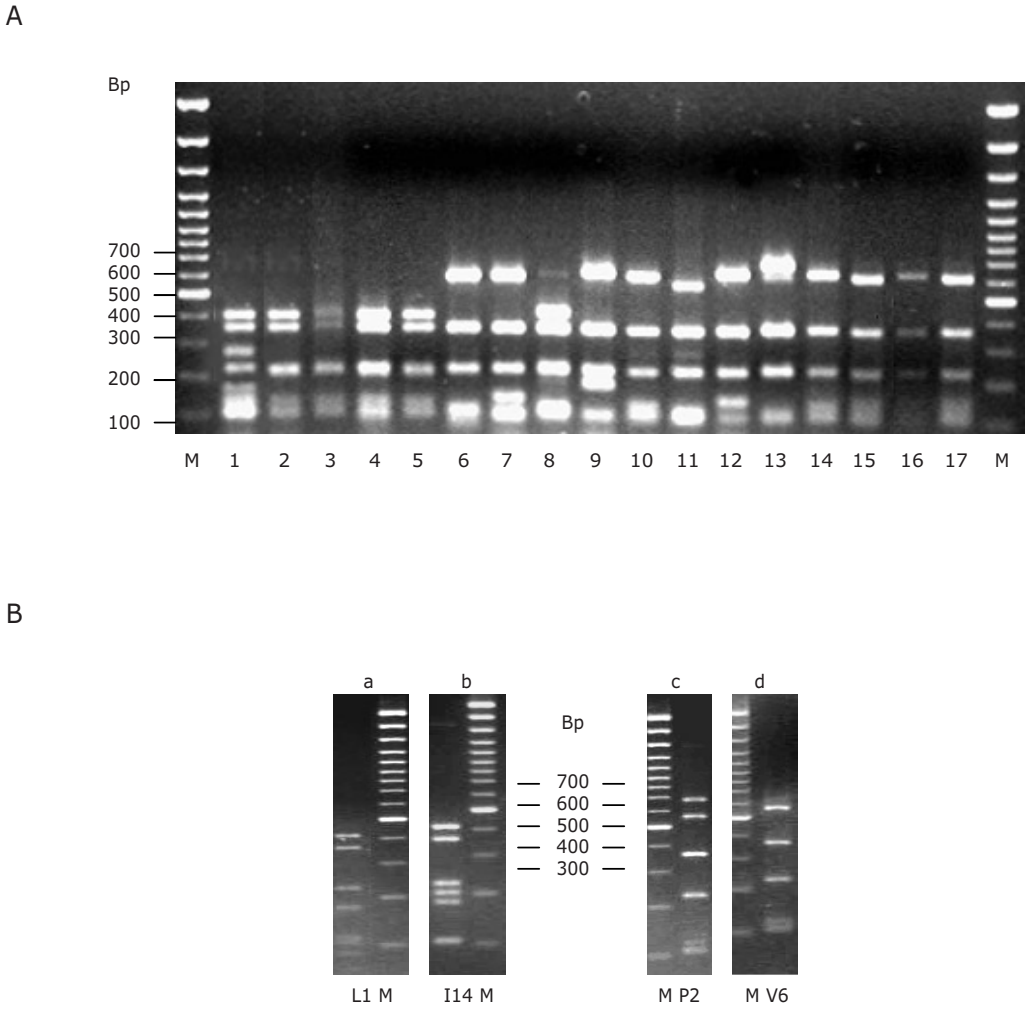

FIG. 1 - Agarose gel showing the *Taq*I, restriction profile of PCR amplified ITS1 region of strains used in this work. A: Reference strains *Pseudomonas fluorescens* biotype A (lane 1), *P. fluorescens* biotype B (lane 2), *P. fluorescens* biotype C (lane 3), *P. fluorescens* biovar 3 (lane 4), *P. fluorescens* biotype G (lane 5), *Pseudomonas cichorii* (lane 6), *Pseudomonas alcaligenes* (lane 7), *Pseudomonas putida* (lane 8), *Pseudomonas mendocina* (lane 9), *Pseudomonas asplenii* (lane 10), *Pseudomonas aeruginosa* (lane 11), *Pseudomonas stutzeri* (lane 12), *Pseudomonas fragi* (lane 13), *Pseudomonas syringae* (lane 14), *Pseudomonas agarici* (lane 15), *Pseudomonas chlororaphis* (lane 16), *Pseudomonas viridiflava* (lane 17); Marker Gene Ruler 100 bp MBI Fermentas (lanes M).
B: strains with different profile from reference strains: profile a, strain L1; profile b, strain I14; profile c, strain P2 and profile d, strain V6. M, marker Gene Ruler 100 bp MBI Fermentas.

Molecular characterisation

The belonging to the *Pseudomonas* genus was confirmed by tests with the specific primer PSM_G; all the isolates amplified the typical fragment of 450 bp (results not shown).

The differentiation of the various species within the *Pseudomonas* genus was performed by means of *Taq*I restriction profiles of the ITS1 amplicons, according to Guasp *et al*. (2000) and 16S rRNA gene sequencing. After digestion of 16S-23S (ITS1) amplified from genomic DNA yielded a banding profile that consisted of one to twenty bands ranging from 80 to 700 bp; three bands, 380, 230 and 110 bp, were present in all the strains. Figure 1 shows the different ITS1 restriction profiles; the results of digestion were clustered by UPGMA (Fig. 2). We grouped the isolates into six main clusters.

Cluster I includes 21 isolates that were characterised as *Pseudomonas fluorescens* by API 20NE. Their RFLP of 16S-ITS-23S regions with *Taq*I corresponded to the profile of the reference strains, *Pseudomonas fluorescens* ATCC 17482, *Pseudomonas fluorescens* ATCC 17559, *Pseudomonas fluorescens* DSMZ 50124 and *Pseudomonas fluorescens* ATCC 17518 (Fig. 1A). These results suggest that cluster I represents the species *Pseudomonas fluorescens*. In this cluster all the isolates, mostly from 'ready to use' salad, were fluorescent and showed marked pectinolytic and lecithinase activity. The differentiation of the biovar was performed by mean the comparison of the RFLP profile from the combination of three endonucleases (*Taq*I, *Vsp*I and *Hae*III associated with a cultural test (levan production) according to Scarpellini *et al*. (2004). Six strains were classified as

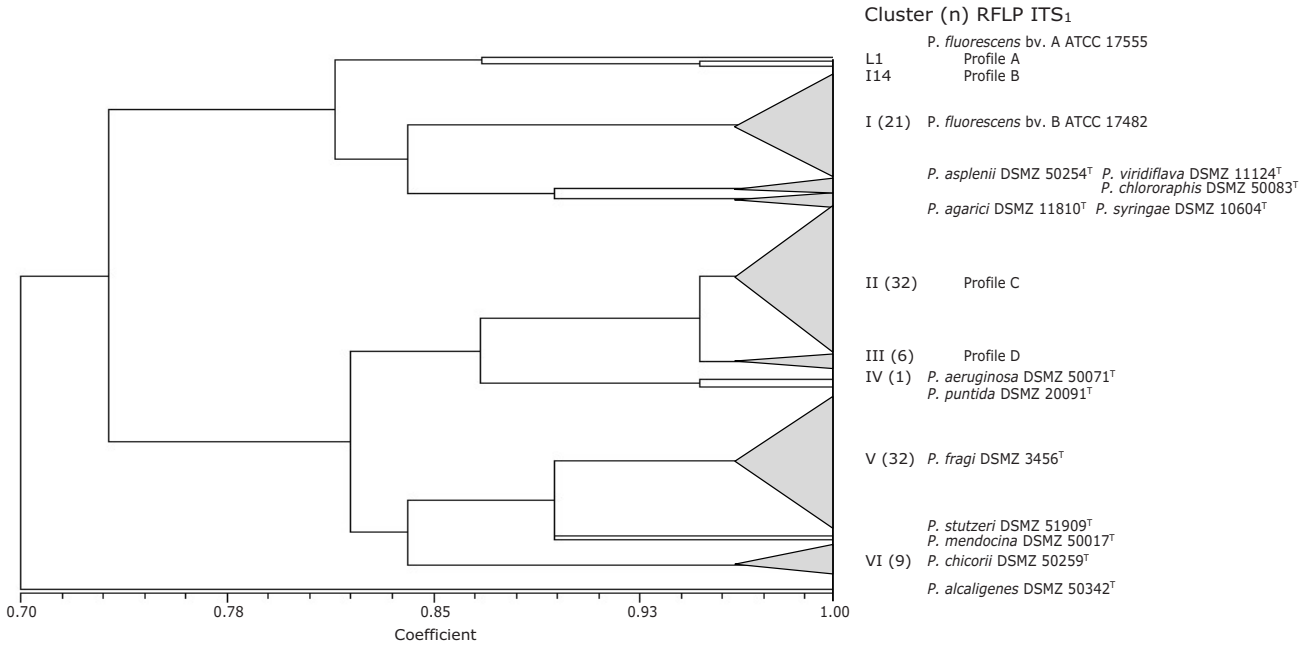

FIG. 2 - Dendrogram based on UPGMA analysis of ITS1 profiles obtained for *Pseudomonas* species.

Pseudomonas fluorescens biotype B: I8, I10, I18, I19, I20, I22 (Fig 1A, lane 2), 7 as *Pseudomonas fluorescens* biotype C: I3, I5, I12, I13, I15, L2, P10 (Fig. 1A, lane 3), 4 as *Pseudomonas fluorescens* biovar 3: I23, C15, V36, V46 (Fig. 1A, lane 4) and 4 as *Pseudomonas fluorescens* biotype G: I7, I11, I16, I21 (Fig. 1A, lane 5) (results not shown).

Cluster II contains 32 strains originated mostly from sushi, all classified as *Pseudomonas putida* with API 20NE. Nevertheless our strains showed a restriction profile that differed from the type strain, *Pseudomonas putida* DSMZ 291^T used as the reference (Fig. 1B, profile C). By means of 16S rRNA gene region sequence analysis we grouped these strains with *Pseudomonas fragi* (accession number gi.10567504). All the isolates of the cluster were proteolytic positive, while 50% possessed either lecithinase or lipase activity. None of the strains showed pectinolytic activity, and amilolytic activity was observed only rarely.

Cluster III includes only 6 isolates and none of the reference strains (Fig. 1B, profile D): 4 strains were isolated from sushi and 2 from ground meat. All six strains were fluorescent, none produced levan, nor did they show pectinolytic activity, but, when present, they had moderate lipolytic and proteolytic activity. Despite the 6 isolates were classified by the API 20NE system as *Pseudomonas putida*. The 16S rRNA gene sequence analysis grouped this cluster close to *Pseudomonas migulae* (accession number gi 3309634).

Cluster IV contains only one strain classified with API 20NE as *Pseudomonas aeruginosa*. It grew at 41 °C and produced phenazine pigment. DNA 16S-23S intergenic spacer region profile was the same as our reference strain, *Pseudomonas aeruginosa* DSMZ 50071^T (Fig. 1A, lane 11) and the 16S rRNA gene region sequence analysis showed the same nucleotide sequence as *Pseudomonas aeruginosa*

TABLE 3 – Distribution of the strains of *Pseudomonas* species in different foods

Isolates	Ready–to–use–salad	Ground raw meat	Sushi	Chicken hamburger	Pasteurised milk	Fresh cheese
P. *fluorescens* biotype B	6					
P. *fluorescens* biotype C	5			1	1	
P. *fluorescens* biovar 3	1	1	2			
P. *fluorescens* biotype G	4					
P. *fragi* (cluster II)	1	8	16	7		
P. *migulae*		2	4			
P. *aeruginosa*	1					
P. *fragi* (cluster V)	2	5	20	1	2	1
P. *chicorii*	1		4	2	1	1
P. *orientalis*	1					
P. *jessenii*					1	

(accession number gi 17932687) with homology with the 16S rRNA gene sequences of 99%.

Cluster V contains 27 isolates classified as *Pseudomonas putida,* 2 as *Pseudomonas* spp. and 2 as *Pseudomonas fluorescens* with the API 20NE system. All these strains showed the same RFLP pattern with *Taq*I, as observed with the reference strain (*Pseudomonas fragi* DSMZ 3456^T) (Fig. 1A, lane 13). The 16S rRNA gene region sequence analysis also grouped this cluster as *Pseudomonas fragi* (accession number gi. 4165419), providing further confirmation that cluster V represents the species *Pseudomonas fragi*. Most of the strains of this cluster were isolated from foods of animal origin; none of them used mannose and mannitol and all showed marked proteolytic and lipolytic activity.

Cluster VI contains 9 fluorescent strains with the same restriction profile as *Pseudomonas cichorii* DSMZ 50259^T (Fig. 1A, lane 6), obtained in the 16S-ITS-23S regions with *Taq*I. The 16S rRNA gene region sequence analysis shows the same nucleotide sequence as *Pseudomonas chicorii* (accession number gi 4165404).

The biochemical characteristics of the strains of each cluster are shown in Table 2.

Strains L1 and I14 profile A and B in Fig. 1B respectively showed different RFLP profiles (Fig.1B) and the complete sequencing of the 16S rRNA gene region grouped them with *Pseudomonas jessenii* (accession number gi. 3212001) and *Pseudomonas orientalis* (accession number gi. 3142686) respectively.

The distribution of the different species in the foodstuffs is shown in Table 3.

Pectinolytic activity appears typical of isolates of vegetable origin, particularly in *Pseudomonas fluorescens* biovar B (cluster I) that is the dominant species in ready to use salad. Some slight pectinolytic activity was also found in *Pseudomonas chicorii* isolated from vegetables. The *Pseudomonas fluorescens* biotype B possessed moderate amylase activity, and this was also noted in some strains of *Pseudomonas chicorii* and *Pseudomonas fragi*, predominantly isolated from sushi. Clusters II and V of *Pseudomonas fragi* contained the highest number of isolates from animal origin foodstuffs; both clusters had marked lipolytic and proteolytic activity.

In milk and dairy products an equal distribution of *Pseudomonas fragi*, *Pseudomonas cichorii*, *Pseudomonas fluorescens* and *Pseudomonas jessenii* was observed:

DISCUSSION

The classification of the *Pseudomonas* genus by means of physiological and biochemical characteristics is useful, but it is not enough to distinguish the *Pseudomonas* species: genomic studies are needed to confirm the exact taxonomic position of *Pseudomonas* spp. group 1.

Our results confirm that a correct identification and characterisation of the *Pseudomonas* species of group 1 can only be achieved by combining cultural, biochemical and molecular tests.

Our study underlining the differences between the phenotype identification results and those obtained by molecular tests. With biochemical tests (API 20NE) our strains were classified like *Pseudomonas fluorescens* and *Pseudomonas putida*, one strains as *Pseudomonas aeruginosa*, and no one as *Pseudomonas fragi*; while the analysis of the electrophoretic profiles obtained following RPFL ITS1 and the DNA sequencing allowed the identification of other species and the recovery of the VI clusters.

Pseudomonas fragi appears the species with the higher level of genetic polymorphism in fact, we recognised two different clusters (II and V). The tested enzymatic activities are accord to the provenience of isolates. Indeed the marked pectinolytic activity of the *Pseudomonas fluorescens* members of cluster I support their role in the processes leading to vegetable product alterations. Instead, lypolytic activity, found in isolates classified as *Pseudomonas fragi,* reveals the important involvement of this species in meat spoilage processes. The lypolityic activity appear very strong in isolates of cluster V, on the contrary the strain of cluster II are characterised by a major proteolytic activity

The presence of *Pseudomonas* in pasteurised milk is consequent to post-processing contamination, and is responsible for spoilage.

Nevertheless these results cannot be considered fully complete, and a true assessment of the correlation between species and origin requires the analysis of a far greater number of products.

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