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PROTEIN FINGERPRINTING AS A COMPLEMENTARY TOOL FOR THE CLASSIFICATION OF *Pseudomonas* BACTERIA

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Abstract: A collection of total 42 bacterial strains belonging to the genus *Pseudomonas* were characterised based on protein fingerprinting using sodium dodecyl sulphate polyacrylamide gel electrophoregrams of cell-free extracts. Densitometrical analysis revealed unique and distinct profiles characteristic of the studied species. This comparison differentiated the isolates into four main clusters and twelve subclusters. The obtained protein patterns have proved to be an effective and reliable method both for the classification of bacteria and for showing similarities and variability among them.

Key Words: Protein Fingerprinting, SDS-PAGE, Electrophoresis, Identification, *Pseudomonas*

INTRODUCTION

Pseudomonas bacteria are common Gram-negative, chemoorganotrophic microorganisms widely distributed throughout the world [1]. The main interest in these microbes is due to their importance regarding the degradation of xenobiotics [2], their action as plant pathogens [3], as well as their potential to

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Abbreviation: SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
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stimulate plant growth [4-6]. Hence, the search for new isolates and their identification are desirable from a biotechnological point of view. It is therefore highly relevant to also establish which features of pseudomonads could allow us to distinguish between them and help us to select only those strains, which are useful. Nevertheless, several general phenotypic and genotypic approaches to the classification of these bacteria have already been pursued. The classical identification relies mainly on morphological, physiological and biochemical criteria, but this approach is time-consuming and often gives ambiguous results [7]. Such problems with distinguishing at the subspecies level between rhizospheral fluorescent pseudomonads based on fatty acid methyl ester (FAME) analysis were already reported in our previous paper [8]. Due to this fact, the development and use of new methods that improve the identification and detection of these microbes is advisable. Genotypic techniques, i.e. sequencing of highly conserved 16S rDNA genes (ribotyping) and randomly amplified polymorphic DNA (RAPD) have already been successfully applied in microorganism classification [9-11]. The identification of isolates can also be performed through spectral analysis using Fourier transformation infrared spectroscopy (FTIR) [12-14] or mass spectrometry [15, 16]. Other modern methods are based on SDS-PAGE of cell-free extracts or of cell wall proteins (so-called protein fingerprinting) [17-19], also used for typing certain pseudomonad bacteria [20].

In the present study, we characterised *Pseudomonas* isolates by means of SDS-PAGE of total cellular protein profiles, a method not previously used for environmental *Pseudomonas*. In addition, we evaluated the usefulness of protein fingerprinting as a complementary analysis for the identification of the examined pseudomonads.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

A total number of 42 pseudomonad isolates were used in this study (Table 1) [21-26]. Those isolates are deposited at the Microbial Culture Collection of Agricultural Microbiology Department, Agricultural University of Wrocław, and are available on request. All strains were maintained as frozen stocks at -70°C in a medium containing 50 % glycerol, whereas the working cultures were prepared from those stock cultures by two consecutive transfers into King B medium for single-colony purification [27]. Next, a liquid $^{1}/_{10}$ Trypticase Soy Broth medium (Difco, Detroit, USA) adjusted to pH 7.0, was used for the cultivation of bacteria [28]. Each 30-mL sterile medium was inoculated with an appropriate suspension of bacterial cells taken from 3-day old slants. The cultures were cultivated in 100-mL Erlenmeyer flasks and aerated by rotary shaking (150 rpm) at 28±1°C. The bacterial suspensions were standardised by measuring their optical density at 600 nm. 3 mL of such 72-hr old cultures were

then harvested by centrifugation (10000×*g*, 5 min) and the pellet obtained was washed with distilled water, and recentrifuged. The protease inhibitor phenylmethane-sulfonyl-fluoride (Sigma Chemical Co., St. Louis, USA) was added to a final concentration of 1 mM. The samples were frozen at -70°C and kept until used for further analyses.

Extraction of total proteins from bacterial cells

The frozen samples were first quickly defrosted for 2 min in a warm heated water bath (30°C), and were then frozen again at -70°C (20 min). Such a procedure allows partly disrupted bacterial cells to be obtained. Each sample was centrifuged for 5 min at 10000×*g*, and the pellet obtained was resuspended in 200 μL of CelLytic™ B-II Bacterial Cell Lysis/Extraction Reagent (Sigma) [29]. The suspension was incubated for 30 min at room temperature. Afterwards, the sample was again recentrifuged and 80 μL from each sample was transferred into a new 1.5-mL Eppendorf tube. Then, 20 μL of sample treatment buffer (125 mM Tris-HCl, pH 6.8; 2 % (w/v) SDS, 8 % (v/v) glycerol, 0.0001 % (w/v) bromophenol blue; 5 % (v/v) β-mercaptoethanol) was added and the whole mixture was vortexed to ensure good homogenization. Such prepared samples were kept on a boiling water bath for 4 min, and again recentrifuged (10000×*g*, 10 s) in order to remove any accidentally-remaining whole cells or their fragments, and to obtain cell-free extracts.

Standard conditions for SDS-PAGE of cell-free protein extracts (protein fingerprinting)

SDS-PAGE was performed by the method described by Laemmli [30], modified according to Hames [31]. Proteins were analysed on 1.5-mm thick and 80-mm long gels run in a dual vertical slab unit PAGE'R-1B (PPH Marker, Wrocław, Poland). From each sample, 25 μL of extract was loaded on a polyacrylamide gel. The separation gel (10 %) and staking gel (5 %) were prepared from an acrylamide monomer solution (Carl Roth, Karlsruhe, Germany). The protein molecular masses were calculated on the basis of comparison with the following standards [in kDa]: β-galactosidase (116), phosphorylase B (97), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (21), and lysozyme (14). Electrophoresis was carried out at a constant current of 35 mA through the stacking gel, and at 60 mA through the separation gel at room temperature. After electrophoresis, the gels were rinsed out for 30 min in an isopropanol-acetic acid-water mixture (1:3:6, by volume), then for 5 min in methanol-acetic acid-water (3:1:6). Such impregnated gels were stained for 3 hrs in 1 % (w/v) Coomassie Brilliant Blue R-250. Afterwards, the gels were destained in a methanol-acetic acid-water (3:1:6) solution until protein bands became clearly visible [32].

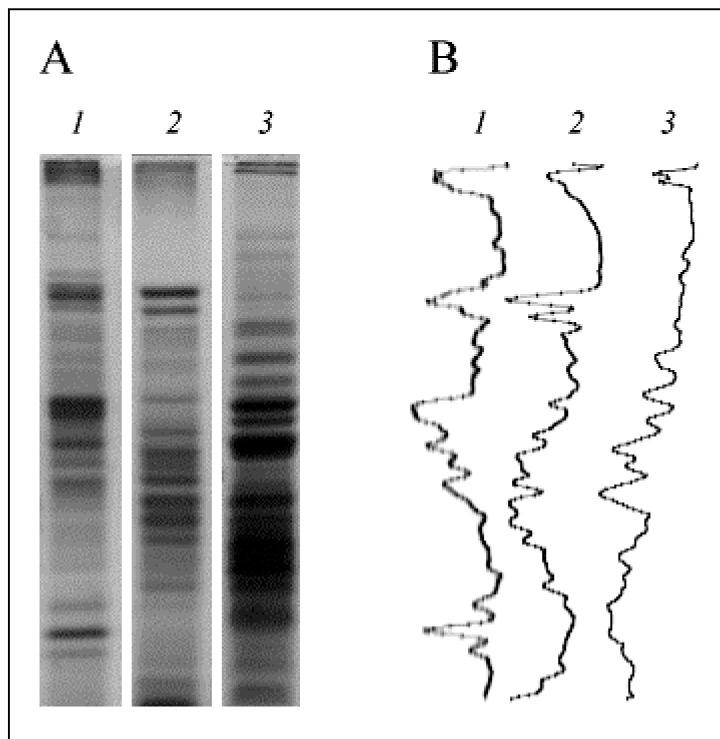


Fig. 1. SDS-PAGE of total cell-free proteins from pseudomonad bacteria (A). Relative line image plots (densitograms) generated by image analysis (B). Lanes: **1** - *P. putida* 01G3; **2** - *P. fluorescens* Pf 5; **3** - *P. fluorescens* R 210.

Statistical analysis of protein electrophoregrams

Each of the electrophoregrams was scanned and digitised with an Agfa SnapScan® 1236S computer scanner, at 36-bit colour and 9600 dots per inch (dpi) resolution. Using graphical shareware software Paint Shop Pro® version 4.12 (JASC Inc., Eden Prairie, USA), the gel images were standardised and exported as bitmaps into Band Leader Application version 2.01 (TechKnowledge, Tel Aviv, Israel), with which they were converted into simple *x/y* plots, normalised and smoothed out (Fig. 1). Next, those generated protein profile line images were exported to Excel version 97 (Microsoft Inc., Seattle, USA) and consecutively converted into numerical sequences. All the data were consolidated as a xls file. Afterwards, the data were processed using Statistica for Windows version 5.1 (StatSoft Ltd., London, UK). Dendrograms were constructed for proper matrices using Euclidean distances and Ward's amalgamation algorithm, resulting in a clustering of highly similar protein

patterns together. The classification into clusters was done on the basis of 250 selected central pixels. Sets of pixels from the start and front of the electrophoregrams were not taken into consideration due to the presence of non-specific bands (a migration of high-molecular-weight proteins or an occurrence of low-molecular-weight proteins as a result of non-specific lysis in the presence of SDS). Values of Euclidean distances lower than 2.1 were omitted and consequently not shown on the dendrogram.

RESULTS AND DISCUSSION

The analysis of whole-cell protein extracts of a total of 42 strains of *Pseudomonas* by one-dimensional SDS-PAGE revealed a set of clearly different profiles. The computer-assisted numerical processing of these patterns using cluster analysis with Euclidean distances yielded a dendrogram, which consisted of several groups at similarity levels ranging from 18 to 100 % of the maximum distance (Fig. 2). Based on the electrophoretic mobility of cellular proteins, the *Pseudomonas* strains studied were grouped into four main clusters (I-IV) – drawn at *ca.* 32 % of the maximum distance. Each group was additionally discriminated into minor clusters (at *ca.* 20 % of the maximum distance) grouping those protein profiles of the highest similarity (Table 1). Within group I, four subclusters (I a-d) represented by 12 isolates were distinguished. Those strains that have already been classified as distinct species, like *P. chlororaphis* (PGS 12) and *P. putida* (01 G3), were instantly separated first and foremost as a separate cluster (I a). It could be noted that subcluster I a was distinctly separated from other subclusters within this group. The Euclidean distance value with respect to those subclusters was about 4.3, whereas the distance between those two species only amounted to about 1.6. In order to demonstrate the results clearly, the dendrogram was simplified and only differences between clusters exceeding 2.1 were taken into consideration. With this threshold value in view, values of Euclidean distances below that were passed over. Remaining subclusters within this group were formed mostly by isolates belonging to the *P. fluorescens* bacteria, although *P. marginalis* PPs 21 was also included into cluster I b. Group II, which showed the highest similarity to group I, consisted of a total of 9 strains from *P. fluorescens* and *P. marginalis* species. Within this group, two subclusters (II a, b) were shown as closely related sets with little distance between them. The third group had 12 tested microbial strains composed of four individual subclusters (III a-d). Those isolates, which have been previously classified as *P. fluorescens* biotype C or F, were only found within this group. Curiously enough, only one isolate PsR 114 was found in the separate subcluster III c, whereas the clearly distinguished subcluster III d consisted of three strains (PPs 15, PsR 81 and PsR 28). Within this subgroup, bacteria previously identified as *P. fluorescens* without a designated biotype were included. The last cluster IV of the lowest similarity [the highest (100 %)

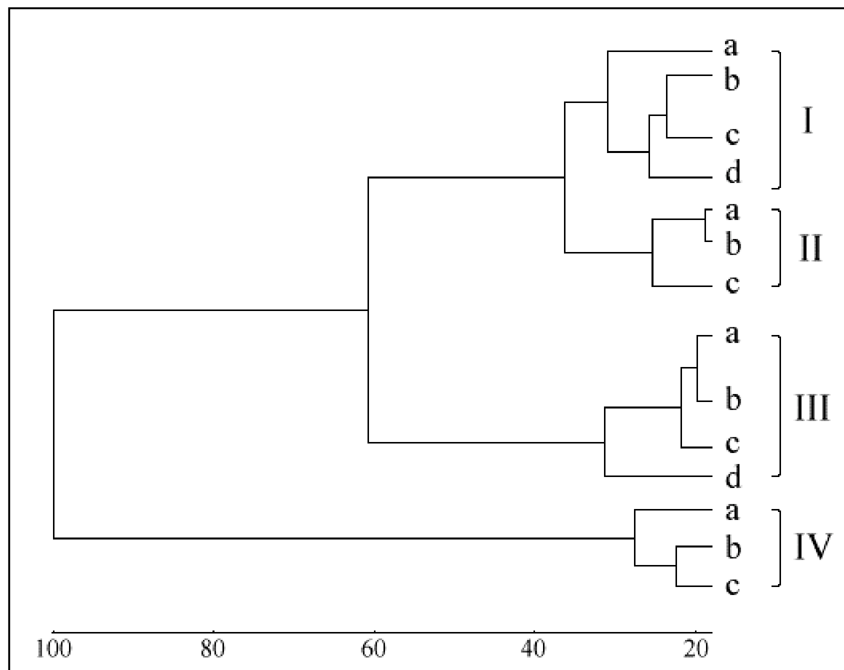
Euclidean distance] to the previous groups, was divided into three subgroups that consisted of a total of 9 strains. *P. fluorescens* biotype G as well as the

Tab.1. *Pseudomonas* strains used in this study

Cluster	Strain	Name	Habitat, country code[Ref.]
I a	PGS 12	*P. chlororaphis*	Soil, NL[21]
	01G3	*P. putida*	-, F[22]
I b	PPs 21	*P. marginalis*	Wheat rhizosphere, PL[23]
	PsR 21	*P. fluorescens*	Rape seed rhizosphere, PL[23]
	PsR 82	*P. fluorescens*	Rape seed rhizosphere, PL[23]
I c	PPs 11	*P. fluorescens*	Wheat rhizosphere, PL[23]
	PsR 41	*Pseudomonas* sp.	Rape seed rhizosphere, PL[23]
	PsR 26	*P. fluorescens*	Rape seed rhizosphere, PL[23]
	PsR 67	*P. fluorescens*	Rape seed rhizosphere, PL[23]
	PPs 20	*P. fluorescens*	Wheat rhizosphere, PL[23]
I d	PsR 2	*P. fluorescens*	Rape seed rhizosphere, PL[23]
	PsR 6	*P. fluorescens*	Rape seed rhizosphere, PL[23]
II a	PsR 120	*Pseudomonas* sp.	Rape seed rhizosphere, PL[23]
II b	B 6/4	*P. fluorescens* biotype G	Soil, Antarctica[24]
	PsR 116	*P. fluorescens*	Rape seed rhizosphere, PL[23]
II c	O 336	*P. marginalis*	Broad-bean rhizosphere, PL[23]
	WCS 374	*P. fluorescens* biotype G	Potato rhizosphere, NL[25]
	P 221	*P. fluorescens*	Wheat rhizosphere, PL[23]
	PPs 16	*P. fluorescens*	Wheat rhizosphere, PL[23]
	PPs 96	*P. fluorescens*	Wheat rhizosphere, PL[23]
	Ps 122	*P. fluorescens*	Broad-bean rhizosphere, PL[23]
III a	88 f	*P. fluorescens*	Alfalfa rhizosphere, PL[23]
	Ps 85	*P. fluorescens* biotype F	Broad-bean rhizosphere, PL[23]
III b	Pf 5	*P. fluorescens*	Cotton rhizosphere, USA[26]
	PsR 121	*P. fluorescens* biotype C	Rape seed rhizosphere, PL[23]
	PsR 37	*P. fluorescens*	Rape seed rhizosphere, PL[23]
	PsR 78	*P. fluorescens*	Rape seed rhizosphere, PL[23]
	PsR 25	*P. fluorescens*	Rape seed rhizosphere, PL[23]
	PsR 35	*Pseudomonas* sp.	Rape seed rhizosphere, PL[23]
III c	PsR 114	*P. fluorescens* biotype C	Rape seed rhizosphere, PL[23]
III d	PPs 15	*P. fluorescens*	Wheat rhizosphere, PL[23]
	PsR 81	*P. fluorescens*	Rape seed rhizosphere, PL[23]
	PsR 28	*P. fluorescens*	Rape seed rhizosphere, PL[23]
IV a	R 210	*P. fluorescens* biotype G	Rape seed rhizosphere, PL[23]
	PsR 86	*Pseudomonas* sp.	Rape seed rhizosphere, PL[23]
	PsR 91	*P. fluorescens* biotype G	Rape seed rhizosphere, PL[23]
IV b	PsR 102	*P. aureantiaca*	Rape seed rhizosphere, PL[23]
	PsR 45	*P. fluorescens*	Rape seed rhizosphere, PL[23]
IV c	PsR 79	*Pseudomonas* sp.	Rape seed rhizosphere, PL[23]

PsR 99	*Pseudomonas* sp.	Rape seed rhizosphere, PL[23]
PsR 84	*Pseudomonas* sp.	Rape seed rhizosphere, PL[23]
PsR 89	*Pseudomonas* sp.	Rape seed rhizosphere, PL[23]

strain PsR 86 were grouped in the separate cluster IV a, unrelated to the two remaining subsets (IV b, c). *P. aureantiaca* PsR 102 was also included here (cluster IV b), whereas strains unidentified at species level were placed in



cluster IV c. Moreover, no correlation between the protein profiles of the isolates studied and the plants from which they were obtained was observed.

Fig. 2. Grouping of the pseudomonad strains studied using cluster analysis (Euclidean distances, Ward's amalgamation algorithm) based on whole-cell protein profiles. Individual clusters consisted of 2 (I a), 3 (I b), 5 (I c); 2 (I d), 1 (II a), 2 (II b), 6 (II c), 2 (III a), 6 (III b), 1 (III c), 3 (III d), 3 (IV a), 2 (IV b), and 4 (IV c) strains, respectively. The scale represents similarity levels above 18 % of the maximum Euclidean distance [real (absolute) values ranged from 2.1 to 14.2]. For a more detailed description of the dendrogram see the text and Table 1.

The results presented here led to the subdivision of *P. fluorescens* into several clusters, which is a good reflection of the heterogeneity of this species as described in the literature. Moreover, the protein profiles were also compared with the protein profile database available in our laboratory. So far, this

database includes more than 300 protein patterns mostly belonging to soilborne bacteria. Such comparisons allow a confirmation of the unique protein profile characteristic of the studied pseudomonads. A clear distinction between the protein profiles from bacteria belonging to the biotype G and those from biotypes C and F was also observed. Apart from this observation, considerable diversity among the profiles was obtained from pseudomonads of biotype G, also indicating the necessity of further studies. On the other hand, protein profiles from particular strains of the *P. marginalis* and *P. aureantiaca* species were put into distinct clusters. In addition, this technique showed some differences between equivocal strains. It was also found that the observed protein patterns were generally stable even if the strains were transferred several times from slants to Petri dishes. These findings suggest a possible application of such a method to monitor individual, single strains during e.g. environmental studies. Thus, SDS-PAGE might be a valuable and at the same time cheaper alternative solution supplementing modern fluorescent techniques, like green fluorescence protein (GFP) labelling.

In contrast to results reported earlier by Gottlieb [23], we propose some more accurate classification of the studied therein pseudomonads. Cluster analysis based on protein fingerprinting revealed that the strains previously identified as *P. fluorescens* without precise definition might be placed in certain biotypes:

- PPs 16, PPs 96, Ps 122, P 221 and PsR 116 - are related to biotype G;
- PsR 78, PsR 25, and PsR 37 - are closely related to biotype C;
- 88 f - *P. fluorescens* biotype F.

Moreover, protein patterns obtained from bacteria previously determined by phenotypic analysis of utilised carbon sources (BIOLOG) [23] as *P. marginalis*, were located among microbes belonging to *P. fluorescens*. Thus, it would appear that either already given systematic names should be taken into consideration again and the organisms reclassified, or SDS-PAGE is not a sufficient method for discrimination between *P. fluorescens* and *P. marginalis*. It was also found that cluster IV consisted of several strains (PsR 86, PsR 79, PsR 99, PsR 84 and PsR 89) unlike the remaining isolates and having unique, dissimilar phenotypic traits [23]. These profiles were markedly different from those obtained from *P. fluorescens* and other species therein analysed for comparison. Owing to these findings, Gottlieb properly surmised that those strains belong to completely distinct species, perhaps not having been described in the literature as yet [23]. Our results confirmed clearly this assumption. Regarding strain PsR 102 (reported as *P. aureantiaca*), the obtained profile suggested that it is more related to *P. fluorescens* biotype G than to the others tested in this study. Consequently, this strain was found among isolates grouped within cluster IV and finally reclassified. Thus, its systematic position remains unknown.

However, certain differences between our results and those reported in [23] have appeared and, therefore, care should be taken in interpreting the data to

reveal reliable conclusions. It is certain that neither of the methods individually applied can provide a definitive classification of the strains.

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REFERENCES

1. Palleroni, N.J. Introduction to the family *Pseudomonadaceae*. In**: The Prokaryotes**. Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H. (eds). Springer Verlag, New York (1992) 3071-3085.
2. Harayama, S. and Timmis, K.N. Catabolism of aromatic hydrocarbons by *Pseudomonas*. In: **Genetics of Bacterial Diversity**. Hopwood, D.A. and Chater, K.F. (eds.). Academic Press, London (1989) 151-174.
3. Schroth, M.N., Hildebrand, D.C. and Panopoulos, N. Phytopathogenic pseudomonads and related plant-associated pseudomonads. In**: The Prokaryotes**. Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H. (eds.). Springer-Verlag, Berlin (1991).
4. Davison, J. Plant beneficial bacteria. **Bio/Technology** 6 (1988) 282-286.
5. Kloepper, J.W., Lifshitz, R. and Zablotowicz, R.M. Free living bacterial inocula for enhancing crop productivity. **Trend Biotechnol.** 7 (1989) 39-43.
6. Glick, B.R. The enhancement of plant growth by free-living bacteria. **Can. J. Microbiol.** 41 (1995) 109-117.
7. Sørheim, R., Torsvik, V.L. and Goksøyr, J. Phenotypical divergences between populations of soil bacteria isolated on different media. **Microb. Ecol.** 17 (1989) 181-192.
8. Żarnowski, R., Pietr, S.J., Quilez, J.F., Cabrera, E. and Barrero, A.F. The unique cyclopropane fatty acid composition of rhizospheral *Pseudomonas fluorescens* isolates. **Cell. Mol. Biol. Lett.** 5 (2000) 145-150.
9. Hartung, J.S. Molecular probes and assays useful to identify plant pathogenic fungi, bacteria, and marked biocontrol agents. In: **Plant-Microbe Interactions and Biological Control**. Boland, G.J. and Kuykendall, L.D. (eds.). Marcel Dekker Inc. New York (1998) 393-413.
10. DeParrasis, J. and Roth, D.A. Nucleic acid probes for identification of phytobacteria: identification of genus-specific 16S RNA sequences. **Phytopathology** 80 (1990) 618-621.

11. McDermott, J.M., Brändle, U., Dutly, F., Haemmerli, U.A., Keller, S., Müller, K.E. and Wolfe, M.S. Genetic variation in powdery mildew of barley: development of RAPD, SCAR, and VNTR markers. **Phytopathology** 84 (1994) 1316-1321.
12. Amiel, C., Mariey, L., Denis, C., Pichon, P. and Travert, J. FTIR spectroscopy and taxonomic purpose: contribution to the classification of lactic acid bacteria. **Lait** 81 (2001) 249-255.
13. Rodriguez-Saona, L.E., Khambaty, F.M., Fry, F.S. and Calvey, E.M. A novel approach for the rapid discrimination of bacterial strains by Fourier-transform near-infrared spectroscopy. **Proc. SPIE** 4206 (2001) 22-31.
14. Rodriguez-Saona, L.E., Khambaty, F.M., Fry, F.S. and Calvey, E.M. Rapid detection and identification of bacterial strains by Fourier transform near-infrared spectroscopy. **J. Agric. Food Chem.** 49 (2001) 574-579.
15. Li, T.-Y., Liu, B.-H. and Chen, Y.-C. Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. **Rapid Commun. Mass Spectrom.** 14 (2000) 2393-2400.
16. Claydon, M.A., Davey, S.N., Edwards-Jones, V. and Gordon, D.B. The rapid identification of intact microorganisms using mass spectrometry. **Nature Biotechnol.** 14 (1996) 1584-1586.
17. Hertel, C., Ludwig, W., Pot, B., Kerster, K. and Schleifer, K.H. Differentiation of lactobacilli occurring in fermented milk products by using oligonucleotide probes and electrophoretic protein profiles. **Syst. Appl. Microbiol.** 16 (1993) 463-467.
18. Tsakolidou, E., Manolopoulou, E., Kabaraki, E., Zoidou, E., Pot, B., Kersters, K. and Kalantzopoulos, G. The combined use of whole-cell protein extracts for the identification (SDS-PAGE) and enzyme activity screening of lactic bacteria isolated from traditional Greek dairy products. **Syst. Appl. Microbiol.** 17 (1994) 444-458.
19. Péter, O. and Bretz, A.G. Polymorphism of outer surface proteins of *Borrelia burgdorferi* as a tool for classification. **Zbl. Bakt.** 277 (1992) 28-33.
20. Lambert, B., Leyns, F., van Rooyen, L., Gosselé, F., Papon, Y. And Swings, J. Rhizobacteria of maize and their antifungal activities. **Appl. Environ. Microbiol.** 53 (1987) 1866-1871.
21. Georgakopoulos, D.G., Hendson, M., Panopoulos, N.J. and Schroth, M.N. Cloning of a its phenazine biosynthetic locus of *Pseudomonas aureofaciens* PGS12 and analysis of expression in vitro with the ice nucleation reporter gene. **Appl. Environ. Microbiol.** 60 (1994) 2931-2938.
22. Chablain P.A., Zgoda, A.L., Sarde, C.-O. and Truffaut, N. Genetic and molecular organisation of the alkylbenzene catabolism operon in the psychrotrophic strain *Pseudomonas putida* 01G3. **Appl. Environ. Microbiol.** 67 (2001) 453-458.

23. Gottlieb, M. Physiological traits of rhizosphere bacteria from the genus *Pseudomonas* and their capability of wheat seedling colonisation (in Polish). PhD thesis. Agricultural University, Wrocław (2001).
24. Pietr, J.S. Antagonistic activity of saprophytic bacteria from *Bacillus* and *Pseudomonas* genera against cucumber phytopathogenic *Fusarium* fungi (in Polish). **Zesz. Nauk. Akad. Roln. we Wrocławiu: Rozprawy** 64 (1987) 1-54.
25. Geels, F.P. and Schippers, B. Reduction of yield depression in high frequency potato cropping after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp. **Phytopathol. Zeit.** 108 (1983) 207-238.
26. Kraus, J. and Loper, J.E. Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of *Pythium* damping-off of cucumber. **Phytopathology** 82 (1992) 264-271.
27. King, E.O., Ward, M.K. and Raney, D.E. Two simple media for the demonstration of pyocyanin and fluorescein. **J. Lab. Clin. Med.** 44 (1954) 301-305.
28. Difco Manual of Dehydrated culture media and reagents for microbiology (10th ed.). Difco Laboratories, Detroit, MI (1994) 1027.
29. Mehigh, R. Lysis of *E.coli* purification of soluble recombinant proteins using CelLytic-B™ and CelLytic-B™ II. **Sigma Origins** 3 (2001) 15-16.
30. Laemmli, U.K. Cleaving of the structural proteins during the assembly of the head of bacteriophage T4. **Nature (London)** 227 (1970) 680-685.
31. Hames, B.D. One-dimensional polyacrylamide gel electrophoresis. In**: Gel electrophoresis of proteins. A practical approach**. Hames, B.D. and Rickwood, D. (eds.) Oxford University Press, New York (1995) 1-147.
32. Wilson, C.M. Staining of proteins on gels: comparison of dyes and procedures. **Methods Enzymol.** 91 (1983) 236-247.