J. Biol. Control, 16(1): 37-42, 2002

Differentiation of *Pseudomonas* strains through PAGE banding pattern

R. NADAKUMAR, S. BABU, A. KANDAN, T. RAGUCHANDER and R. SAMIYAPPAN
Department of Plant Pathology, Tamil Nadu Agricultural University
Coimbatore 641 003, Tamil Nadu, India
Email: raguchander@hotmail.com

ABSTRACT: **Eleven *Pseudomonas* isolates of ten-plant species collected from all over Tamil Nadu were compared for their total cell proteins separated through sodium dodecyl sulphate poly acrylamide gel electrophoresis. All the isolates showed marked variations among themselves and between fluorescent and non-fluorescent groups. However, similarity was observed with respect to three protein bands with molecular weight of 46, 43 and 32kDa. Data were scored based on the presence or absence of protein bands and cluster analysis was performed. The isolates from same location *viz.*, Coimbatore (PF1, PB2, COP1 and COT1), Sankaran Kovil (PSK1 and PSK2) and host (rice) showed greater level of similarity and occupied same cluster groups.**

KEY WORDS: Cell protein, electrophoresis, *Pseudomonas*, SDS-PAGE

Pseudomonas spp. make up a diverse group of bacteria, that can generally be found in all geographical conditions. During the last 25 years, extensive research has illustrated the latent potential of exploiting certain *Pseudomonas* species for the biocontrol of several plant diseases (Backman *et al.*, 1997). However, their biocontrol efficacy varies greatly with the pathogen. The proteins and enzymes of *Pseudomonas* spp. are partly responsible for their effectiveness against particular disease or pathogen. Electrophoretic separation of these proteins provide information about the cellular protein profile pattern and based on the similarity of banding pattern of protein, one can assess the similarity within the species or strains and can correlate with their biocontrol activity. Separation of total cell protein through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is currently used in bacterial identification (Lambert *et al.*, 1987; Kersters, 1985). Protein fraction of the cell envelope have been used to characterize the *Xanthomonas* spp. (Santhos and Dianese, 1985; Bouzar *et al.*, 1994; Thaveechai and Schaad, 1986), *Pseudomonas* spp. (Dristig and Dianese, 1990; He *et al.*, 1983) and more importantly to screen the anti-fungal rhizobacteria isolated from different localities and soil types (Leyns *et al.*, 1990). As *Pseudomonas* belong to gram negative bacteria, the outermembrane proteins and the lipopolysaccharides (LPS) of outermembrane are apparently involved in the interaction between plant and pathogens (Minsavage and Schaad, 1983). The antifungal activity of some of the isolates used in this study viz., PF1, PB2, COT1 and COT2 were proven against fungal, bacterial and viral pathogens, both under

greenhouse and field conditions (Vidhyasekaran *et al.*, 1997; Kandan *et al.*, 2001; Nandakumar *et al.*, 2001). The objective of this study was to compare the protein profile of those fluorescent and non-fluorescent pseudomonads isolated from different parts of Tamil Nadu and to describe the relationship between the cell proteins, geographical locations and correlate the biocontrol activity.

MATERIALS AND METHODS

Isolation of *Pseudomonas* isolates

Isolates of *Pseudomonas* used in this study were either isolated from the rhizosphere of respective host or obtained from culture collection of Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. The culture represents ten plant species from 10 different places of Tamil Nadu (Table 1). For isolation, one gram of rhizosphere soil adhering to the root surface was collected and transferred to a 250ml conical flask containing 100ml of sterile distilled water. After thorough shaking for 15minutes in a mechanical shaker, serial dilutions were made. One ml each of $10^{-5}$and 10^{-6} dilutions was pipetted out and poured into sterile Petri-dishes. Then King's agar medium B (KMB) was poured, rotated and the dishes were incubated at room temperature (28± 2°C) for 24h (King *et al.*, 1954). Fluorescent and non-fluorescent colonies were identified and characterized (Stanier *et al.*, 1966). The bacterial cultures were maintained on KMB temporarily at 4 °C and for long term storage in 30 percent glycerol at –80 °C.

Bacterial cultivation and protein separation

From the stock culture, fresh cultures were prepared on plates of KMB. One loopful of 48h grown fresh culture was inoculated into 50ml of KMB broth and incubated in a mechanical shaker under constant shaking at 150rpm for 48h at room temperature (25±2°C). The bacterial cells at its logarithmic phase of growth were harvested by centrifugation at 6000 rpm for 10 minutes. Cells were washed twice in cold Tris-buffer (3.3mM, pH 7.4), again resuspended in Tris-buffer (Tris-HCl 10mM, 0.75M sucrose, pH 7.4) and incubated for 10 minutes on ice and vortexed. The resulting cell suspension was incubated for 30minutes at 4°C before centrifugation at 10000rpm for 20minutes. The supernatant was used as total cell protein source for electrophoresis (Dristig and Dianese, 1990). The total protein was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) of total cell proteins

SDS-PAGE was carried out by the method described by Laemmli (1970). A stacking gel with 4 per cent acrylamide was layered on top of a 12 per cent separating gel in a Sigma slab gel apparatus. Samples with equal amount of protein (50 mg) were dissolved in sample buffer and denatured by boiling for 4 min before loading into the gel. Medium range molecular weight marker (Genei, India) was used as standard. Electrophoresis was initiated with 20mA current and then increased to a constant 45mA for approximately 2h. Gels were stained in a solution containing 40 per cent methanol (v/v), 10 per cent acetic acid (v/v) and 1 percent Commassie brilliant blue overnight. Gels were destained in 40 percent methanol (v/v), 10 percent acetic acid (v/v). Approximate molecular weight of desired proteins in kiloDaltons (kDa) was determined by comparing the distance between the gel and the protein bands to logarithmic transformation of molecular weight plotted against the distance travelled by standards (Weber and Osborn, 1969).

Diversity analysis

Diversity analysis among 11 isolates of *Pseudomonas* was analysed using either presence or absence of protein bands in SDS-PAGE analysis. Similarity among the isolates was assessed based on Jaccard's coefficient (Jaccard, 1908). Based on the dissimilarity coefficients, dendrogram was constructed using Unweighted Paired Grouping Average (UPGMA) method and the isolates were grouped into clusters.

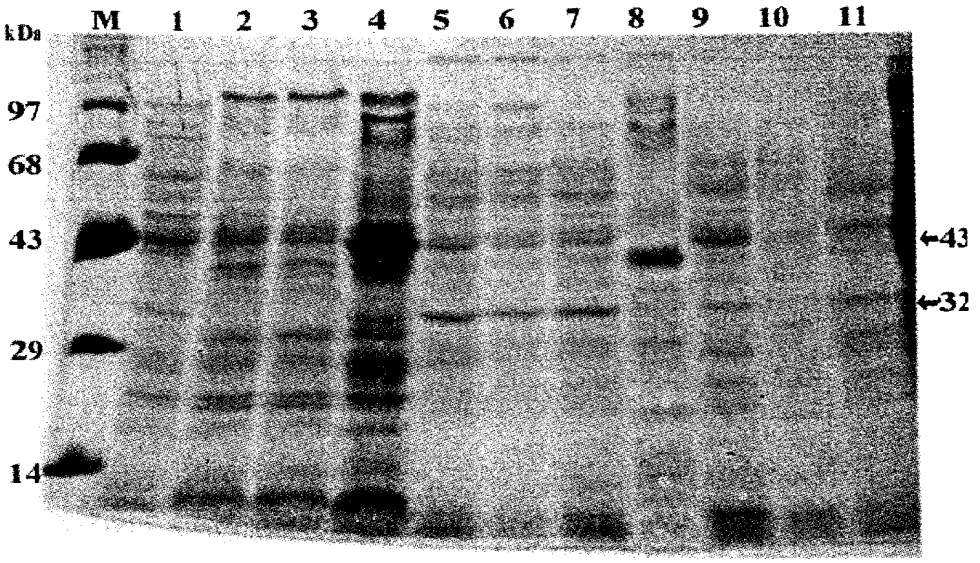
RESULTS AND DISCUSSION

Out of 11 isolates tested, four isolates (PF1, PB2, COP1 and COT1) were found to be fluorescent and other seven were non-fluorescent (Table 1).

Table 1. Details of *Pseudomonas* isolates used

Isolate	Host	Location	Nature
PF1	Blackgram	Coimbatore	Fluorescent
PB2	Rice	Coimbatore	Fluorescent
COP1	Rice	Coimbatore	Fluorescent
COT1	Tomato	Coimbatore	Fluorescent
ALR	Groundnut	Alyiar nagar	Non-fluorescent
PMTP	Sugarcane	Mettupalayam	Non-fluorescent
PYR5	Lime	Yercaud	Non-fluorescent
PSV2	Onion	Srivilliputhur	Non-fluorescent
PSK1	Jasmine	Sankarn Kovil	Non-fluorescent
PSK2	Chrysanthemum	Sankarn Kovil	Non-fluorescent
PTRY4	Banana	Trichy	Non-fluorescent

Pseudomonas spp. isolated from different geographical locations present more differences than similarities in their electrophoretic pattern of total cell proteins. Since the protein must reflect the physiological state of the cell rather than morphological structure, it appears reasonable to expect a variation in the type of protein bands obtained, depending upon the physiological state of the cell at the time of harvesting (Glynn and Reid, 1969). All the isolates of *Pseudomonas* spp. showed



Lane M: Protein Marker. 1: PF1. 2: PB2, 3: COP1, 4: COT1, 5: ALR, 6: PMTP 7: PYR5, 8: PSV2, 9: PSK1, 10:PSK2, 11: PTRY4

Fig. 1. SDS-PAGE analysis of *Pseudomonas* isolates

an average of 15 protein bands varying from 10 kDa to 100 kDa with large number of discrete bands (Fig. 1), however, major differences were detected among them. Generally, the banding pattern was clear and distinct in fluorescent isolates as compared to non-fluorescent ones. Despite clear variation observed in their banding pattern, similarity was also observed among the isolates with respect to two or three protein bands. A unique 43 kDa protein band was seen in all isolates except in PSK1, 32 kDa protein found in all isolates except those from rice ecosystem and 46 kDa protein in PF1, PB2 COP1, COT1, ALR3, PMTP, PYR5, PSV2 and PSK1.

Dristig and Dianese (1990) compared 65 isolates representing three biovars 1, 2 and 3 of *Pseudomonas solanacearum* isolated from seven host plants and five different regions of Brazil and found that the 37 and 39 kDa proteins common in several strains of biovars 1 and 3 and were not found in biovar 2. The presence of common protein among the specific microbial community can be used as biochemical marker to identify the isolates. Further the proteins in outer membrane of bacteria are reported to be apparently involved in interaction between the plants and pathogen (Minsavage and Schaad, 1983). Hence these three proteins perhaps may serve as biochemical marker to identify the rhizosphere *Pseudomonas* isolates and its biocontrol activity. Besides these similarities, some isolates shared a few common proteins. For example, PB2, COP1, COT1 showed identical protein profile with molecular weight of 99, 90, 45, 35, 30, 20 kDa proteins and shared similarities with PF1 by having 20 and 18 kDa in common. A 29kDa protein was found in ALR3, PMTP, PYR5, PSV2 and PSK1 and 28kDa protein in PYR, PSV2 and PSK1 isolates. Other examples of high similarity can be detected among the two groups of *Pseudomonas* as fluorescent and non-fluorescent. Further the isolates from the same location (PF1, PB2, COP1 and COT1) and host (PB2 and COP1) showed high similarity among themselves.

Table 2. Jaccard's similarity coefficient for 11 *Pseudomonas* isolates based on the presence or absence of protein bands in SDS-PAGE analysis

Isolates	PF1	PB2	COP1	COT1	ALR	PMTP	PYR5	PSV2	PSK1	PSK2	PTRY4
PF1	1.00										
PB2	0.33	1.00									
COP1	0.36	0.90	1.00								
COT1	0.44	0.44	0.38	1.00							
ALR	0.53	0.35	0.38	0.37	1.00						
PMTP	0.50	0.40	0.43	0.26	0.71	1.00					
PYR5	0.39	0.32	0.33	0.27	0.56	0.63	1.00				
PSV2	0.31	0.17	0.18	0.26	0.41	0.38	0.37	1.00			
PSK1	0.35	0.21	0.16	0.37	0.30	0.26	0.47	0.26	1.00		
PSK2	0.20	0.29	0.21	0.17	0.17	0.19	0.35	0.12	0.50	1.00	
PTRY4	0.50	0.24	0.25	0.20	0.50	0.57	0.63	0.22	0.41	0.27	1.00

Each *Pseudomonas* isolate exhibited distinct and characteristic protein pattern suggesting that these differences probably reflect the diversification in geographical distributions of the isolates and host species. In our study, similarity in banding pattern was observed between fluorescent and non-fluorescent group, location and host from which the isolates were obtained. In diversity analysis, the similarity matrix generated using Jacard's co-efficient, revealed that strains 2 and 3 were similar among themselves with a similarity value of 0.90 and least similarity was noticed between strains 8 and 10 with similarity value of 0.18 (Table 2). Based on the dendrogram constructed using Unweighted Paired Grouping Average (UPGMA) method, the strains were classified into four groups I, II, III and IV (Fig. 2).

Cluster composition was maximum in the first cluster, which had five strains *viz*, PF1, ALR, PMTP, PYR5, PTRY4. Cluster II consisted of a solitary individual PSV2. Cluster III and IV consisted of the isolates from same location Coimbatore (PB2, COP1 and COT1) and Sankaran Kovil (PSK1 and PSK2). Dristig and Dianese (1990) obtained a similar banding pattern in different isolates of *Pseudomonas solanacearum* isolates from potato, tomato and eggplant and also a clear difference between fluorescent and non-fluorescent group of isolates. The isolates PF1, PB2, COP1 and COT2 isolated from Coimbatore and sharing higher similarity showed biocontrol activity against *Rhizoctonia solani, Pyricularia oryzae* and tomato spotted wilt virus (Vidhyasekaran *et al.*, 1997; Kandan *et al.*, 2002; Nandakumar *et al.*, 2001). Except PF1 and the other three strains come under same cluster III and had the common proteins. Hence these isolates can be used as standard isolates for comparing the other *Pseudomonas* isolates for assessing their biocontrol activity with respect to their geographical distribution and the host plants from which the isolates were isolated.

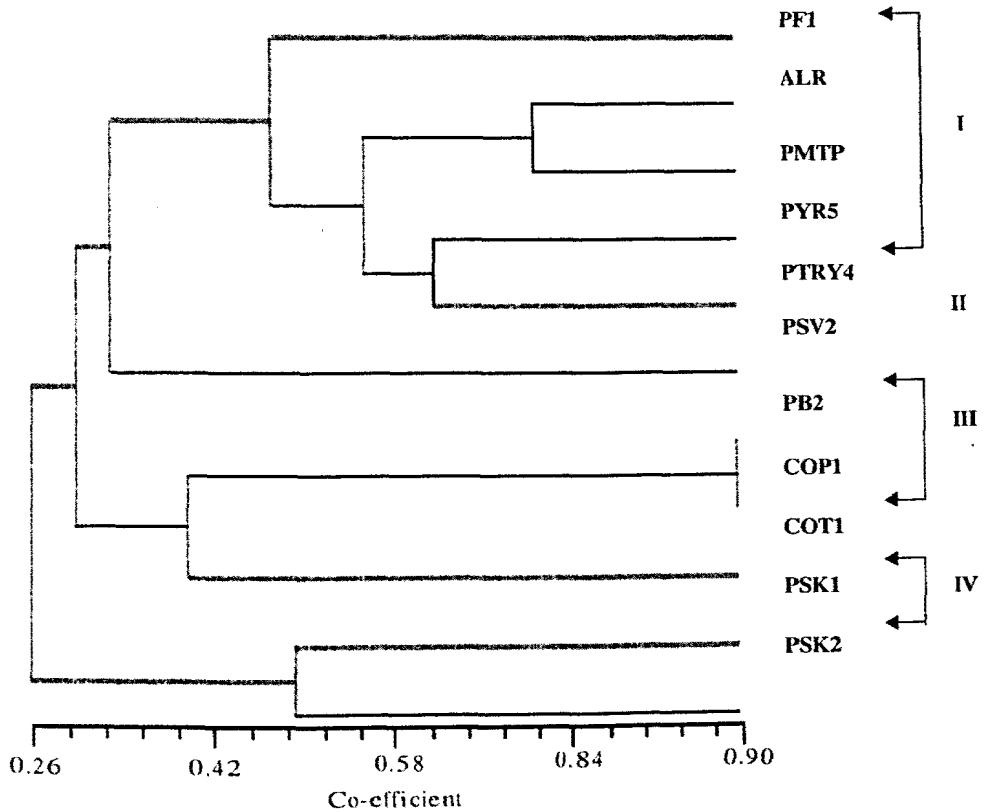


Fig. 2. Dendrogram analysis of 11 *Pseudomonas* isolates generated by cluster analysis of dissimilarity coefficient of protein bands

REFERENCES

Backman, P. A., Wilson, M. and Murphy, J. F. 1997. Bacteria for biological control of plant disease. pp 95-109. In: Rechcigal, N. A. and Rechcigl, J. E. (Eds.). *Environmentally Safe Approaches to Crop Disease Control.*, Lewis publishers, Boca Raton, Florida.

Bouzar, H., Jones, J. B., Minsacage, G. V., Stall, R. E. and Scott, J. W. 1994. Proteins unique to phenotypically distinct groups of *Xanthomonas campertris* pv. *vesicatoria* revealed by silver staining. *Phytopathology*, **84**: 39-44.

Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Annals of Biochemistry*, **72**: 248-254.

Dristig, M. C. G. and Dianese, J. C. 1990. Characterization of *Pseudomonas solanacearum* biovars based on membrane protein patterns. *Phytopathology*, **80**: 641-646.

Glynn, N. N. and Reid, J. 1969. Electrophoretic patterns of soluble fungal proteins and their possible use as taxonomic criteria in the genus *Fusarium*. *Canadian Journal of Botany*, **47**: 1823-1831.

He, L. Y., Srequeira, L. and Kelman, A. 1983. Characteristics of strains of *Pseudomonas solanacerum* from China. *Plant Disease*, **67**: 1357-1361.

Jaccard, P. 1908. Nouvellers rescherches sur la distribution florate. *Bull. Soc. Vaud. Sci. Nat.* **44**: 223-270.

Kandan, A., Ramiah, R., Radja Commare, R., Nandakumar, A., Raguchander, T. and Samiyappan, R. 2001. Induction of phenyl propanoid metablosim by *Pseudomonas fluorescens* against tomato spotted wilt virus in tomato. *Folia Microbiologica*, **47 (2)**: 121-129.

Kersters, K. 1985. Numerical methods in the classification of a bacteria by protein electrophoresis. pp. 337-368 In: Jones, D. and Priest, F.G. (Eds.). *Computer-Assisted Bacterial Systematics*. Academic press. London.

King, E. O., Ward, M. K. and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine*, **44**: 301-307.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

Lambert, B., Leyns, F., Joos, H., Tenning, P., Vas Rijsbergen, R., Van Outryue, F., Zhao, Y., Swings, J. and Van Montagu, M. 1987. Rhizobacteria of maize and their antifungal activities. *Applied and Environmental Microbiology*, **53**:1866-1871.

Leyns, F., Lambert, B., Joos, H. and Swings, J. 1990. Antifungal bacteria from different crops, pp. 437-444. In: De Honby (Ed.). *Biological control of soil-borne plant pathogens*. CAB International, UK.

Minsavage, G. V. and Schaad, N. W. 1983. Characterization of membrane proteins of *Xanthomonas camperstris* pv. *campestris*. *Phytopathology*, **73**: 747-755.

Nandakumar, R., Babu, S., Viswanathan, R., Raguchander, T. and Samiyappan, R. 2001. Induction of systemic resistance in rice against sheath blight disease by *Pseudomonas fluorescens*. *Soil biology and Biochemistry*, **33**: 603-611.

Santhos, R. M. D. B. and Dianese, J. C. 1985. Comparative membrane characterization of *Xanthomonas campestris* pv. *cassavae* and *X. campestris* pv. *manihotis*. *Phytopathology*, **75**: 581-587.

Stanier, R. Y., Palleroni, N. J. and Metraux, J. P. 1966. The aerobic pseudomonads. A taxonomic study. *Journal of General Microbiology*, **43**: 159-171.

Thaveechai, N. and Schaad, N. W. 1986. Serological and electrophoresis analysis of a membrane protein extract of *Xanthomonas campestris* pv. *campestris* from Thailand. *Phytopatholgy*, **76**: 139-147.

Vidhyasekaran, P., Rabindran, R., Muthamilan, R., Nayar, K., Rajappan, K., Subramanian, N. and Vasumathi, K. 1997. Development of powder formulation of *Pseudomonas fluorescens* for control of rice blast. *Plant Pathology*, **46**: 291-297.

Weber, K. and Osborn, M. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyarcrylamide gel electrophoresis. *Journal of Biological Chemistry*, **244**: 4406-4412.