J. Turk. Phytopath., Vol. 29, No. 2-3, 105-111, 2000 ISSN 0378 - 8024

Characterization and numerical taxonomy of *Pseudomonas tolaasii* isolates using antimicrobial susceptibility patterns

Nurettin ŞAHİN* **A. Üsame TAMER**** **Cem AZERİ****

* Muğla University, Faculty of Arts and Sciences, Department of Biology, 48187 Kötekli, MUĞLA

** Celal Bayar University, Faculty of Arts and Sciences, Department of Biyology, Muradiye, MANİSA

ABSTRACT

The susceptibilities of *Pseudomonas tolaasii* isolates (n= 160) isolated from sporophores of the *Agaricus bisporus* to 20 antimicrobial agents were determined by disk-diffusion method. *P. tolaasii* NCPPB 2192^T and NCPPB 2325 were used as reference strains. All *P. tolaasii* isolates were sensitive to tobramycine and kanamycine. Antimicrobial susceptibility pattern of isolates showed two distinct phenotypic group. Tetracycline sensitivity and glucose utilization were the most diagnostic characters between the phenotypic groups. The results showed that in addition to normal identification methods it is possible to differentiate some of the varieties of *P. tolaasii* with the help of antimicrobial susceptibility tests.

Key words: *Pseudomonas tolaasii*, antibiotics, numerical taxonomy

INTRODUCTION

Bacterial brown blotch (Tolaas 1915) caused by *Pseudomonas tolaasii* (Paine 1919) and a variant sometimes referred to as *P. gingeri* (Rainey *et al.*, 1992) is considered as the most common and serious bacterial disease on cultivated mushrooms throughout the world. Under some environmental conditions still not well-determined, but influenced by temperature and relative humidity, the bacterium can become pathogenic and provoke the brown blotch disease (Soler-Rivas *et al.*, 1999). Taxonomy of the *P. tolaasii* group is not fully resolved (Rainey *et al.*, 1992, Wells *et al.*, 1995). It is described in the Section V of Bergey's Manual of Systematic Bacteriology (Palleroni 1984) due to the natural relationships with well characterized species of the genus Pseudomonas are largely unknown.

The aim of the present study is numerical taxonomy of bacterial pathogens responsible for the brown discoloration on the *Agaricus bisporus* using antimicrobial susceptibility and biochemical tests data.

MATERIALS and METHODS

Isolation of bacteria

Sporophores of the *Agaricus bisporus* obtained from Manisa and İzmir province showing brown or reddish-brown blotches were used. Isolation of bacteria from altered caps or stipes was performing on King's medium B (KB) following the usual procedures (Lelliot and Stead, 1987). Pure cultures were screened for pathogenicity and for the "white line" reaction (Wong and Preece, 1979). For this purpose authentic strains of *P. tolaasii* and *P. "reactans"* NCPPB 1311^T were used.

Characterization & susceptibility testing

All the bacterial isolates were assayed for their ability to form a precipitate (white line reaction) in KB medium with *P."reactans"* NCPPB 1311^T. Auxanographic features were determined in API 20 E galleries (BioMerieux, Fr.).

Antimicrobial susceptibility of the isolates and reference strains was tested on plates of Mueller-Hinton agar (Difco) pre-inoculated with the test organism and dried (Bauer *et al.*, 1966; NCCLS, 1990). Antimicrobial susceptibility test discs (Oxoid) were placed on the agar surface. Following discs were used: Erythromycin 15 µg (E); Streptomycin 10 U (S); Polymyxin B 300 IU (PB); Penicillin G 10 IU (P); Kanamycin 30 µg (K); Tetracycline 30 µg (TE); Sulphamethoxazole-Trimethroprin 25 µg (SXT); Neomycin 30 µg (N); Chloramphenicol 30 µg (C); Doxycycline 30 µg (DO); Gentamycin 10 µg (CN); Carbenicillin 100 µg (CAR); Bacitracin 10 IU (B); Tobramycin 10 µg (TOB); Cefalexin 30 µg (CL); Ampillicin 10 µg (AMP); Rifampicin 5 µg (RD); Vancomycin 30 µg (VA); Nalidixic acid 30 µg (NA); Novobiocin 30 µg (NB).The plates were evaluated after 24 and 48 hours of incubation at 27 °C. If the area surrounding a disc (≥ 16 mm diameter) was free from bacterial growth, it was recorded that the organism was sensitive to that antibiotic. Tests were performed in triplicate. All susceptibility tests could be read without difficulty after 24h of incubation. Pure bacterial cultures were stored at -20 °C in glycerol. Reference strains of *P. tolaasii* (NCPPB 2192^T and NCPPB 2325) obtained from the National Collection of Plant Pathogenic Bacteria, UK and were used as controls.

Numerical taxonomy

Numerical analysis of the data obtained from antibiotic susceptibility and carbon utilization tests was performed by using the simple matching coefficient. The results of

tests that were positive or negative for all of the strains were eliminated from subsequent numerical analysis. The data matrix was used to estimate the strain similarities, with calculation of the simple matching coefficient, and cluster analysis was computed into phenogram by using complete linkage clustering method (Sneath and Sokal, 1973). Isolated strains were grouped under eighth clusters (close groups) and type strains. All analyses were carried out with the TAXON-X program (Chun, 1995).

RESULTS and DISCUSSION

Susceptibilities of isolated and two reference strains of *Pseudomonas tolaasii* (NCPPB 2192^T and NCPPB 2325) to antibiotics were determined. All strains were sensitive to tobramycine and kanamycine. Resistance for all investigated strains was noted for 11 antibiotics: rifampicin, cefalexin, ampicillin, carbenicillin, bacitracine, erythromycine, chloramphenicol, penicillin G, vancomycin, nalidixic acid, and novobiocin. Penicillinase production was detected all strains tested. The results of other differential antibiotic susceptibility patterns and some phenotypic properties were shown in Table 1. Similar results were reported by Richardson, 1993 and Vantomme *et al.*, 1987. Most of the reported strains of *P. tolaasii* is tetracycline sensitive, but more than 40 percent of isolated strains were resistant to this antibiotic. Our findings were supported this observation (Table 1). Although, antibiotic usage was not offical for mushroom cultivation in Türkiye the resistance pattern relatively high. This results may be strong evidence

Table 1. Differential characteristics of isolates based on their antimicrobial susceptibility pattern and some phenetic properties

	Type strains		**Phenotypic Clusters**		
	P. tolaasii NCPPB 2192	*P. tolaasii* NCPPB 2325	A4 -1	M1 & M2	Others
No. of strains	1	1	20	40	100
Gelatin hydrolysis	+	-	+	+	+
Glucose assimilation	-	+	+	-	-
Rhamnose assimilation	-	-	-	+	-
Resistance to antibiotics ($\mu g\ ml^{-1}$)					
Doxycycline (30)	S	S	S	50%	40%
Streptomycine (10)	S	S	R	50%	40%
Tetracycline (30)	S	S	S	R	40%
Polymyxine B (300)	R	R	S	-	40%
Gentamycine (10)	S	S	S	-	20%
SXT(*) (25)	R	R	S	R	R
Neomycine (30)	S	S	S	-	20%

S. Sensitive (≥ 16 mm diameter), **R**: Resistance, (*). Sulphamethoxazole-Trimethroprin
NCPPB: National Collection of Plant Pathogenic Bacteria, Hertsfordshire, U.K.

that probable transfer of resistance plasmids between Pseudomonas strains and other compost microflora.

Two main phenotypic cluster-groups, showed in Figure 1, were obtained after a computer-assisted numerical analysis from antibiotic susceptibility data. Group A; contains the type (NCPPB 2192 & NCPPB 2325) and tetracycline sensitive strains of *P. tolaasii* (B8-2, I-1, I-2, A4-1). Group B contains tetracycline resistant strains (M-1, M-2, I-3, I-4). Cluster-groups A and B except strains A4-1 and I-4 were defined at the 85% and 75% similarity (S) levels respectively. Strains A4-1 and I-4 do not share significant (S ≥75%) relationships with any of he cluster-groups (Fig. 1).

Glucose and gelatin is the most seperative carbon source for biochemical characterization. None of the strains utilized mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose and urea tested in API galleries.

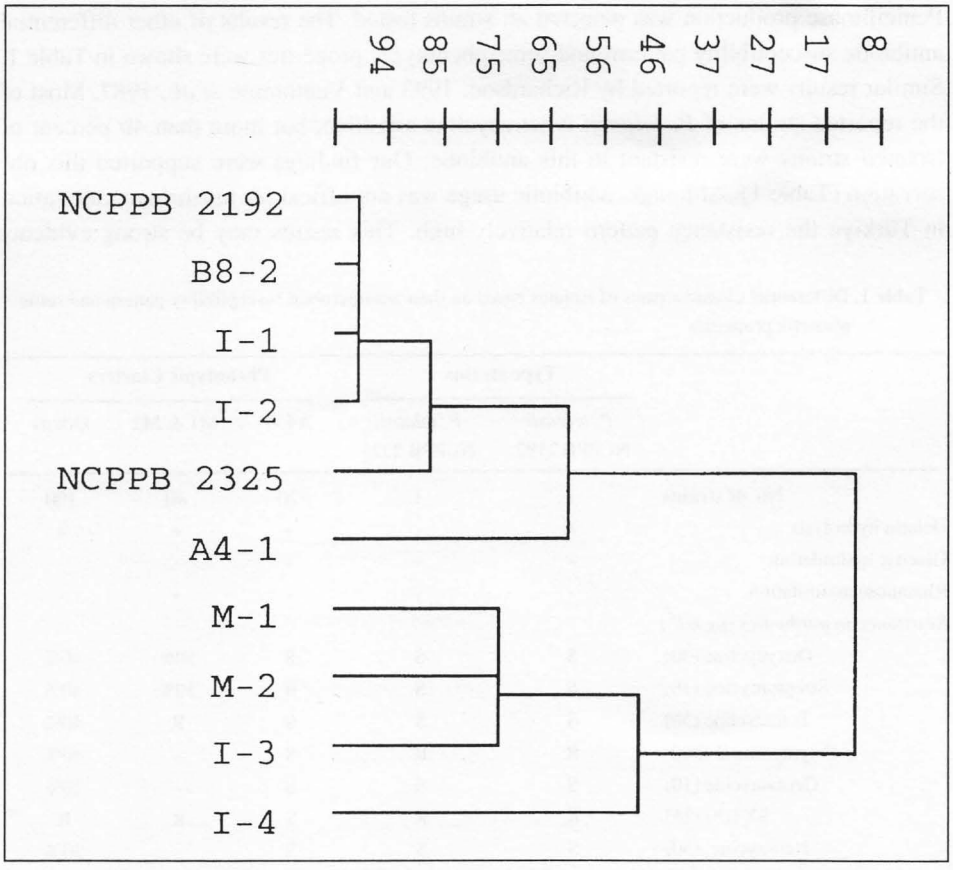

Figure 1. Phenogram showing the positions of *P. tolaasii* strains based on the antibiotic susceptibility tests. The scale shows the similarities in %.

Our study demonstrated clearly that antibiotic susceptibility patterns may be useful in distinguishing between similar strains of pathogenic *P. tolaasii*, occuring on cultivated mushrooms (Fig. 1). This method is more economical and will take a shorter time than classical tets. But, for a high reliable result, should be used with other taxonomical data.

The resistance or susceptibility to inhibitors are also generally stable characters and can serve as diagnostic aids. Furthermore, the patterns of susceptibility to antibiotics can be useful in distinguishing similar species from each other as well as the strains of the same species (Trüper and Schleifer, 1992). Studies done by Tamer and Bursalioglu, (1984); El-Banna, (1989); Şahin and Tamer, (1998) have shown that strains can be clearly differentiated based on the their antibiotic susceptibility pattern.

As a result, in addition to normal determination methods, it is possible to separate some of the varieties of *P. tolaasii* with the help of this technique. This technique may be useful for the detection of negative "white line reacting" strains of *P. tolaasii*.

ÖZET

Pseudomonas tolaasii İZOLATLARININ ANTİMİKROBİYAL DUYARLILIK PROFİLLERİ KULLANILARAK KARAKTERİZASYONU VE NUMERİK TAKSONOMİSİ

Agaricus bisporus sporoforlarından izole edilen (n= 160) *Pseudomonas tolaasii* suşlarının 20 antimikrobiyal maddeye karşı duyarlılıkları disk-diffüzyon metodu ile belirlendi. *P. tolaasii* NCPPB 2192^T ve NCPPB 2325 referans suşlar olarak kullanıldı. Tüm *P. tolaasii* izolatları tobramycin ve kanamycine duyarlıdır. İzolatların antibiyotik duyarlılık profilleri iki farklı fenotipik grubu gösterdi. Tetrasiklin duyarlılığı ve glukoz kullanımı fenotipik gruplar arasında en tanımlayıcı karakterler olarak belirlendi. Sonuçlar gösterdi ki, normal identifikasyon metodlarına ek olarak antimikrobiyal duyarlılık testleri yardımıyla *P. tolaasii*'nin bazı varyeteleri ayırd edilebilir.

Anahtar kelimeler: *Pseudomonas tolaasii*, antibiyotikler, numerik taksonomi

ACKNOWLEDGEMENTS

We are grateful to Dr. N. Sante Iacobellis (Universita degli studi della Basilicata, Italy) for helpful discussion and providing some *Pseudomonas tolaasii* strains used in this study. This work was partly supported by grants TBAG/AY-44 from TÜBITAK and AF077 from the Celal Bayar University.

LITERATURE CITED

BAUER, A. W., KIRBY, W. M. M., SHERRIS, J. C., TURCK M., 1966. Antibiotic susceptibility testing by a standardized single disk method. **Amer. J. Clinic. Pathol. 45**: 493-497.

CHUN, J., 1995. Computer assisted classification and identification of actinomycetes. Ph.D. thesis. University of Newcastle upon Tyne, Newcastle, UK.

EL-BANNA, T., 1989. Characterization of some unclassified Pseudomonas species. Ph. D. thesis. Universitat Hannover, Germany and University of Tanta, Egypt.

LELLIOT, R. A., STEAD, D. E., 1987. Methods for the diagnosis of bacterial diseases of plants. Methods in Plant Pathology, Vol 2. T. F. Precee ed. Blackwell Sci. Pub., Oxford, UK, 216 pp.

NCCLS-National Committee for Clinical Laboratory Standards, 1990. Performance standards for antimicrobial disc susceptibility tests. 4th Ed., M2-A4, Vol. 10, No: 7. Villanova, Pa.

PAINE, S.G., 1919. Studies in bacteriosis II. A brown blotch disease of cultivated mushrooms. **Annals of Appl. Biology 5**: 206-219.

PALLERONI, N. J., 1984. Pseudomonadaceae. In: Bergey's Manual of Systematic Bacteriology, Vol. 1 (N. R. Krieg, J. G. Holt, eds.). Baltimore, Lippincott Williams & Wilkins.

RAINEY, P.B., BRODEY, C.L., JOHNSTONE, K., 1992. Biology of *Pseudomonas tolaasii*, cause of brown blotch disease of the cultivated mushroom. **Advances in Plant Pathology 8**: 95-117.

RICHARDSON, P.N., 1993. Stipe necrosis of cultivated mushroom (*A. bisporus*) associated with a fluorescent Pseudomonad. Plant pathology 42, 27-929.

SNEATH, P. H. A., SOKAL, R. R., 1973. Numerical taxonomy. The principles and practice of numerical calssification. San Fransisco, W. H. Freeman Co.

SOLER-RIVAS, C., JOLIVET, S., ARPIN, N., OLIVIER, J.M., WICHERS, H.J., 1999. Biochemical and physiological aspects of brown blotch disease of *Agaricus bisporus*. **FEMS Microbiol Rev. 23, 5**: 591-614.

ŞAHIN, N., TAMER, A. Ü., 1998. Antimicrobial susceptibility patterns of recently isolated oxalate utilizing bacteria. **Commun. Fac. sci. Univ. Ankara Ser. C, 16**: 1-7.

TAMER, A.Ü., BURSALIOGLU, M., 1984. Bazı *Bacillus* Chon türlerine değişik kemoterapotiklerin etkisi. **Mikrobiyoloji Bülteni, 18**: 119-122.

TOLAAS, A.G., 1915. A bacterial diseases of cultivated mushrooms. **Phytopathology 5**: 51-54.

TRUPER, H.G., SCHLEIFER, K.H., 1992. Prokaryote characterization and identification. In: Balows, A., Trüper, H.G., Dworkin, M., Herder, W., Schleifer, K-H. (Eds). The Prokaryotes, 2nd ed: A handbook on the biology of bacteria; ecophysiology, isolation, identification, applications. Springer-Verlag, New York.

VANTOMME, R., OVERSTIJNS, A., GOOR, M., KERSTERS, K., DE LEY, J., 1987. Routine diagnosis and sensitivity to chemical compounds of phytopathogenic and saprophytic Pseudomonads from cultivated mushrooms. *Proc. of the 13th Int. Congress on the Sci. and Cultivation of Edible Fungi*, 701-710.

WELLS, J.M., SAPERS, G.M., FETT, W.F., BUNERFIELD, J.D., JONES, J.B., BOUZAR, H., MILLER, F.C., 1995. Postharvest discoloration of the cultivated mushroom *Agaricus bisporus* caused by *Pseudomonas tolaasii*, *P. "reactans"* and *P. "gingeri"*. **Phytopathology 86**: 1098-1104.

WONG, W. C., PREECE, T. F., 1979. Identification of *Pseudomonas tolaasii:* The white line in agar and mushroom tissue block rapid pitting tets. **J. of Appl. Bacteriol, 47**: 401-407.