Phenotypic and Genotypic Diversity of *Pseudomonas tolaasii* and White Line Reacting Organisms Isolated from Cultivated Mushrooms

By M. GOOR,¹* R. VANTOMME,² J. SWINGS,¹ M. GILLIS,¹
K. KERSTERS¹ AND J. DE LEY¹

Laboratorium voor Microbiologie en Microbiële Genetica, Rijksuniversiteit Gent¹
and Onderzoekscentrum voor Fytobacteriosen, Sektie II, IWONL², Ledeganckstraat 35,
B-9000 Gent, Belgium

(Received 11 September 1985; revised 28 February 1986)

We compared 21 bacterial strains isolated in Belgium from cultivated *Agaricus bisporus*, *Pleurotus ostreatus* and *Psalliota edulis* showing typical brown blotch symptoms, with 12 culture collection strains of *Pseudomonas tolaasii*, nine *P. agarici* strains causing drippy gill, four '*P. gingeri*' strains causing ginger blotch, three *Pseudomonas* strains responsible for mummy disease, three saprophytic *P. fluorescens*, four *P. aeruginosa* and three '*P. reactans*' strains. All strains were characterized by 147 auxanographic API tests (API 50CH, API 50AO, API 50AA) and by 128 (for 14 strains, 55) additional biochemical, serological and phytopathological features. The results were analysed by numerical methods. Taking into account also results obtained by gel electrophoresis of soluble proteins and by DNA:DNA hybridizations, we were able to differentiate seven groups, corresponding respectively to *P. aeruginosa* (phenon I), *P. fluorescens* biovar II (phenon II), '*P. gingeri*' (phenon III), *P. tolaasii*, including nine of our own isolates (phenon IV), the so-called white line reacting organisms, containing 11 of our own isolates (phenon V), two mummy disease isolates (phenon VI) and *P. agarici* (phenon VII). *P. tolaasii* formed a homogeneous group, containing both virulent and avirulent strains. The saprophytic white line reacting organisms of phenon V were, despite their phenotypic similarity, heterogeneous genotypically and in their gel electrophoresis patterns. A determinative scheme for the differentiation of *P. tolaasii*, the white line reacting organisms and the other *Pseudomonas* species occurring on mushrooms is proposed.

INTRODUCTION

Brown blotch disease (Tolaas, 1915), the most serious and commonly spread disease on cultivated mushrooms, is caused by *Pseudomonas tolaasii* (Paine, 1919). The appearance of sunken chocolate-brown lesions on the caps and stipes of affected mushrooms is responsible for considerable quality losses. As much as 7–10% of the total Belgian mushroom production may be lost due to this disease. Three other members of the genus *Pseudomonas* induce diseases on cultivated mushrooms: *P. agarici*, causing drippy gill (Young, 1970); '*P. gingeri*', causing ginger blotch (Wong *et al.*, 1982) and an unidentified *Pseudomonas* species responsible for mummy disease (Schisler *et al.*, 1968). Moreover, the occurrence of many saprophytic pseudomonads has been reported frequently on mushrooms and in their environment (Olivier *et al.*, 1978; Zarkower *et al.*, 1984). Some of these saprophytic *Pseudomonas* were capable of inducing a 'white line reaction' with *P. tolaasii* and were designated as 'reacting organisms' (Wong & Preece, 1979) or 'inducers' (I strains) (Zarkower *et al.*, 1984). Few authors have discussed the taxonomic position of *Pseudomonas* strains associated with mushrooms. From the existing literature (Young, 1970; Fahy, 1981), the differentiation of *P. tolaasii* and *P. agarici* was possible, whereas differentiation between the other pseudomonads associated with mushrooms posed more problems. According to Fahy (1981) *P. tolaasii* comprised both saprophytic and pathogenic isolates whereas other

authors (Wong & Preece, 1979; Zarkower *et al.*, 1984) stated that the white line reacting organisms were clearly distinct from *P. tolaasii*. Zarkower *et al.* (1984) advocated the use of the white line test as a diagnostic tool for the identification of *P. tolaasii*.

In this study we concentrated on *P. tolaasii* and on the white line reacting organisms. Our aims were as follows: (i) to characterize 46 culture collection strains of *Pseudomonas* isolated mostly from cultivated mushrooms, using 275 auxanographic and other phenotypic tests; (ii) to identify 21 strains isolated in Belgium from cultivated mushrooms with brown blotch symptoms by comparing their phenotypic features with those of the culture collection strains; (iii) to determine the diversity within *P. tolaasii* and the white line reacting organisms by numerical analysis of phenotypic features and by comparing their soluble protein gel electrophoretic patterns; (iv) to determine the genotypic relationships of *P. tolaasii* and the white line reacting organisms by DNA:DNA hybridizations; and (v) to select diagnostic features for a simple and accurate identification of *P. tolaasii* and the other *Pseudomonas* species associated with cultivated mushrooms.

METHODS

Bacterial strains, media and inocula. Samples from the mushroom species *Agaricus bisporus*, *Psalliota edulis* and *Pleurotus ostreatus* showing typical brown blotch lesions were collected in different farms in Belgium. The blotched outer tissue layers were suspended in sterile physiological saline (0.85% NaCl). Two drops of each suspension were plated on *Pseudomonas* agar F (PAF) containing 1% (w/v) tryptone, 1% (w/v) proteose peptone, 1% (w/v) glycerol, 0.15% (w/v) MgSO_4 , 0.15% (w/v) K_2HPO_4 and 2.5% (w/v) agar. Single fluorescent colonies were selected and streaked to obtain pure cultures. Different colony forms appearing after subculturing were labelled t1, t2, t3. Twenty-one isolates, together with 46 culture collection strains, were included in this study (Table 1). Type strains are denoted by a superscript T. The legend to Table 1 explains the use of single and double inverted commas for certain names. Inoculation of all liquid media was performed with two drops of a bacterial suspension containing about 10^9 cells per ml physiological saline and made from a 24–48 h-old starter culture on PAF slants. Unless otherwise stated, all tests were incubated at 28 °C.

Pathogenicity tests. The method of Olivier *et al.* (1978) was applied on the mushroom variety *Agaricus bisporus* Somycel 53. Two drops of a bacterial suspension containing approximately 10^9 cells per ml sterile distilled water were applied to each mushroom cap. Controls were made with sterile water. Each bacterial culture was tested at least twice. Results were read after 1 and 2 d incubation at 23 °C. Inoculations were also performed on mushroom beds in farming conditions.

White line test. This was done on all strains according to Wong & Preece (1979). In PAF medium a characteristic white precipitation band between opaque white colonies of authentic *P. tolaasii* strains and translucent colonies of the so-called white line reacting organisms was formed. Wong & Preece (1979) recommended this test as a reliable method to monitor *P. tolaasii* strains in mushroom crops. In our study, strain NCPPB 1311 was used to detect *P. tolaasii*, whereas *P. tolaasii* NCPPB 2192^T was also used to select the white line reacting organisms. The formation of a white line was followed during 6 d at 23 °C.

Serology. The antiserum (241/1) used for slide agglutinations was prepared at the Unité de Phytopathologie, Centre d'Études des Phytobactérioses (Section I), Université Catholique de Louvain-la-Neuve, Belgium, against the type strain of *P. tolaasii* NCPPB 2192^T. A bacterial suspension (0.5 ml) containing approximately 10^9 cells ml^{-1} was treated with formalin according to Laroche & Verhoyen (1981) and injected intravenously into a rabbit twice in 4 d. Blood was collected 4 d after the last injection. The titre of the antiserum reached 1/320 using the agglutination test against whole cells.

API systems. In the auxanographic API systems (API System, France) growth tests were done on 49 sugars and derivatives (50CH), 49 organic acids (50AO) and 49 amino acids and amines (50AA) (see Tables 2 and 3). Strains were grown on nutrient agar slants containing 0.01 M-phosphate buffer (pH 6.8) for 48 h at 28 °C. The basal medium for the carbon assimilation tests was prepared by the addition of 5 ml of a filter-sterilized solution of 8.04% (w/v) Yeast Nitrogen Base (Difco) and 0.6% (w/v) Yeast Extract Powder (Oxoid) to 55 ml sterilized 0.1 M-phosphate buffer (pH 7.0) containing 0.2 g Oxoid agar no. 1. The medium was mixed and held in a 43 °C water bath. A loopful of bacteria was taken from the slant and suspended in 0.01 M-phosphate buffer (pH 7.0). A 3 ml portion of this cell suspension containing approximately 1.5×10^9 cells ml^{-1} was added to the basal medium. The test strips were inoculated as indicated by the manufacturer. The final concentration of the substrates was 0.2% carbohydrates or organic acids, and 0.1% amino acids or amines. Growth was observed after 1, 2, 4 and 7 d. The production of a diffusible fluorescent pigment was noted after 4 and 7 d.

Other morphological, biochemical and physiological tests. Gram reaction, cell morphology and motility were

observed on cells not older than 24 h. Colony morphology was observed after 2 d on YPGA (0.5% yeast extract, 0.5% peptone, 1% glucose and 2% agar; pH 7.2). Oxidase activity was tested by the method of Kovács (1956). Catalase activity was determined by mixing a loopful of bacteria in a drop of 10% (v/v) H_2O_2 . Pigment production was observed on the media of King *et al.* (1954). Temperature sensitivity was checked in nutrient broth, incubated in water baths at 33, 37, 39 and 41 °C (± 0.2 °C). Nitrate reduction was tested after 2 d in nutrient broth containing 0.1% KNO_3 . Salt tolerance was tested in nutrient broth containing 3, 4, 5, 6 and 7% (w/v) NaCl. Resistance to 23 antibiotics was tested on YPGA medium according to Bauer *et al.* (1966), using the following discs (Oxoid): 30 µg nalidixic acid, 10 U penicillin G, 10 µg erythromycin, 10 µg ampicillin, 10 µg streptomycin, 10 µg gentamicin, 2 µg lincomycin, 30 µg chloramphenicol, 200 µg nitrofurantoin, 30 µg novobiocin, 10 µg fusidic acid, 10 U bacitracin, 10 µg methicillin, 10 µg colistin sulphate, 30 µg tetracycline, 100 µg sulphafurazole, 25 µg cephaloridine, 30 µg neomycin, 300 U polymyxin B, 30 µg kanamycin, 25 µg mecillinam, 30 µg cephradine, 10 µg tobramycin.

The following additional tests were done on 53 strains (see Table 1). Alkalinization of 14 organic acids was tested in a medium consisting of 0.5% NaCl, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% $\text{NH}_4\text{H}_2\text{PO}_4$, 0.1% K_2HPO_4 , 0.0025% bromothymol blue and 0.2% of the filter-sterilized sodium salt of the following organic acids: DL-tartrate, D-galacturonate, propionate, fumarate, succinate, DL-malate, DL-lactate, D-gluconate, acetate, benzoate, formate, citrate, oxalate, malonate. Glucose utilization was determined according to Hugh & Leifson (1953). Acid production from 22 carbon sources was tested in the same medium without agar. The following carbohydrates and related compounds were added aseptically to a final concentration of 1% (w/v): D-arabinose, raffinose, starch, dextrin, salicin, dulcitol, α -D-melezitose, L-rhamnose, D-lactose, sucrose, D-maltose, D-trehalose, D-fructose, melibiose, inulin, adonitol, *meso*-inositol, D-xylose, D-glucose, D-ribose, D-mannose, D-mannitol. D-Lactose, raffinose, soluble starch, inulin, dextrin, *meso*-inositol, salicin and dulcitol were sterilized separately by autoclaving for 10 min at 120 °C; the other compounds were filter-sterilized. The test was read after 1, 2 and 7 d. Gelatin liquefaction was followed for 3 weeks in nutrient broth tubes containing a charcoal gelatin disc (Oxoid). Hydrolysis of casein was followed for 7 d on nutrient agar with 10% (w/v) skim milk powder. The methods of Møller (1955) were used to determine L-arginine dihydrolase and L-lysine- and L-ornithine hydrochloride decarboxylase activities. Production of levan was followed according to Lelliott *et al.* (1966). β -Galactosidase activity was checked as described by MacFaddin (1976, method 2). Starch hydrolysis was tested after 1 week on nutrient agar plates containing 0.8% soluble starch. Lipase activity was tested according to Sierra (1957), using Tween 60 and Tween 80. Indole, H_2S and acetoin production and the formation of reducing substances from sucrose were determined as described by Dye (1968). DNAase activity was followed on Oxoid DNase agar medium (code CM321). After 7 d incubation, 1 M-HCl was used to precipitate the non-hydrolysed DNA. Acidification, alkalinization, reduction, coagulation and peptonization in litmus milk were followed for 14 d in 10% skim milk powder in distilled water to which 0.06% soluble litmus was added (pH 6.5). The hydrolysis of aesculin was followed during 1 week according to the method of Sneath (1956). Growth factor requirements were determined after 1 week in a medium consisting of 0.01% NaCl, 0.001% FeCl_3 , 0.05% $(\text{NH}_4)_2\text{HPO}_4$, 0.1% glucose with or without 0.3% yeast extract. Sensitivity towards different chemical compounds [0.01% sodium nitrite, 0.01% potassium nitrite, 0.01% and 0.2% hexamethylenetetramine, 0.01% streptomycin sulphate, 0.01% kasugamycin technical (60% pure), 0.01% CGA 78039 WP50, 0.01% CGA 78039 ammonium salt, 0.2% propyl gallate, 0.01% formaldehyde (35% pure), 0.2% 4-hydroxyethylbenzoate sodium salt, 0.2% sodium benzoate and 0.2% quinine sulphate] was investigated on YPGA medium. Growth was followed for 2 weeks.

Numerical analysis. Each feature was considered as a separate unit character. Pigmentation and colony morphology were coded, in two or more exclusive states. Codes from 1 (negative) to 7 (strongly positive after 1 d incubation) corresponding to rate and strength of growth were attributed to the auxanographic features. Other characters were coded as 1 (−), 4 (±) and 7 (+). Missing tests were coded as 0 (zero). The similarity coefficient, derived from the Canberra-metric dissimilarity coefficient, was calculated with an adapted version of the computer program of Bonham-Carter (1967). Strains were clustered using the unweighted average pair-group method (UPGMA) (Sneath & Sokal, 1973) with the Clustan 1C program of Wishart (1978) on the Siemens 7541 (BS 2000) computer of the Centraal Digitaal Rekencentrum of the Rijksuniversiteit Gent.

Polyacrylamide gel electrophoresis of soluble cell proteins. Cells were grown in Roux flasks for 48 h on nutrient agar. The preparation of cell free extracts, polyacrylamide gel electrophoresis and photography of the stained gels were as described by Kersters & De Ley (1975). Each protein extract was run at least in duplicate. For some strains the entire procedure was repeated.

DNA: DNA hybridizations. Cells were grown in Roux flasks on nutrient agar or PAF medium for 48 h and harvested and washed in 0.01 M-phosphate buffer (pH 7.0). High M_r DNA was prepared by the method of Marmur (1961) and stored in 0.1 × SSC at −80 °C (SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0). The degree of genome similarity (expressed as % *D*, degree of duplexing) was determined from the initial renaturation rate (De Ley *et al.*, 1970). Each hybridization experiment was done at least in triplicate.

Table 1. *Strains examined*

Name as received	Strain no.	Isolated from	Place of isolation	Year of isolation	Isolated by	Pathogenicity test*	White line reaction† with:			Agglutination reaction‡	Phenon
							NCPBB 2192†	NCPBB 1311	“P. tolaasii”		
<i>P. aeruginosa</i>	CCEB 4817§	—	—	1966	Merck, Sharp and Dohme	—	—	—	—	—	I
<i>P. aeruginosa</i>	NCPBB 2195	<i>Agaricus bisporus</i>	UK	1968	D. S. Gandy	—	—	—	—	—	I
<i>P. aeruginosa</i>	NCPBB 2196	<i>Agaricus bisporus</i>	UK	1968	D. S. Gandy	—	—	—	—	—	I
<i>P. aeruginosa</i>	NCPBB 2197	<i>Agaricus bisporus</i>	UK	1968	D. S. Gandy	—	—	—	—	—	I
<i>P. agarici</i>	NCPBB 2289†	<i>Agaricus bisporus</i>	New Zealand	1969	J. M. Young	+	—	—	—	—	VII
<i>P. agarici</i>	NCPBB 2290	<i>Agaricus bisporus</i>	New Zealand	1968	J. M. Young	—	—	—	—	—	VII
<i>P. agarici</i>	NCPBB 1996	<i>Agaricus bisporus</i>	UK	1963	L. Baker	—	—	—	—	—	VII
<i>P. agarici</i>	NCPBB 1999†	<i>Agaricus bisporus</i>	UK	1966	L. Baker	—	—	—	—	—	VII
<i>P. agarici</i>	NCPBB 1999†2	<i>Agaricus bisporus</i>	UK	1966	L. Baker	—	—	—	—	—	VII
<i>P. agarici</i>	NCPBB 2471†1	<i>Agaricus bisporus</i>	UK	1972	M. Bateson	—	—	—	—	—	VII
<i>P. agarici</i>	NCPBB 2471†2	<i>Agaricus bisporus</i>	UK	1972	M. Bateson	—	—	—	—	—	VII
<i>P. agarici</i>	NCPBB 2472	<i>Agaricus bisporus</i>	UK	1972	M. Bateson	—	—	—	—	—	VII
“ <i>P. agarici</i> ”	NCPBB 2304	<i>Agaricus bisporus</i>	UK	1970	M. J. Hurst	—	—	—	—	—	—
<i>P. fluorescens</i>	MMCA 40†	Pre-filter tank	UK	1951	M. E. Rhodes	—	—	—	—	—	—
(= ATCC 13525†)											
<i>P. fluorescens</i>	NCPBB 1794†1§	—	—	1966	K. Klinge	—	—	—	—	—	II
<i>P. fluorescens</i>	NCPBB 1794†2§	—	—	1966	K. Klinge	—	—	—	—	—	II
<i>P. fluorescens</i>	NCPBB 1795§	Pleural liquid	—	1966	O. Jessen	—	—	—	—	—	II
“ <i>P. gingeri</i> ”	NCPBB 3146†1§	<i>Agaricus bisporus</i>	UK	1981	T. F. Preece	—	—	—	—	+	III
“ <i>P. gingeri</i> ”	NCPBB 3146†2§	<i>Agaricus bisporus</i>	UK	1981	T. F. Preece	—	—	—	—	+	III
“ <i>P. gingeri</i> ”	NCPBB 3147†1§	<i>Agaricus bisporus</i>	UK	1981	T. F. Preece	—	—	—	—	—	III
“ <i>P. gingeri</i> ”	NCPBB 3147†2§	<i>Agaricus bisporus</i>	UK	1981	T. F. Preece	—	—	—	—	—	III
“ <i>P. reactans</i> ”	NCPBB 3149†1§	<i>Agaricus bisporus</i>	UK	1979	T. F. Preece	—	+	—	—	—	V
“ <i>P. reactans</i> ”	NCPBB 3149†2§	<i>Agaricus bisporus</i>	UK	1979	T. F. Preece	—	+	—	—	—	V
“ <i>P. reactans</i> ”	NCPBB 3149†3§	<i>Agaricus bisporus</i>	UK	1979	T. F. Preece	—	+	—	—	—	V
<i>P. tolaasii</i>	NCPBB 2192†	<i>Agaricus bisporus</i>	UK	1965	D. S. Gandy	+	—	+	+	+	IV
<i>P. tolaasii</i>	NCPBB 741†1	<i>Agaricus bisporus</i>	Netherlands	1951	H. C. Bels-Konig	+	—	+	+	+	IV
<i>P. tolaasii</i>	NCPBB 741†2	<i>Agaricus bisporus</i>	Netherlands	1951	H. C. Bels-Konig	+	—	+	+	+	IV
<i>P. tolaasii</i>	NCPBB 1116	<i>Agaricus bisporus</i>	UK	1961	L. A. E. Baker	+	—	+	+	+	IV
<i>P. tolaasii</i>	NCPBB 2194	<i>Agaricus bisporus</i>	Canada	1969	D. S. Gandy	+	—	+	+	+	IV
<i>P. tolaasii</i>	NCPBB 2325†1	<i>Agaricus bisporus</i>	Italy	1970	G. L. Ercolani	+	—	+	+	+	IV
<i>P. tolaasii</i>	NCPBB 2325†2	<i>Agaricus bisporus</i>	Italy	1970	G. L. Ercolani	+	—	+	+	+	IV

Binomials which are not in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) are given in single inverted commas (‘ ’); names of misidentified strains are given in double inverted commas (‘ ’). †, Type strain; †1, †2, †3, different colony types from the same mother culture. Culture collections: ATCC, American Type Culture Collection, Rockville, Md., USA; CCEB, Culture Collection of Entomogenous Bacteria, Prague, Czechoslovakia; LMG, Culture Collection Laboratorium Microbiologie, Gent, Belgium; MMCA, Medical Microbiology Culture Collection Aarhus, University of Aarhus, Denmark; NCPBB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK.

White line reaction† with:

<i>P. tolaasii</i>	NCPPB 2412	<i>Agaricus bisporus</i>	New Zealand	1968	D. R. W. Watson	+	—	—	+	+	IV
" <i>P. tolaasii</i> "	NCPPB 1311	<i>Agaricus bisporus</i>	UK	1962	G. E. Jones	—	+	—	—	—	V
" <i>P. tolaasii</i> "	NCPPB 2193	<i>Agaricus bisporus</i>	UK	1965	D. S. Gandy	—	—	—	—	—	—
" <i>P. tolaasii</i> "	NCPPB 2413†	<i>Agaricus bisporus</i>	New Zealand	1968	D. R. W. Watson	—	—	—	—	—	—
" <i>P. tolaasii</i> "	NCPPB 2413‡	<i>Agaricus bisporus</i>	New Zealand	1968	D. R. W. Watson	—	—	—	—	—	—
" <i>P. washingtoniae</i> "	NCPPB 967§	—	—	1944	—	—	+	—	—	—	V
<i>Pseudomonas</i> sp. (‘mummy disease’)	NCPPB 2208	<i>Agaricus bisporus</i>	UK	1976	P. Roberts	—	—	—	—	+	—
<i>Pseudomonas</i> sp. (‘mummy disease’)	NCPPB 2874	<i>Agaricus bisporus</i>	USA	1959	—	—	—	—	—	—	VI
<i>Pseudomonas</i> sp. (‘mummy disease’)	NCPPB 2875	<i>Agaricus bisporus</i>	UK	1976	P. Roberts	—	—	—	—	—	VI
<i>Pseudomonas</i> sp.	LMG 6634	<i>Agaricus bisporus</i>	Belgium	1981	R. Vantomme	+	—	—	—	+	IV
<i>Pseudomonas</i> sp.	LMG 6635	<i>Agaricus bisporus</i>	Belgium	1981	R. Vantomme	+	—	—	—	—	IV
<i>Pseudomonas</i> sp.	LMG 6636	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	+	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6637¶	Casing layer	Belgium	1982	R. Vantomme	+	—	—	+	—	IV
<i>Pseudomonas</i> sp.	LMG 6637‡	Casing layer	Belgium	1982	R. Vantomme	—	—	—	—	—	IV
<i>Pseudomonas</i> sp.	LMG 6638¶	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	+	—	—	+	—	IV
<i>Pseudomonas</i> sp.	LMG 6638‡	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	IV
<i>Pseudomonas</i> sp.	LMG 6639¶	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	IV
<i>Pseudomonas</i> sp.	LMG 6639‡	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	IV
<i>Pseudomonas</i> sp.	LMG 6640¶	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	+	—	—	+	—	IV
<i>Pseudomonas</i> sp.	LMG 6640‡	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	IV
<i>Pseudomonas</i> sp.	LMG 6641¶	<i>Psalliota edulis</i>	Belgium	1982	R. Vantomme	+	—	—	+	—	IV
<i>Pseudomonas</i> sp.	LMG 6641‡	<i>Psalliota edulis</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	IV
<i>Pseudomonas</i> sp.	LMG 6642	<i>Agaricus bisporus</i>	Belgium	1981	R. Vantomme	+	—	—	+	—	IV
<i>Pseudomonas</i> sp.	LMG 6643	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6644	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	+	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6645	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	+	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6646	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6647	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	+	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6648	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	+	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6649	<i>Psalliota edulis</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6650	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	+	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6651	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	—	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6652	<i>Psalliota edulis</i>	Belgium	1982	R. Vantomme	—	+	—	—	—	—
<i>Pseudomonas</i> sp.	LMG 6653	<i>Agaricus bisporus</i>	Belgium	1982	R. Vantomme	—	+	—	—	—	V
<i>Pseudomonas</i> sp.	LMG 6654¶§	<i>Pleurotus ostreatus</i>	Belgium	1983	R. Vantomme	—	—	—	—	—	IV
<i>Pseudomonas</i> sp.	LMG 6654‡§	<i>Pleurotus ostreatus</i>	Belgium	1983	R. Vantomme	+	—	—	+	+	IV

* Phytopathogenicity on *Agaricus bisporus* according to the method of Olivier *et al.* (1978).

† White line reaction by the method of Wong & Preece (1979).

‡ Agglutination reaction with antiserum prepared against *P. tolaasii*, NCPPB 2192†.

§ These 14 strains were characterized phenotypically by the 147 auxanographic API tests and by 55 conventional tests, and not by the full range of 275 tests (see text).

|| Strains inducing superficial yellow lesions (ginger blotch) but never typical brown blotch symptoms.

¶ ‘*P. reactans*’ is the name for the white line reacting organisms, used by Wong & Preece (1979). This name has not been validly published and is not included in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) or in Validation Lists subsequently published in the *International Journal of Systematic Bacteriology*. These ‘*P. reactans*’ strains are catalogued as *P. fluorescens* in the NCPPB.

RESULTS

Phenotypic characters and numerical analysis

After numerical analysis of the 275 phenotypic features by the unweighted average pair-group method, seven phenons could be delineated (Fig. 1). Phenon I comprised the four strains of *P. aeruginosa* and seemed to be quite homogeneous, with a mean similarity of 93%. Phenons II and III consisted respectively of three strains of *P. fluorescens* biovar II and all four strains of '*P. gingeri*'. The main group was formed by phenon IV, comprising eight named *P. tolaasii* strains and 15 organisms isolated from Belgian mushroom farms. All the culture collection strains of *P. tolaasii*, including the type strain NCPPB 2192^T, were isolated from *Agaricus bisporus*, whereas the isolates from Belgium came from a broader spectrum, namely *Agaricus bisporus* (10 strains), *Psalliota edulis* (2 strains), *Pleurotus ostreatus* (2 strains) and casing layer (1 strain). Regardless of their different origin all these strains formed a very tight phenotypic group (mean similarity 89% S). Phenon V consisted of eleven organisms isolated from cultivated mushrooms in Belgium, one misnamed "*P. tolaasii*" strain (NCPPB 1311) (Wong & Preece, 1979), '*P. washingtoniae*' NCPPB 967, together with three different colony types of the reference strain of '*P. reactans*' NCPPB 3149. The latter name, however, has never been validly published, and is a *nomen nudum*. Phenon VI comprised two of the three mummy disease organisms, NCPPB 2874 and NCPPB 2875. Eight of the nine *P. agarici* strains clustered together in phenon VII. Some strains did not belong in any of the seven phenons: one of our own isolates from *Psalliota edulis* (LMG 6652), the type strain of *P. fluorescens* MMCA 40^T, three misnamed "*P. tolaasii*" strains NCPPB 2193 and NCPPB 2413t1 and t2, the mummy disease organism NCPPB 2208 and one misnamed "*P. agarici*" strain NCPPB 2304. The two types t1 and t2 of NCPPB 2413 differed only in their colony morphology. An extensive description of the different phenotypic characteristics for each phenon is given in Tables 2 and 3. These tables show that each phenon could be distinguished by several phenotypic tests. The two most similar phenons, i.e. phenon IV and phenon V, differed in four features: growth on L-arabinose (–, +), rhamnose (–, +), 5-ketogluconate (+, –) and acid production from rhamnose (–, +).

Pathogenicity, white line reaction and serology

The results of the pathogenic reaction on *Agaricus bisporus* Somycel 53, the white line reactions with *P. tolaasii* NCPPB 2192^T and NCPPB 1311, and the agglutination with the antiserum prepared against the type strain of *P. tolaasii* NCPPB 2192^T are summarized in Table 1. Eight *P. tolaasii* culture collection strains (belonging to phenon IV), nine of our own isolates from phenon IV and strain *P. aeruginosa* NCPPB 2197 (phenon I), induced brown blotch symptoms on *Agaricus bisporus*. The same strains, with the exception of NCPPB 2197 and isolates LMG 6634 and LMG 6635, also gave a white line precipitation band with the white line reacting organism NCPPB 1311 (phenon V). The antiserum prepared against *P. tolaasii* NCPPB 2192^T agglutinated all strains of phenon IV with the exception of LMG 6639t1. Cross reactions occurred with '*P. gingeri*' NCPPB 3146t1, NCPPB 2208 (a mummy disease organism) and LMG 6652 (from *Psalliota edulis*). All strains of phenon V, together with the ungrouped strain LMG 6652, formed a white line precipitation band with the type strain of *P. tolaasii* NCPPB 2192^T. The former strains were further characterized by a negative pathogenic and serological reaction. All strains not mentioned above were negative in all three tests.

Comparison of the gel electrophoretic protein patterns

Normalized photographs of the electrophoregrams of representative members of each phenotypic group are shown in Fig. 2. Phenons I, III, IV, VI and VII seemed to be very homogeneous with respect to their gel electrophoretic protein patterns. However, phenon II and particularly phenon V were extremely heterogeneous. Within phenon V, seven strains (LMG 6636, LMG 6643, LMG 6645, LMG 6647, LMG 6648, LMG 6651 and LMG 6653) could be differentiated from the others by the presence of a heavy protein band in their gel electrophoregrams. All remaining strains of this phenon showed a low degree of similarity in their gel electrophoretic patterns. The strains occupying a separate position in the phenotypic

Fig. 1. Sorted and shaded matrix of similarity coefficients (1 - Canberra-metric dissimilarity coefficient, expressed as %) of 67 *Pseudomonas* strains mainly isolated from cultivated mushrooms, based on 275 phenotypic features (14 of the strains were characterized by only 202 of the 275 tests - see Methods and Table 1). Type strains according to the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) are marked with an asterisk. The symbol ● indicates that the strain gave brown blotch symptoms on *Agaricus bisporus* Somycel 53 after artificial inoculation according to the method of Olivier *et al.* (1978).

Table 2 (continued)

	<i>P. aeruginosa</i>	LMG 6652	<i>P. fluorescens</i> biovar II	' <i>P. gingeri</i> '	<i>P. fluorescens</i> biovar I	NCPPB 2193	<i>P. tolaasii</i>	NCPPB 2413t1/t2	White line reacting organisms	NCPPB 2208	'Mummy disease' isolates	NCPPB 2304	<i>P. agarici</i>
Growth in API 50AA on:													
Glycine	100	0	0	50	0	0	17	0	25	0	0	0	0
L-Norleucine	0	0	0	0	100	0	4	0	50	0	100	0	0
DL-Norvaline	25	0	0	0	0	0	22	0	19	0	0	0	0
DL-2-Aminobutyrate	0	0	33	25	0	0	48	50	50	0	0	0	0
L-Methionine	0	0	33	0	0	0	48	0	69	0	0	0	0
L-Tryptophan	100	0	33	0	100	100	100	100	81	0	0	0	0
Trigonelline	0	100	100	0	0	0	0	50	0	100	0	0	0
L-Ornithine	100	100	100	100	100	100	100	100	100	0	50	0	75
L-Lysine	100	100	100	100	100	100	87	100	100	0	100	100	13
L-Citrulline	0	0	67	100	0	100	83	100	100	0	0	0	0
DL-Kynurenine	50	0	0	0	0	0	70	50	56	0	0	0	0
DL-3-Aminobutyrate	50	0	67	75	0	0	22	50	6	0	0	0	0
DL-5-Aminovalerate	100	100	100	100	100	100	96	100	81	100	0	100	25
2-Aminobenzoate	100	0	0	0	0	100	87	100	69	0	0	0	0
Acetamide	100	0	0	0	0	0	0	0	0	0	0	0	0
Sarcosine	100	100	100	100	100	100	100	100	100	100	100	100	63
Butylamine	0	100	0	0	0	0	0	0	0	0	0	0	0
Amylamine	0	100	33	0	0	0	0	0	19	0	0	0	0
Ethanolamine	100	100	100	100	100	100	30	100	94	100	0	0	38
Histamine	100	100	0	100	0	100	0	100	0	0	0	0	0
Glucosamine	0	0	100	100	100	100	100	100	100	100	100	100	63
Colonies on YPGA medium:													
White	0	100	0	0	100	100	77	0	44	0	100	0	63
Cream	25	0	100	100	0	0	23	100	56	0	0	0	38
Yellow-brown	75	0	0	0	0	0	0	0	0	0	0	0	0
Yellow	0	0	0	0	0	0	0	0	0	100	0	100	0
Smooth	0	0	100	100	100	100	91	100	94	100	100	100	100
Irregular	100	100	67	100	0	100	59	0	56	100	100	0	25
Raised to convex	100	100	33	100	NT	100	95	100	81	100	100	100	100
Diameter < 3 mm	50	0	100	100	100	100	100	100	100	0	100	100	100
Cells:													
Elongated	0	0	0	0	0	0	0	0	13	100	0	0	0
Filamentous	0	0	0	0	100	0	4	0	6	100	0	0	0
Diameter ≤ 2 µm	100	100	0	75	0	100	86	100	50	0	50	100	88
Sensitivity to:													
Chloramphenicol (30 µg)	0	0	0	0	0	0	0	0	19	0	50	0	0
Novobiocin (30 µg)	0	0	0	0	0	0	22	0	6	0	0	0	0
Fusidic acid (10 µg)	0	0	0	0	100	0	9	0	6	0	0	0	0
Gentamicin (10 µg)	0	0	0	50	0	0	0	0	0	0	0	0	88
Tobramycin (10 µg)	0	0	0	75	0	0	0	0	0	0	0	0	100
Neomycin (10 µg)	0	0	0	100	0	0	9	0	0	0	0	100	88
Streptomycin (10 µg)	0	0	67	50	0	0	9	0	13	0	0	100	100
Nalidixic acid (30 µg)	0	100	0	0	100	100	83	100	75	0	0	0	0
Kanamycin (30 µg)	0	100	100	100	100	100	74	50	81	100	100	100	100
Tetracycline (30 µg)	50	100	100	100	100	100	100	100	100	100	50	100	100
Fluorescence on:													
King A medium	100	0	0	0	0	0	0	0	6	0	0	0	0
King B medium	100	100	33	25	100	100	100	100	100	0	0	0	100

Table 2 (continued)

	<i>P. aeruginosa</i>	LMG 6652	<i>P. fluorescens</i> biovar II	<i>P. gingeri</i>	<i>P. fluorescens</i> biovar I	NCPPB 2193	<i>P. tolaasii</i>	NCPPB 2413t1/t2	White line reacting organisms	NCPPB 2208	'Mummy disease' isolates	NCPPB 2304	<i>P. agarici</i>
Reduction of nitrate	100	100	100	0	0	0	0	0	0	0	0	0	0
Growth in the presence of NaCl:													
3%	100	100	100	100	100	100	100	100	81	100	100	100	0
4%	100	100	67	0	100	100	100	100	69	100	100	100	0
5%	100	100	0	0	0	100	4	0	0	0	100	0	0
6%	100	100	0	0	0	0	0	0	0	0	0	0	0
7%	100	0	0	0	0	0	0	0	0	0	0	0	0
Growth at:													
33°C	100	100	100	100	100	100	100	100	94	100	100	0	100
37°C	100	100	0	0	0	0	0	0	0	100	0	0	0
39°C	100	0	0	0	0	0	0	0	0	0	0	0	0
41°C	100	0	0	0	0	0	0	0	0	0	0	0	0

The following phenotypic features were present in all 67 strains: rounded, motile Gram-negative rods; moderate to extensive growth on YPGA; oxidase and catalase activity; growth in the API system on glycerol, D-ribose, D-glucose, D-mannitol, D-arabitol, D-gluconate, propionate, heptanoate, caprylate, pelargonate, caprate, malonate, succinate, fumarate, glutarate, DL-lactate, DL-glycerate, DL-3-hydroxybutyrate, L-malate, pyruvate, 2-oxoglutarate, citrate, D- α -alanine, L- α -alanine, L-leucine, L-isoleucine, L-valine, L-serine, L-threonine, L-phenylalanine, L-tyrosine, L-histidine, L-aspartate, L-glutamate, L-arginine, L-proline, betaine, β -alanine, DL-4-aminobutyrate, diaminobutane, spermine.

The following phenotypic features were absent in all 67 strains: sensitivity to 10 U penicillin G, 10 μ g erythromycin, 10 μ g ampicillin, 2 μ g lincomycin, 200 μ g nitrofurantoin, 10 U bacitracin, 10 μ g methicillin, 10 μ g colistin sulphate, 25 μ g cephaloridine, 300 U polymyxin B, 100 μ g sulphafurazole, 25 μ g mecillinam, 30 μ g cephradine; growth in the API system on D-arabinose, L-xylose, methyl β -D-xyloside, L-sorbose, dulcitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, aesculin, salicin, D-cellobiose, maltose, lactose, D-melibiose, inulin, D-melezitose, D-raffinose, starch, glycogen, β -gentiobiose, D-turanose, D-tagatose, D-fucose, L-fucose, oxalate, pimelate, suberate, glycolate, 2-hydroxybenzoate, 3-hydroxybenzoate, D-mandelate, L-mandelate, isophthalate, L-cysteine, D-tryptophan, creatine, 3-aminobenzoate, 4-aminobenzoate, urea, ethylamine, tryptamine, benzylamine.

analysis (Fig. 1), i.e. LMG 6652, MMCA 40^T, NCPPB 2193, NCPPB 2413t1 and t2, NCPPB 2208 and NCPPB 2304, also showed aberrant protein gel electrophoregrams.

DNA : DNA hybridizations

DNA : DNA hybridization experiments revealed that within phenon IV all strains tested (NCPPB 2192^T, NCPPB 741t1, LMG 6641t1 and LMG 6641t2) were closely related (DNA binding 98–100% *D*). A clear genotypic diversity existed among strains from phenon V (DNA binding 41–90% *D*). Strains LMG 6643 and LMG 6647, both belonging in phenon V and displaying identical protein electrophoregrams, were also genotypically highly related (90% *D*). DNA of the strains of phenon V that had different gel electrophoretic patterns, i.e. NCPPB 1311, NCPPB 967, NCPPB 3149t1 and LMG 6646, yielded DNA binding values of respectively 52%, 68%, 70% and 81% *D* when hybridized with DNA of strain LMG 6643. DNA of '*P. reactans*' NCPPB 3149t1 hybridized with DNA of strains NCPPB 1311, NCPPB 967 and LMG 6646 yielded values of 58%, 62% and 63% *D*, respectively. Within phenon V the lowest DNA binding value was found between strains NCPPB 1311 and LMG 6646 (41% *D*).

Fig. 2. Normalized photographs of the protein gel electrophoregrams of representative members of the different gel electrophoretic groups. Lanes 1 and 2, *P. aeruginosa* CCEB 481^T and NCPPB 2197; lanes 3 and 4, *P. fluorescens* biovar I MMCA 40^T and *P. fluorescens* biovar II NCPPB 1794t1; lanes 5 and 6, '*P. gingeri*' NCPPB 3146t1 and t2; lane 7, a misnamed "*P. tolaasii*" NCPPB 2193; lanes 8–13, *P. tolaasii* LMG 6641t1, LMG 6641t2, NCPPB 2192^T, LMG 6654t1, NCPPB 2412 and LMG 6634; lane 14, a misnamed "*P. tolaasii*" NCPPB 2413t1; lanes 15–21, the white line reacting organisms '*P. reactans*' NCPPB 3149t1, a misnamed "*P. tolaasii*" NCPPB 1311, '*P. washingtoniae*' NCPPB 967, and our own isolates LMG 6649, LMG 6646, LMG 6643 and LMG 6647; lanes 22–24, 'mummy disease' isolates NCPPB 2874, NCPPB 2875 and NCPPB 2208; lane 25, a misnamed "*P. agarici*" NCPPB 2304; lanes 26–28, *P. agarici* NCPPB 2289^T, NCPPB 2471t1 and t2.

Hybridization between strains of phenons IV and V gave values ranging from 31% *D* (between strains NCPPB 2192^T and LMG 6643) to 44% *D* (between strains NCPPB 2192^T and LMG 6646). DNA binding values between strains from phenon IV or phenon V and representative strains of the other phenons did not exceed 50% *D*. This was also the case for the two misnamed "*P. tolaasii*" strains NCPPB 2193 and NCPPB 2413t1, which showed a DNA binding value of respectively 46% and 32% *D* with *P. tolaasii* NCPPB 2192^T.

DISCUSSION

Pseudomonas tolaasii

In our opinion *P. tolaasii* should be restricted to phenon IV, comprising 23 closely related strains including the type strain *P. tolaasii* NCPPB 2192^T. The high relatedness of the strains was shown by phenotypic analysis, gel electrophoretic comparisons and DNA:DNA hybridizations. From this viewpoint *P. tolaasii* corresponded to group A of Wong & Preece (1979) and to the blotching group of Zarkower *et al.* (1984), but was not congruent with the *P. tolaasii* group defined by Fahy (1981), which included both blotching and non-pathogenic strains. The latter comprised strains clustering in our phenon V. A complete description of the phenotypic features of *P. tolaasii* is given in Tables 2 and 3.

In *Bergey's Manual of Systematic Bacteriology*, Palleroni (1984) referred for the definition of *P. tolaasii* to the original description by Paine (1919), comprising 22 features which – unfortunately – were also common to many other *Pseudomonas* species. All strains of our phenon IV showed these features, except for the alkalization of litmus milk. From the features given in the original description by Paine (1919) the pathogenicity on mushrooms seemed to be the only reliable one to identify *P. tolaasii*. Of the 23 *P. tolaasii* strains (phenon IV), six were avirulent on

Table 3. *Phenotypic features of 53 Pseudomonas strains, mostly from cultivated mushrooms*

The percentage frequency of present features in phenons and unclustered strains is given. Results for the tests common to all 53 strains are given in the footnote. NT, Not tested.

Feature	Phenon . . . No. of strains . . .	<i>P. aeruginosa</i> LMG 6652	<i>P. fluorescens</i> biovar I	NCPB 2193	<i>P. tolaasii</i>	NCPB 2413t1/t2	White line reacting organisms	NCPB 2208	'Mummy disease' isolates	NCPB 2304	<i>P. agarici</i>
	I 3	- 1	- 1	- 1	IV 21	- 2	V 12	- 1	VI 2	- 1	VII 8
Oxidative glucose metabolism (O/F)	100	100	100	100	100	100	100	100	100	100	75
H ₂ S production from cysteine	0	0	0	0	0	0	0	0	100	100	100
Arginine dihydrolase	100	100	100	100	100	100	100	100	100	0	0
Levan production from sucrose	0	0	100	0	0	0	0	0	0	0	0
Hydrolysis of:											
Casein	100	100	100	100	76	0	67	0	0	NT	0
Tween 80	100	100	100	100	100	100	92	100	100	100	100
Acid production from:											
Dulcitol	0	100	0	0	0	0	0	0	0	0	0
D-Arabinose	100	0	0	0	0	0	25	100	0	0	0
L-Rhamnose	0	0	0	0	0	0	100	0	0	0	0
D-Lactose	0	0	0	0	0	0	17	0	0	0	0
Sucrose	0	0	100	0	0	0	0	0	0	0	0
D-Trehalose	0	100	0	0	0	0	58	0	0	0	0
D-Fructose	33	0	0	100	29	100	25	100	100	0	63
Melibiose	100	100	0	0	0	0	58	100	0	0	0
Inulin	0	100	0	0	0	0	25	0	0	0	0
Adonitol	0	0	100	0	19	100	75	0	0	0	0
meso-Inositol	0	0	0	0	20	0	50	0	0	0	75
D-Xylose	100	100	100	100	90	100	100	100	100	100	0
D-Glucose	100	100	100	100	100	100	100	100	100	0	88
D-Ribose	100	100	100	100	100	100	100	100	100	0	100
D-Mannose	100	100	100	100	100	100	100	100	100	0	87
D-Mannitol	100	100	100	100	57	100	92	0	100	100	100
Litmus milk:											
Acid production	0	100	0	0	52	0	75	0	0	0	0
Reduction	0	0	0	0	29	50	17	0	0	0	0
Alkalinization	0	0	100	100	19	50	8	100	100	100	100
Coagulation	100	0	0	0	14	0	75	0	0	0	0
Peptonization	100	0	0	0	0	0	0	0	0	0	0
Alkalinization of:											
Sodium acetate	100	100	100	100	100	100	100	0	0	0	25
Sodium benzoate	100	0	0	0	0	0	8	0	0	0	0
Sodium formate	100	0	0	0	0	0	8	0	0	0	0
Sodium citrate	100	100	100	100	100	100	100	100	0	100	100
Sodium tartrate	0	0	0	0	0	0	25	0	100	0	0
D-Galacturonic acid	0	0	0	0	0	0	0	100	100	0	0
Propionic acid	100	100	0	0	57	100	8	0	0	0	0
Sensitivity to:											
Sodium nitrite (0.01%)	0	0	100	0	48	0	58	0	0	0	0
Potassium nitrite (0.01%)	0	0	0	100	57	50	8	0	0	0	0
Hexamethylenetetramine (0.01%)	0	0	0	0	0	0	0	0	0	100	0
Hexamethylenetetramine (0.2%)	100	0	100	100	71	100	25	100	100	100	100

Table 3 (continued)

	<i>P. aeruginosa</i>	LMG 6652	<i>P. fluorescens</i> biovar I	NCPPB 2193	<i>P. tolaasii</i>	NCPPB 2413t1/t2	White line reacting organisms	NCPPB 2208	'Mummy disease' isolates	NCPPB 2304	<i>P. agarici</i>
Streptomycin sulphate (0.01%)	0	0	0	100	33	0	8	100	100	100	100
Kasugamycin 60% (0.01%)	0	0	0	100	76	50	58	100	100	100	100
CGA 78039 WP50 (0.01%)	50	0	100	100	81	50	25	100	100	100	88
CGA 78039 ammonium salt (0.01%)	50	0	0	100	52	0	0	0	100	100	100
Formaldehyde 35% (0.01%)	0	0	0	0	24	0	0	0	0	100	25
Propyl gallate (0.2%)	0	100	100	100	100	100	100	100	100	100	100
4-Hydroxyethylbenzoate, sodium salt (0.2%)	0	100	100	100	100	100	100	100	100	100	100
Sodium benzoate (0.2%)	0	0	0	0	9	50	0	0	0	100	0
Quinine sulphate (0.2%)	0	0	100	100	67	100	92	100	100	100	100

The following phenotypic features were present in all 53 strains: hydrolysis of gelatin and Tween 60; alkalization of fumarate, succinate, malate, lactate and malonate (sodium salts).

The following phenotypic features were absent in all 53 strains: DNAase activity; ornithine- and lysine decarboxylase activity; hydrolysis of starch and aesculin; production of indole; reducing substances from sucrose; acetoin production; requirement for growth factors; β -galactosidase activity; acid production from raffinose, starch, dextrin, salicin, α -D-melezitose, D-maltose; alkalization of gluconate and oxalate (sodium salts).

Table 4. White line reaction, pathogenicity and slide agglutination of *P. tolaasii* strains from phenon IV

No. of strains from phenon IV	White line reaction against NCPPB 1311	Pathogenicity on <i>Agaricus bisporus</i>	Slide agglutination with antiserum against NCPPB 2192 ^T
15	+	+	+
2	—	+	+
5	—	—	+
1 (LMG 6639t1)	—	—	—

Agaricus bisporus. All these avirulent strains were obtained as colony variants upon subculturing of virulent forms on PAF medium. The existence of different colony types, correlating with virulence differences, was also observed by Olivier *et al.* (1978) and by Cutri *et al.* (1984). In our work no gel electrophoretic or major phenotypic differences could be found between the virulent and avirulent forms. Three authors have extended the phenotypic description of *P. tolaasii* (Young, 1970; Fahy, 1981; Zarkower *et al.*, 1984). Our results differed in five features from the 36 described by Young (1970), namely acid production on arabinose, rhamnose and melibiose, lipase activity and growth on 6% NaCl. Five *P. tolaasii* strains examined by Fahy (1981), namely NCPPB 741, NCPPB 1116, NCPPB 2192^T, NCPPB 2194 and NCPPB 2325, were included in our analysis; the agreement between our results and these of Fahy (1981) was over 95%. Our data agreed completely with those obtained by Zarkower *et al.* (1984) on four *P. tolaasii* strains (NCPPB 741, NCPPB 1116, NCPPB 2192^T and NCPPB 2325).

Wong & Preece (1979) advocated the use of the white line test for the identification of *P. tolaasii*. From Table 4 it is clear that all strains giving a white line reaction with NCPPB 1311 also gave a pathogenic reaction on *Agaricus bisporus* and agglutinated with the antiserum against

Table 5. *Phenotypic features useful for the differentiation of the pseudomonads occurring on mushrooms*

+, All strains positive; —, all strains negative.

Feature	No. of strains . . .	<i>P. aeruginosa</i>	<i>P. gingeri</i>	<i>P. tolaasii</i>	White line reacting organisms	'Mummy disease' isolates	<i>P. agarici</i>
	4	4	23	16	3	8	
Growth on:							
Erythritol, sorbitol	—	+	+	+	—	—	—
L-Arabinose	—	—	—	+	+	—	—
L-Rhamnose	—	—	—	+	—	—	—
L-Arabitol	—	—	+	+	—	—	—
2-Ketogluconate	+	—	+	+	+	—	—
<i>n</i> -Valerate, <i>n</i> -caproate	+	+	+	+	+	—	—
D-Tartrate	—	—	—	—	+	—	—
Histamine	+	+	—	—	—	—	—

P. tolaasii NCPPB 2192^T. This confirmed the reliability of the white line reaction for the identification of virulent *P. tolaasii* as stated by Wong & Preece (1979) and Zarkower *et al.* (1984). It should be stressed, however, that a negative white line reaction does not rule out the possibility of pathogenic (2 strains) or non-pathogenic (6 strains) *P. tolaasii* (Table 4). The latter group contained one strain (LMG 6639t1) showing no pathogenicity on *Agaricus bisporus*, no white line reaction and no agglutination with the antiserum. Nevertheless this strain undoubtedly belonged in phenon IV as shown by its phenotypic and gel electrophoretic characteristics (Fig. 1, Fig. 2).

In *Bergey's Manual of Systematic Bacteriology* (Palleroni, 1984) *P. tolaasii* is included in Section V of the genus *Pseudomonas*. This section comprises a number of species whose taxonomic position is not well established. In the case of *P. tolaasii*, however, DNA:rRNA hybridizations (De Vos *et al.*, 1985) have clearly shown that it belongs in Section I of the genus *Pseudomonas* (Palleroni, 1984) and that it is thus a real member of *Pseudomonas sensu* De Vos & De Ley (1983). The taxonomic relationships of *P. tolaasii* with the other members of this section remain unclear. By comparing our phenotypic data with those of Stanier *et al.* (1966), *P. tolaasii* could best be placed in biotype G (biovar V) of *P. fluorescens*. However, this biotype is very heterogeneous and consists of doubtful *P. fluorescens* strains. Because of the lack of a comprehensive comparative study encompassing reference strains from the genus *Pseudomonas*, in particular members of *P. fluorescens*, we could not draw definite taxonomic conclusions.

Misnamed "*P. tolaasii*" strains

Our phenotypic analysis (Fig. 1), protein gel electrophoretic study (Fig. 2) and DNA:DNA hybridizations indicated that the "*P. tolaasii*" strains NCPPB 2193, NCPPB 2413t1 and t2, and NCPPB 1311 were not authentic *P. tolaasii* strains, as defined in the preceding section. Wong & Preece (1979) had already included strain NCPPB 2193 as a member of their saprophytic group C pseudomonads. Strains NCPPB 2413t1 and t2 were not examined by Wong & Preece (1979) but were clearly different from the *P. tolaasii* strains of phenon IV (Tables 2 and 3). Strain NCPPB 1311 was a white line reacting organism clustering in phenon V.

The white line reacting organisms

In our study the white line reaction against the type strain of *P. tolaasii* was not limited to strains from one single phenotypic group. Indeed strain LMG 6652, which remained phenotypically and gel electrophoretically ungrouped, was also a white line reacting organism. However, here we restrict comment to the so-called white line reacting organisms of phenon V, encompassing '*P. washingtoniae*', '*P. reactans*' strains, one misnamed '*P. tolaasii*' NCPPB 1311, and 11 of our own isolates. Phenon V was phenotypically rather homogeneous and corresponded to group B of Wong & Preece (1979) and to set 1 of Zarkower *et al.* (1984: Table 2). The phenotypic description of our white line reacting organisms can be found in Tables 2 and 3. However, this group was not very homogeneous in its gel electrophoretic properties or DNA:DNA homologies. The low DNA relatedness of the white line reacting organisms with *P. tolaasii* precludes their inclusion in this species. Preece & Wong (1982) used the name '*P. reactans*' for the white line reacting organisms, but gave no description. This name has no standing in nomenclature according to the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1975). Because of the heterogeneity within this group we are reluctant to draw any taxonomic conclusions.

Phenotypic differentiation of the Pseudomonas species occurring on mushrooms

Our results show that the unidentified *Pseudomonas* species of mummy disease, the three pathogenic taxa *P. tolaasii*, *P. agarici* and '*P. gingeri*', the saprophytic *P. aeruginosa* and the white line reacting organisms can be easily differentiated from each other by several phenotypic tests. A key comprising the most useful diagnostic tests is given in Table 5.

We thank API System, La Balme-les-Grottes, Montalieu-Vercieu, France, for supplying API galleries and Ing. A. Overstijns, Provinciaal Onderzoek- en Voorlichtingscentrum voor Land- en Tuinbouw (Beitem-Rumbeke) for research facilities. J. D. L. is indebted to the Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw (IWONL) and the Fonds voor Geneeskundig Wetenschappelijk Onderzoek (FGWO) for research and personnel grants. K.K., J.S. and M.Go. are indebted to the Nationaal Fonds voor Wetenschappelijk Onderzoek (NFWO).

REFERENCES

- BAUER, A. W., KIRBY, W. M. M., SHERRIS, J. C. & TURCK, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology* **45**, 493-497.
- BONHAM-CARTER, G. F. (1967). *Fortran IV Program for Q-mode Cluster Analysis of Nonquantitative Data Using IBM 7090/7094 Computers*. Kansas Geological Survey Computer Program. Lawrence, USA: University of Kansas.
- CUTRI, S. S., MACAULEY, B. J. & ROBERTS, W. P. (1984). Characteristics of pathogenic non-fluorescent (smooth) and non-pathogenic fluorescent (rough) forms of *Pseudomonas tolaasii* and *Pseudomonas gingeri*. *Journal of Applied Bacteriology* **57**, 291-298.
- DE LEY, J., CATTOIR, H. & REYNAERTS, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *European Journal of Biochemistry* **12**, 133-142.
- DE VOS, P. & DE LEY, J. (1983). Intra- and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *International Journal of Systematic Bacteriology* **33**, 487-509.
- DE VOS, P., GOOR, M., GILLIS, M. & DE LEY, J. (1985). Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. *International Journal of Systematic Bacteriology* **35**, 169-184.
- DYE, D. W. (1968). A taxonomic study of the genus *Erwinia*. I. The '*amylovora*' group. *New Zealand Journal of Science* **11**, 590-607.
- FAHY, P. C. (1981). The taxonomy of the bacterial plant pathogens of mushroom culture. *Mushroom Science* **11**, 293-311.
- HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *Journal of Bacteriology* **66**, 24-26.
- KERSTERS, K. & DE LEY, J. (1975). Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. *Journal of General Microbiology* **87**, 333-342.
- KING, E. O., WARD, W. K. & RANEY, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44**, 301-307.
- KOVÁCS, N. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature, London* **178**, 703.
- LAPAGE, S. P., SNEATH, P. H. A., LESSEL, E. F., SKERMAN, V. B. D., SEELIGER, H. P. R. & CLARK, W. A. (editors) (1975). *International Code of Nomenclature of Bacteria*. Washington, DC: American Society for Microbiology.

- LAROCHE, M. & VERHOYEN, M. (1981). Identification sérologique des phytobactéries en Belgique. *Mededelingen van de Faculteit van de Landbouwwetenschappen, Rijksuniversiteit Gent* **46/3**, 757–771.
- LELLIOTT, R. A., BILLING, E. & HAYWARD, A. C. (1966). A determinative scheme for the fluorescent plant pathogenic pseudomonads. *Journal of Applied Bacteriology* **29**, 470–489.
- MACFADDIN, J. F. (1976). *Biochemical Tests for Identification of Medical Bacteria*. Baltimore: Williams & Wilkins.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *Journal of Molecular Biology* **3**, 208–218.
- MØLLER, V. (1955). Simplified test for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta pathologica et microbiologica scandinavica* **36**, 158–172.
- OLIVIER, J. M., GUILLAUMES, J. & MARTIN, D. (1978). Study of a bacterial disease of mushroom caps. *Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria, Angers*, pp. 903–916.
- PAINE, S. G. (1919). Studies in bacteriosis. II. A brown blotch disease of cultivated mushrooms. *Annals of Applied Biology* **5**, 206–219.
- PALLERONI, N. J. (1984). Genus I. *Pseudomonas* Migula 1894. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 141–199. Edited by N. R. Krieg. Baltimore/London, Ontario: Williams & Wilkins.
- PREECE, T. F. & WONG, W. C. (1982). Quantitative and scanning electron microscope observations on the attachment of *Pseudomonas tolaasii* and other bacteria to the surface of *Agaricus bisporus*. *Physiological Plant Pathology* **21**, 251–257.
- SCHISLER, L. C., SINDEN, J. W. & SIGEL, E. M. (1968). Etiology of mummy disease of cultivated mushrooms. *Phytopathology* **58**, 944–948.
- SIERRA, G. (1957). A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek* **23**, 15–22.
- SKERMAN, V. B. D., MCGOWAN, V. & SNEATH, P. H. A. (1980). Approved Lists of Bacterial Names. *International Journal of Systematic Bacteriology* **30**, 225–420.
- SNEATH, P. H. A. (1956). Cultural and biochemical characteristics of the genus *Chromobacterium*. *Journal of General Microbiology* **15**, 70–98.
- SNEATH, P. H. A. & SOKAL, R. R. (1973). *Numerical Taxonomy. The Principles and Practice of Numerical Classification*. San Francisco: W. H. Freeman.
- STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966). The aerobic pseudomonads: a taxonomic study. *Journal of General Microbiology* **43**, 159–271.
- TOLAAS, A. G. (1915). A bacterial disease of cultivated mushrooms. *Phytopathology* **5**, 51–54.
- WISHART, D. (1978). *Clustan User Manual*, 3rd edn. Interuniversity Research Councils Series, Report no. 47.
- WONG, W. C. & PREECE, T. F. (1979). Identification of *Pseudomonas tolaasii*: the white line test in agar and mushroom tissue block rapid pitting tests. *Journal of Applied Bacteriology* **47**, 401–407.
- WONG, W. C., FLETCHER, J. T., UNSWORTH, B. A. & PREECE, T. F. (1982). A note on ginger blotch, a new bacterial disease of the cultivated mushroom, *Agaricus bisporus*. *Journal of Applied Bacteriology* **52**, 43–48.
- YOUNG, J. M. (1970). Drippy gill – a bacterial disease of cultivated mushrooms caused by *Pseudomonas agarici* n. sp. *New Zealand Journal of Agricultural Research* **13**, 977–990.
- ZARKOWER, P. A., WUEST, P. J., ROYSE, D. J. & MYERS, B. (1984). Phenotypic traits of fluorescent pseudomonads causing bacterial blotch of *Agaricus bisporus* mushrooms and other mushroom-derived fluorescent pseudomonads. *Canadian Journal of Microbiology* **30**, 360–367.