***Pseudomonas costantinii* sp. nov., another causal agent of brown blotch disease, isolated from cultivated mushroom sporophores in Finland**

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Pathogenic bacteria are frequently associated with mushroom sporophores exhibiting brown blotch disease symptoms. These bacteria belong mainly to *Pseudomonas tolaasii* or occasionally to '*Pseudomonas reactans*'. Although a group of isolates originating from some Finnish mushroom farms satisfied the two characteristic criteria for diagnosis of infection with *P. tolaasii* (i.e. yielding a typical brown blotch symptom on *Agaricus bisporus* sporophores and producing a typical white line in agar when streaked towards the '*P. reactans*' LMG 5329 inducing strain), results based on numerical taxonomy, siderotyping, DNA–DNA hybridizations and 16S rDNA phylogenetic analyses supported the view that these isolates constituted a novel species within the genus *Pseudomonas*, *Pseudomonas costantinii*. The type strain is PS 3a^T (= CFBP 5705^T = HAMBI 2444^T).

Keywords: *Pseudomonas costantinii* sp. nov., *Pseudomonas tolaasii*, polyphasic taxonomy, brown blotch disease, *Agaricus bisporus*

INTRODUCTION

Various species belonging to the genus *Pseudomonas* play a key role in mushroom growing, as they are involved in the major steps of the cultivation process. Already present in the compost, pseudomonads play a predominant role at casing, i.e. promoting the reproductive phase of growth of the cultivated mushrooms: several studies have ascertained the key role of *Pseudomonas putida* in triggering fruit-body induction (Rainey, 1991; Fermor *et al.*, 2000). Besides the evident beneficial effect of pseudomonads in mushroom cultivation, several pathogens belonging to the genus *Pseudomonas* that endanger cultivated mushroom

crops and their quality have been described. Drippy gill, a bacterial disease affecting mushroom sporophores and caused by *Pseudomonas agarici*, was described by Young (1970). Most frequently, blotch diseases have been reported in cultivated mushrooms. These are mainly due to *Pseudomonas tolaasii*, causing brown blotch disease (Tolaas, 1915; Paine, 1919), and '*Pseudomonas gingeri*', responsible for ginger blotch disease (Wong *et al.*, 1982). Wells *et al.* (1996) described a mild light-brown discoloration with slight tissue collapse in the cultivated mushroom *Agaricus bisporus* that was attributed to '*Pseudomonas reactans*', another common inhabitant of the mushroom hyphosphere but so far considered to be saprophytic (Wong & Preece, 1979; Goor *et al.*, 1986). *P. tolaasii* is the major and most extensively studied pathogen of cultivated mushrooms. Its predominance in pathogenesis is due to its wide distribution, affecting several mushroom hosts, including *A. bisporus*, *Agaricus bitorquis*, *Pleurotus ostreatus* and *Pleurotus eryngii* (Bradbury, 1987), and causing substantial losses to mushroom cultivation (Soler-Rivas *et al.*, 1999). The identification of infection of mushrooms with *Pseudomonas tolaasii* essentially relies on two features: (i)

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Additional biochemical data for *P. costantinii* and other pseudomonads are available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>).

Abbreviation: PVD, pyoverdine.

The GenBank accession numbers for the 16S rDNA sequences of strains PS 3a^T, LMG 2342^T and LMG 5329 are AF374472, AF255336 and AF255337, respectively.

induction of a brown blotch symptom on mushroom sporophores and (ii) production of a white precipitate in agar when the pathogenic form of the bacterium is streaked towards a '*Pseudomonas reactans*' strain (Wong & Preece, 1979; Zarkower *et al.*, 1983; Cutri *et al.*, 1984). Since its description by Paine in 1919, the taxonomy of *P. tolaasii* has been revised only once, on the basis of numerical analyses of physiological and phenotypic features, and the results of DNA–DNA hybridization studies (Goor *et al.*, 1986).

During 1997 and 1998, brown blotch symptoms that could have been due to damage inflicted by *P. tolaasii* were observed on sporophores harvested from some Finnish mushroom farms. In a previous study, some bacterial isolates from mushrooms affected by brown blotch disease were ascribed to a distinct siderovar of *P. tolaasii* (siderovar 2) based on the pyoverdines (PVDs) synthesized by these strains under iron-starved conditions (Munsch *et al.*, 2000). Further studies on the two siderovars of *P. tolaasii*, including gene probing (*tll*; Rainey *et al.*, 1993), ribotyping, analysis of internally transcribed spacer ITS1 sequences and determination of DNA fingerprints, consistently differentiated the studied strains into two distinct groups (Munsch *et al.*, 2002): *P. tolaasii* and *P. tolaasii*-like. As well as comparing these groups, the aim of these studies was to examine their affiliation with a group of well-characterized *Pseudomonas* spp., to allow a more precise taxonomic assignment of the *P. tolaasii*-like strains.

We relied on a polyphasic approach, including numerical taxonomy of phenotypic tests, fatty-acid profiling, siderotyping, DNA–DNA hybridizations, G + C content determination and 16S rDNA sequence comparisons, to differentiate the *P. tolaasii*-like isolates from other members of the genus *Pseudomonas*. On the basis of our results, we propose a novel species within the pseudomonads, *Pseudomonas costantinii*, which is to include strains that cause brown blotch disease on the cultivated mushroom *A. bisporus*.

METHODS

Bacterial strains. A core of 23 strains was used in this study: it comprised 12 typical *P. tolaasii* strains, six *P. tolaasii*-like strains, two '*P. reactans*' strains and three '*P. gingeri*' strains (Table 1). Bacteria were routinely grown on King's B medium (King *et al.*, 1954). Thirty-one reference strains from the CFBP (Collection Française de Bactéries Phytopathogènes, INRA Angers, France) collection were also included in this study (Fig. 1).

White-line production and host pathogenicity tests. Tests for the production of white lines in agar were performed according to Wong & Preece (1979). All of the *P. tolaasii* or *P. tolaasii*-like strains listed in Table 1 were streaked parallel to '*P. reactans*' LMG 5329 (LMG, Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium); '*P. reactans*' CFBP 5712 was tested towards *P. tolaasii* CFBP 2068^T and *P. tolaasii*-like strain PS 3a^T for white-line production. Pathogenicity tests were carried out on mushroom sporophores, as described by Munsch (1994).

Electron microscopic observations. An overnight culture of *P. tolaasii*-like strain PS 3a^T in King's B medium was adsorbed onto a carbon-coated copper grid, with collodion as a support. The bacterial sample was prepared and negatively stained with aqueous 2% uranyl acetate according to Alatosava (1987) and examined by using a JEOL TEMSCAN 100 CX II electron microscope, at the Department of Electron Optics, Oulu University.

Phenotypic characterization and numerical analysis. Twenty conventional tests for *Pseudomonas* identification were performed on the 23 core and 31 reference strains, as described previously (Gardan *et al.*, 1992; Achouak *et al.*, 2000). Assimilation of carbon sources by the strains was also studied, using the Biotype 100 system according to the manufacturer's instructions (bioMérieux). The strips were incubated at 25 °C; any turbidity or colour changes in the cupules were recorded after 4 days. A total of 119 phenotypic features were included in the numerical taxonomic analysis of the strains. The distance matrix was calculated by using the Jaccard coefficient; cluster analysis was done by using the unweighted pair group with arithmetic means (UPGMA) method (Sneath & Sokal, 1973).

Antimicrobial susceptibility. Antimicrobial-sensitivity assays were performed for nine *P. tolaasii* (CFBP 1404, LMG 2342^T, CFBP 5703, CFBP 4058, CFBP 4030, LMG 6641, CFBP 5713, CFBP 5714 and CFBP 5710) and four *P. tolaasii*-like (PS 3a^T, CFBP 5706, CFBP 5709 and CFBP 5707) strains, by using ATB PSE strips (bioMérieux) according to the manufacturer's instructions.

Siderotyping methods. The siderotyping methods used here were based on PVD characterization, as described by Meyer *et al.* (1997, 1998). PVD/IEF profiles were determined for those *Pseudomonas* strains that were included in the DNA–DNA hybridization study. *P. tolaasii* LMG 2342^T and *P. tolaasii*-like strain PS 3a^T were tested for their uptake capacity towards 32 structurally different PVDs isolated from the other *Pseudomonas* strains tested. The indicated uptake data correspond to mean values from at least duplicate independent experiments.

Analysis of cellular fatty acids. Cultures of strain PS 3a^T and *P. tolaasii* LMG 2342^T grown on trypticase soy agar for 24 h at 25 °C were collected from four plates, washed three times with sodium phosphate buffer (10 mM, pH 7.0) and freeze-dried. Whole-cell fatty acids were analysed as fatty acid methyl esters, after transmethylation had been done as described by Nikkilä *et al.* (1996). GC analyses were carried out using a Hewlett Packard (HP) series 6890 gas chromatograph, equipped with a HP-5MS (30 m × 0.25 mm × 0.25 µm) column, and a mass-selective detector (model HP 5973). Data analyses were realized with the HP CHEMSTATION software. The fatty acid methyl esters were identified by their mass (Wiley mass spectra library database) and by their retention times. Lactic acid, dihydrosterulic acid, 9,10-methylenehexadecanoic acid and hydroxy fatty acid methyl ester standards were obtained from Larodan Fine Chemicals.

DNA–DNA hybridization, thermal stability of reassociated DNA and G + C content determination. Extraction and purification of DNA were performed based on the procedures of Brenner *et al.* (1982). The DNA of *P. tolaasii*-like strain PS 3a^T was labelled *in vitro* by random priming with a Megaprime Kit (tritium-labelled nucleotides; Amersham). The S1 nuclease/trichloroacetic acid method described by Grimont *et al.* (1980) was used in our studies, with a

Table 1. Bacterial strains used in this study

<i>Pseudomonas</i> sp.	Strain no.*	Source, date and place of isolation
<i>P. tolaasii</i>	LMG 2342 ^T (= CFBP 2068 ^T)	<i>A. bisporus</i> , 1965, UK
	LMG 6641	<i>Agaricus</i> sp., 1982, Belgium
	CFBP 1404 (= NCPPB 1116)	<i>A. bisporus</i> , 1961, UK
	CFBP 4030 (= NCPPB 2325)	<i>A. bisporus</i> , 1969, Italy
	CFBP 4568 (= ICMP 6957)	<i>A. bisporus</i> , 1979, USA
	CFBP 4058 (= ICMP 6955)	<i>A. bisporus</i> , 1979, New Zealand
	CFBP 5704	<i>A. bisporus</i> , 1998, Netherlands
	CFBP 5703	<i>A. bisporus</i> , 1998, Netherlands
	CFBP 5713	<i>A. bisporus</i> , 1998, France
	CFBP 5714	<i>A. bisporus</i> , 1998, Germany
	CFBP 5710	<i>Agaricus</i> sp., 1998, Finland
<i>P. tolaasii</i> -like	PS 3a ^T	<i>A. bisporus</i> , June 1997, Finland
	PS 3a NP	Non-pathogenic form of PS 3a ^T
	CFBP 5706	<i>A. bisporus</i> , January 1998, Finland
	CFBP 5709	<i>A. bisporus</i> , February 1998, Finland
	CFBP 5707	<i>A. bisporus</i> , May 1998, Finland
	CFBP 5708	<i>A. bisporus</i> , May 1998, Finland
' <i>P. reactans</i> '	LMG 5329	<i>A. bisporus</i> , 1979, UK
	CFBP 5712	<i>A. bisporus</i> , USA
' <i>P. gingeri</i> '	CFBP 2810 (= NCPPB 3146)	<i>A. bisporus</i> , 1981, UK
	CFBP 5715	<i>A. bisporus</i> , UK
	CFBP 5711	<i>A. bisporus</i> , USA; Wells <i>et al.</i> (1996)

* NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, Hertfordshire, UK; ICMP, International Collection of Micro-organisms from Plants, Plant Diseases Division, DSIR Mount Albert Research Centre, Auckland, NZ.

reassociation temperature of 70 °C. The temperature (T_m) at which 50 % of the reassociated DNA became hydrolysable by the S1 nuclease was determined according to the method of Crosa *et al.* (1973). The G+C content of the DNA of strain PS 3a^T was determined from the denaturation curve by using the equation of Marmur & Doty (1962). DNA extracted from *E. coli* K-12 CIP 54-117 was used as a control (G+C content = 50.6 mol %).

16S rDNA sequence determination. The universal primers fd1 and rd1, targeting bacterial 16S rRNA genes, described by Weisburg *et al.* (1991) were used to obtain a PCR product of approximately 1.5 kb, by using the protocol of Laguerre *et al.* (1994). The PCR product was sequenced directly by the dideoxynucleotide chain termination method using a model 377 automated DNA sequencer (Applied Biosystems). Three of the sequencing primers used here were described by Dabboussi *et al.* (1999); the remaining four were designed from conserved regions within the 16S rRNA gene sequences of members of the genus *Pseudomonas*. These four primers were For 350 (5'-AGGTCTTCGGATTGTAAAGC-3'), For 348 (5'-CAACCCTTGTCCTTAGTTAC-3'), For 352 (5'-TCGTAGTCCGGATCGCAG-3') and Rev 351 (5'-CTAGCTAATCCGACCTAGG-3'), covering positions 382–401, 1076–1095, 1262–1279 and 218–200, respectively, of the *P. tolaasii* LMG 2342^T 16S rRNA gene sequence (accession no. Z76670). The new sequence data were checked manually for accuracy, based on two or three repetitions.

Phylogenetic analyses. The 16S rDNA sequence of strain PS 3a^T was automatically and then manually aligned by reference to a database of 30 000 already-aligned bacterial

16S rDNA sequences. According to a BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>), the level of similarity between the 16S rDNA sequence of *P. tolaasii*-like strain PS 3a^T and the 16S rDNA sequences of several *Pseudomonas* spp. was 99 %; the highest similarity score was recorded for *P. tolaasii* LMG 2342^T (accession no. AF255336). Phylogenetic trees for the 16S rDNA sequences of PS 3a^T and other members of the genus *Pseudomonas* were constructed according to three different methods [neighbour-joining (BIONJ), maximum-likelihood and maximum-parsimony]. For the neighbour-joining analysis, a distance matrix was calculated by using the Kimura two-correction parameter. Bootstrapping (Felsenstein, 1985) was done using 500 replicates, BIONJ and the Kimura two-correction parameter. BIONJ was done according to Gascuel (1997), and maximum-likelihood and maximum-parsimony data were produced via PHYLIP (Felsenstein, 1993). The phylogenetic trees were drawn by using NJPLOT (Perrière & Gouy, 1996) and CLARIS DRAW for Apple Macintosh. By retaining only the 16S rDNA sequences of members of the genus *Pseudomonas* in the phylogenetic analyses, we were able to include the almost-entire 16S rDNA sequences, corresponding to positions 68–1276 of the PS 3a^T sequence. The phylogenetic tree presented in this study (Fig. 2) resulted from a strict consensus between the neighbour-joining bootstrap analysis (500 replications) and the maximum-likelihood and maximum-parsimony analyses. To save space, only a subset of the total tree generated is presented in Fig. 2. The topology shown is that of the bootstrap tree, as it has been demonstrated that this topology is often better than that of a simple neighbour-joining or maximum-parsimony analysis (Berry

Fig. 1. Dendrogram of the phenotypic distances for 52 of the *Pseudomonas* strains examined in this study.

& Gascuel, 1996). Variable region V6 (Moore *et al.*, 1996) of the 16S rDNA sequence of strain PS 3a^T was also compared to the 16S rDNA GenBank sequences determined for the

type strain of *P. tolaasii* and for *Pseudomonas fluorescens* DSM 50090^T, by using the automatic ALIGN program of Vector NTI (version 6; InforMax).

Fig. 2. Phylogenetic analysis of 16S rDNA sequences of strain PS 3a^T and other *Pseudomonas* spp. The tree shown is a subset of the consensus tree resulting from a neighbour-joining bootstrap analysis (500 replications) and maximum-parsimony and maximum-likelihood analyses (see Methods). Branches that were retrieved by all three methods of analysis are indicated by their neighbour-joining bootstrap values, expressed as a percentage of 500 replications. Strain PS 3a^T (shown in the tree as CFBP 5705^T) formed a robust clade with *P. tolaasii* and a weaker clade with *P. fluorescens* and a few other *Pseudomonas* spp. (subset of the *P. fluorescens* cluster). The tree was rooted using other *Pseudomonas* sequences and is in agreement with the tree of Anzai *et al.* (2000).

RESULTS AND DISCUSSION

Morphological features of *P. tolaasii*-like strain PS 3a^T

Cells of strain PS 3a^T had a single polar flagellum (Fig. 3) and were straight rods of 0.7–0.9 × 2.3–3 µm in size.

Responses to pathogenicity and white-line tests

All of the *P. tolaasii* and *P. tolaasii*-like strains listed in Table 1 yielded the typical white-line precipitate in agar towards '*P. reactans*' LMG 5329. The strains also induced a typical brown blotch symptom when the pathogenicity test was performed on *A. bisporus* sporophores (data not shown) – i.e. they produced dark brown lesions on *A. bisporus* similar to those observed after infection with a typical *P. tolaasii* pathogenic strain after 48 h incubation at 25 °C (Olivier *et al.*, 1978; Wong & Preece, 1979; Munsch *et al.*, 2002). Only the PS 3a NP (non-pathogenic) variant failed to produce a white line when streaked towards '*P. reactans*' LMG 5329. '*P. reactans*' CFBP 5712 and

LMG 5329 produced a white line towards *P. tolaasii* CFBP 2068^T and strain PS 3a^T.

Phenotypic features and numerical taxonomy

The dendrogram displaying the distance relationship among 52 of the *Pseudomonas* strains is shown in Fig. 1. At the distance level of 0.0815, seven phena and 25 unclustered strains could be distinguished: biochemical and physiological features that differentiated the phena are shown in Table 2 and detailed results of the comparative study are available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>).

The five *P. tolaasii*-like strains could be differentiated from all other reference strains tested. Phenon I comprised 10 strains clustered around *P. tolaasii* LMG 2342^T. Phenon II contained two '*P. reactans*' strains, LMG 5329 and CFBP 5712. Phenon III comprised all five *P. tolaasii*-like strains (CFBP 5707, PS 3a^T, CFBP 5706, CFBP 5708 and CFBP 5709) and the type strain of *P. fluorescens* bv. 1, CFBP 2102^T (Fig. 1). Within phenon III, *P. fluorescens* bv. 1 CFBP 2102^T could be distinguished from the *P. tolaasii*-like representatives due to its failure to assimilate histamine and to produce Tween esterase (on Tween 80) and gelatinase (Table 2). The five *P. tolaasii*-like strains could be distinguished from typical representatives of *P. tolaasii* species (phenon I), as they assimilated D-xylose, L-arabinose, benzoate and histamine (Table 2), unlike the *P. tolaasii* strains. Fahy (1981) and Goor *et al.* (1986) indicated similar results for these biochemical tests for strains ascribed to *P. tolaasii*. Also, 30 *P. tolaasii* isolates originating from French mushroom farms exhibited a similar assimilation profile to the strains examined in this study (Munsch, 1994). In the study by Wells *et al.* (1996), *P. tolaasii* strains failed to assimilate L-tartrate and sucrose, similar to the 11 strains of phenon I

Fig. 3. Electron micrograph of cells of strain PS 3a^T. Bar, 1 µm.

Table 2. Characteristics that differentiate *P. tolaasii*-like strains (phenon III) from the six other phenons (numbered in Roman capitals)

Supplementary data for this table can be found at IJSEM Online (<http://ijs.sgmjournals.org>). +, Positive reaction; —, negative reaction; d, strain-dependent reaction with numbers in parentheses indicating the number of strains showing a positive reaction.

Characteristic	Phenon no.						
	I	II	III*	IV	V	VI	VII
Number of strains	11	2	5	2	2	2	2
Assimilation of:							
D-Xylitol	+	+	+	—	—	—	—
5-Keto-D-gluconate	+	—	+	—	—	—	+
Adonitol	+	+	+	—	—	—	—
D-Xylose	—	+	+	+	+	+	—
L-Arabinose	—	+	+	—	+	+	+
meso-Erythritol	+	+	+	+	—	—	—
Benzoate	—	d (1)	+	—	d (1)	—	+
Histamine	—	—	+	+	—	—	+
2-Keto-D-gluconate	+	+	+	—	+	—	+
D-Trehalose	d (8)	+	+	+	+	+	+
L-Tartrate	—	+	—	—	—	—	—
Sucrose	—	—	d (4)	—	+	—	+
Levan formation	—	—	—	—	+	—	+
Nitrate reduction	d (7)	—	—	d (1)	d (1)	—	d (1)

* Excludes *P. fluorescens* bv. 1 CFBP 2102^T.

(Table 2); however, 10% of the strains grew with L-arabinose as substrate, unlike our study where none of the investigated strains assimilated L-arabinose.

Phenon IV contained '*P. gingeri*' CFBP 2810 and CFBP 5715, which were only distantly related to '*P. gingeri*' CFBP 5711 (Fig. 1). Phenons V–VII comprised two strains each (Fig. 1 and Table 2). Interestingly, at a distance level of 0.187, all of the mushroom-associated bacteria clustered together, whether they were pathogenic (inducing brown or ginger blotch diseases) or saprophytic ('*P. reactans*' LMG 5329 and CFBP 5712), with the exception of '*P. agarici*' CFBP 2063^T (the causal agent of drippy gill disease on mushroom sporophores). '*Pseudomonas flectens*' CFBP 3281^T, a non-validated species, clustered with the other strains at a very high distance level (0.89; Fig. 1). Studies by De Vos *et al.* (1985) and Anzai *et al.* (2000) did not consider '*P. flectens*' as a member of the genus *Pseudomonas*. In our study, '*P. flectens*' CFBP 3281^T could be distinguished from other pseudomonads, as it was oxidase-negative and failed to assimilate *cis*-aconitate, D-gluconate, caprate, succinate or fumarate, contrary to the characteristics of the other considered pseudomonads.

All of the *P. tolaasii* and *P. tolaasii*-like strains exhibited the same antibiogram. They were resistant to

Table 3. Cellular-fatty-acid composition of *P. tolaasii*-like strain PS 3a^T and *P. tolaasii* LMG 2342^T

Results are expressed as a mean percentage of total fatty acids and are based on three replicates. The $\pm$ SD values for the results are shown in parentheses.

Fatty acid*	PS 3a ^T	LMG 2342 ^T
C _{10:0 3-OH}	0.8 (0.06)	0.7 (0.3)
C _{12:0}	1.6 (0.09)	1.2 (0.04)
C _{12:0 2-OH}	4.4 (0.16)	4.3 (0.03)
C _{14:0}	0.5 (0.04)	0.3 (0.03)
C _{15:0}	0.2 (0.1)	0.2 (0.1)
C _{16:1}	25.0 (2.2)	24.5 (5.3)
C _{16:0}	20.9 (0.8)	21.7 (1.7)
C _{17:0 cyc}	10.8 (0.4)†	14.9 (4.7)†
C _{17:0}	0.4 (0.2)	0.5 (0.3)
C _{18:1}	31.9 (2.1)‡	26.7 (0.4)‡
C _{18:0}	2.3 (0.3)	1.7 (0.6)
C _{19:0 cyc}	T§	2.3 (1.2)
Unknown	1.5 (0.4)	0.9 (0.2)

* C_{10:0 3-OH}, 3-Hydroxydecanoic acid; C_{12:0}, dodecanoic acid; C_{12:0 2-OH}, 2-hydroxydodecanoic acid; C_{14:0}, tetradecanoic acid; C_{15:0}, pentadecanoic acid; C_{16:1}, hexadecenoic acid; C_{16:0}, hexadecanoic acid; C_{17:0 cyc}, methylene hexadecanoic acid; C_{17:0}, heptadecanoic acid; C_{18:1}, octadecenoic acid; C_{18:0}, octadecanoic acid; C_{19:0 cyc}, methylene octadecanoic acid.

† Significantly different for 2/3 replicates.

‡ Significantly different for 3/3 replicates.

§ T, Trace amount (< 0.1 %).

ticarcillin, ticarcillin/clavulanic acid and colistin, and sensitive to piperacillin/tazobactam, tobramycin, amikacin, netilmicin and ciprofloxacin. Variable reactions for both groups were noticed for the remaining antimicrobials of the ATB PSE strips (ceftazidime, aztreonam, cefepime, imipenem, piperacillin, gentamicin, ofloxacin, fosfomycin and cotrimoxazole).

Analysis of cellular fatty acids

Two hydroxy fatty acids, C_{10:0 3-OH} and C_{12:0 2-OH}, were found in *P. tolaasii* LMG 2342^T and PS 3a^T at about 5% of the total fatty acids; fatty acids with variants of C₁₆ compounds were prevalent in both strains, representing about 46% of the total fatty acids (Table 3). C₁₈ fatty acids were present at about 28% of the total fatty acids in *P. tolaasii* LMG 2342^T and were present at a significantly higher level in strain PS 3a^T, about 34% of the total fatty acids. A detailed study of the fatty acids present in the two strains (Table 3) revealed that PS 3a^T possessed a significantly smaller amount of C_{17:0 cyc} (for two of the three replicates) and a significantly higher amount of C_{18:1} than *P. tolaasii* LMG 2342^T.

The fatty-acid composition determined for *P. tolaasii* LMG 2342^T in this study was similar to data provided

Table 4. pI Values of PVDs of some *Pseudomonas* strains and levels of DNA–DNA reassociation of *P. tolaasii*-like strain PS 3a^T with different *Pseudomonas* strains

Source of unlabelled DNA	Phenon no./no. of unclustered strains*	PVD isoforms pI values	DNA–DNA reassociation value (%)†
<i>P. tolaasii</i> -like strain:			
PS 3a ^T	III	7.7, 5.3‡	100
CFBP 5707	III	7.7, 5.3‡	99
CFBP 5708	III	7.7, 5.3‡	96
CFBP 5706	III	7.7, 5.3‡	89
CFBP 5709	III	7.7, 5.3‡	91
<i>P. tolaasii</i> strain:			
CFBP 2068 ^T	I	9.1, 7.5, 7.3‡	51
CFBP 5704	I	9.1, 7.5, 7.3‡	45
CFBP 5710	I	9.1, 7.5, 7.3‡	48
' <i>P. gingeri</i> ' strain:			
CFBP 2810	IV	7.3, 5.2, 5.0	8
CFBP 5715	IV	—§	23
CFBP 5711	2	7.6, 5.3, 5.0	29
' <i>P. reactans</i> ' CFBP 5712	II	8.3, 7.2	50
<i>P. fluorescens</i> bv. 1 CFBP 2123	9	8.8, 8.6, 7.5, 7.2	49
<i>P. fluorescens</i> bv. 1 CFBP 2102 ^T	III	8.7, 7.3, 7.1	49
' <i>P. salomonii</i> ' CFBP 2022 ^T	3	8.4, 7.3	47
<i>P. corrugata</i> CFBP 2431 ^T	24	—	38
<i>P. fluorescens</i> bv. 2 CFBP 2125	14	7.1	33
<i>P. fluorescens</i> bv. 5 CFBP 2130	17	8.4, 7.4, 5.5	30
<i>P. fluorescens</i> bv. 4 CFBP 2129	15	7.3, 5.3	28
<i>P. aureofaciens</i> CFBP 2133 ^T	VII	8.5, 7.5, 5.1	28
<i>P. fuscovaginae</i> CFBP 2065 ^T	VI	7.3, 5.0, 4.3	23
<i>P. putida</i> CFBP 2066 ^T	21	4.6, 4.2, 4.0	17
<i>P. aeruginosa</i> CFBP 2466 ^T	19	8.8, 8.4, 7.4, 5.1	15
<i>P. cichorii</i> CFBP 2101 ^T	22	7.2, 5.7, 4.5	15
<i>P. marginalis</i> CFBP 3300 ^T	7	ND	10

ND, Not determined.

* Unclustered strain at the 0.0815 distance level (Fig. 1).

† The reassociation values are given as the mean of two determinations.

‡ pI Value data from Munsch *et al.* (2000).

§ —, PVD-deficient.

by Stead (1992). The main differences were the higher amount of C_{18:1}, the smaller amount of C_{16:0} and the absence of the hydroxy fatty acid C_{12:0 3-OH} detected in our study.

The most striking difference between the two strains concerned the cyclopropane fatty acid C_{19:0 cyc}. The amount of this fatty acid present, although quite low (2.3 ± 1.2%), was measurable in *P. tolaasii* LMG 2342^T but fell to the limit of detection in strain PS 3a^T. Similar results could be also observed for *P. tolaasii*-like strains CFBP 5709 and CFBP 5706 (data not shown).

Interestingly, strain PS 3a^T behaved like *P. putida*, *Pseudomonas fuscovaginae*, *Pseudomonas asplenii* and *P. agarici*, the exceptions of *P. fluorescens* subgroup 1a, which did not contain cyclopropane fatty acid

C_{19:0 cyc}. This was contrary to the other mushroom-production-associated fluorescent pseudomonads, '*P. gingeri*', '*P. reactans*' and *P. tolaasii* (Stead, 1992).

The results obtained in this study for the fatty-acid composition of *P. tolaasii* differed from those proposed by Wells *et al.* (1996) for strains of this species; for example, the total amount of cyclopropyl C_{17:0} and C_{19:0} derivatives was twice as high in our study, and the total amount of hydroxy substituted fatty acids was lower. The ratio of saturated to unsaturated fatty acids was 0.45 for strain PS 3a^T and 0.50 for *P. tolaasii* LMG 2342^T (Table 3). According to the study of Wells *et al.* (1996), this ratio was 0.60 for *P. tolaasii* strains; it was 0.72 according to Stead (1992).

The observed variations in the whole-cell fatty-acid composition of *P. tolaasii* may be due to differences in

the growth conditions used in the different studies, such as media composition, pH, temperature and age of the culture. In addition, the methodology used in our study, based on retention-time determination combined with mass-spectra analysis, could also explain some of the differences encountered.

Siderotyping data

P. tolaasii and *P. tolaasii*-like strains could be easily identified by the IEF patterns of their respective PVDs (Table 4 and Munsch *et al.*, 2000). From the results presented in Table 4, it is evident that none of the other *Pseudomonas* strains tested yielded a PVD/IEF profile identical to the one obtained for strain PS 3a^T and its related strains nor to that obtained for the *P. tolaasii* strains. Cross-incorporations (Fig. 4) showed that *P. tolaasii* LMG 2342^T and strain PS 3a^T efficiently recognized only their own PVD and did not incorporate iron complexed to any of the 32 structurally different PVDs synthesized by the other tested *Pseudomonas* strains. The structure of the PVD of strain PS 3a^T has been elucidated recently (Fernandez *et al.*, 2001); this confirmed the novelty of this compound compared to the PVD synthesized by *P. tolaasii* NCPPB 2192^T (Demange *et al.*, 1990) and to other PVDs described so far (Fuchs *et al.*, 2001). PVD siderotyping proved to be a powerful technique in this study, and has been used previously to characterize other *Pseudomonas* species (Achouak *et al.*, 2000; Meyer, 2000).

DNA base content and DNA–DNA hybridization

The G+C content of the DNA of PS 3a^T was 57.5 mol% and fell within the expected range for pseudomonads (Palleroni, 1984; De Vos *et al.*, 1989).

The five *P. tolaasii*-like strains (phenon III; Fig. 1) showed a high level of DNA-relatedness with each other (89–100%, with a mean value of 94.8% and an SD of 4.7%) and were, thus, considered to constitute a homogeneous DNA hybridization group (Table 4). *P. tolaasii*-like strain PS 3a^T exhibited a low level of DNA–DNA reassociation with the three *P. tolaasii* representatives, ranging from 45 to 51% (with a mean value of 47.9% and an SD of 2.7%) (Table 4). DNA–DNA reassociation values between strain PS 3a^T and the other tested reference *Pseudomonas* strains ranged between 10 and 50%. Interestingly, the type strain of *P. fluorescens* bv. 1 (CFBP 2102^T), which clustered with the *P. tolaasii*-like strains in phenon III (Fig. 1), and '*P. reactans*' CFBP 5712 exhibited about 50% relatedness with strain PS 3a^T. DNA–DNA reassociation values of between 23 and 29% (with a mean value of 26.7% and an SD of 3.1%) were observed between strain PS 3a^T and the three '*P. gingeri*' strains, CFBP 2810, CFBP 5711 and CFBP 5715 (Table 4).

DNA–DNA reassociation values of > 70% between bacterial strains are considered to indicate that they

belong to the same species (Wayne *et al.*, 1987; Vandamme *et al.*, 1996). As the five representative *P. tolaasii*-like strains exhibited between 89 and 100% DNA-relatedness with each other, had a relative binding ratio of only 50% with *P. tolaasii* strains and showed no significant DNA–DNA reassociation with the other tested reference *Pseudomonas* species or genomovars (Table 4) these strains were considered to belong to a distinct genomospecies.

16S rRNA gene sequence analysis

A subset of the complete phylogenetic analysis for the 16S rDNA sequences of PS 3a^T and other *Pseudomonas* spp. is reported in Fig. 2; this subset corresponds to the '*P. fluorescens* group' defined by Anzai *et al.* (2000). Comparisons indicated that strain PS 3a^T shared the highest sequence similarity with *P. tolaasii* LMG 2342^T (accession no. AF255336; 99% similarity, 1487 out of 1492 nt). Three sequences are available for the type strain of *Pseudomonas tolaasii* [accession nos Z76670 (LMG 2342^T), D84028 (ATCC 33618^T) and AF255336 (LMG 2342^T)]. Although these three sequences differ slightly (probably because of a few sequencing errors), they grouped in a robust clade, demonstrating that these sequence discrepancies had no influence on the topology of the tree. According to a cladistic analysis, sequence AF255336 seemed to be the most accurate of the *P. tolaasii* sequences. The 16S rDNA sequences for strain PS 3a^T (AF374472) and *P. tolaasii* LMG 2342^T (AF255336) differed in only five nucleotides. The differences determined for the 16S rDNA sequence of PS 3a^T were not located in variable regions V1 helix 6 or in V3 helix 18, but two of the five nucleotide base changes were located in variable region V6 helices P35-1 and P35-2 (Neefs *et al.*, 1990; Moore *et al.*, 1996). The C at position 123, C at position 978, G at position 985, T at position 1104 and G at position 1231 in the 16S rDNA sequence of *P. tolaasii* (starting from the 5' end of sequence AF255336) were replaced by T, T, A, A and A, respectively, in the PS 3a^T 16S rDNA sequence (AF374472). Moreover, the five encountered substitutions were noticeable when the 16S rDNA sequence of PS 3a^T was compared to the other two *P. tolaasii* type strain sequences available in GenBank (Z76670 and D84028). These five distinctive nucleotides were also observed in the 16S rDNA sequences of *P. tolaasii*-like strains PS 3a NP, CFBP 5706 and CFBP 5709 (data not shown). Four of the five base substitutions distinguishing PS 3a^T from the type strain of *P. tolaasii* corresponded to nucleotides observed in the 16S rDNA sequence of *P. fluorescens* DSM 50090^T biotype A (accession no. Z76662). All of the investigated *P. tolaasii*-like strains (PS 3a^T, PS 3a NP, CFBP 5706 and CFBP 5709) had the same V6 region as *P. fluorescens* DSM 50090^T, although their sequences were distinguishable in variable regions V1 and V3 (data not shown).

In summary, all of the presented results, based on phylogenetic, phenotypic and genotypic data, illustrate

Fig. 4. Heterologous PVD-mediated incorporation of ^{59}Fe by *P. tolaasii* LMG 2342^T and *P. tolaasii*-like strain PS 3a^T. Values depicted correspond to the incorporation of ^{59}Fe as measured in *P. tolaasii* LMG 2342^T (solid bars) and *P. tolaasii*-like strain PS 3a^T (open bars) after 20 min incubation (Munsch *et al.*, 2000) and are expressed as a percentage of the incorporation reached with the homologous PVD. Numbers 1 to 34 correspond to the structurally different PVDs tested that originated from the following bacterial strains: 1, *Pseudomonas* sp. E8; 2, *P. syringae* ATCC 19310; 3, *P. fluorescens* 9AW; 4, *P. putida* ATCC 12633; 5, *P. fluorescens* 51W; 6, *P. aeruginosa* Pa6; 7, *P. fluorescens* CCM 2798; 8, *P. fluorescens* CHA0; 9, *P. tolaasii* LMG 2342^T; 10, *P. aeruginosa* ATCC 27853; 11, *P. fluorescens* ii; 12, *P. fluorescens* S88.3; 13, *P. fluorescens* ATCC 17400; 14, *P. fluorescens* 1.3; 15, *Pseudomonas* sp. 267; 16, *P. fluorescens* ATCC 13525^T; 17, *P. aeruginosa* ATCC 15692; 18, *P. fluorescens* 18.1; 19, *P. fluorescens* 12; 20, *P. fluorescens* CFBP 2392; 21, *P. putida* CFBP 2461; 22 *Pseudomonas* sp. ATCC 15915; 23, *Pseudomonas monteilii* CFML 90-54; 24, *Pseudomonas mosselii* CFML 90-77; 25, *Pseudomonas rhodesiae* CFML 92-104; 26, *P. putida* CFML 90-33; 27, *Pseudomonas* sp. CFML 90-40; 28, *Pseudomonas* sp. CFML 90-42; 29, *Pseudomonas* sp. CFML 90-51; 30, *Pseudomonas* sp. CFML 90-52; 31, *Pseudomonas* sp. 7SR1; 32, *Pseudomonas* sp. 2908; 33, *Pseudomonas* sp. A 214; 34, *P. tolaasii*-like PS 3a^T. The 100 % values of the homologous assays corresponded to an incorporation of 8500 c.p.m. for *P. tolaasii* LMG 2342^T and 1060 c.p.m. for *P. tolaasii*-like strain PS 3a^T of the 46000 c.p.m. available in the assays. The c.p.m. values were corrected for the blank values obtained in assays without bacteria.

that the *P. tolaasii*-like strains represent a new species within the genus *Pseudomonas*, for which we propose the name *Pseudomonas costantinii*.

Description of *Pseudomonas costantinii* sp. nov.

Pseudomonas costantinii (cos.tant.i.ni'i. N.L. adj. *costantinii* of Costantin, referring to Julien Costantin, a French mycologist who, in a note dated March 1892 to the 'Société de Biologie', described some superficial brown blotch areas on sporophores).

Cells are Gram-negative straight rods, motile by a single polar flagellum. Produces a fluorescent diffusible greenish pigment on King's B medium. Several-days-old colonies produce sectors which when subcultured prove to be non-pathogenic on mushroom sporophores. Produces one Tween esterase (on Tween 80), are oxidase-positive, arginine-dihydrolase-positive and hydrolyse gelatin. Does not hydrolyse aesculin and does not produce acid from L-tartrate. Nitrates are not reduced. Levan-sucrase-negative, non-pectinolytic and does not degrade polypectates at pH 5 or 8. Does not induce a hypersensitive reaction on tobacco leaves. Utilizes α -D-glucose, β -D-fructose, D-galactose, D-trehalose, D-mannose, D-ribose, L-arabinose, D-xylose, D-arabitol, L-arabitol, xylitol, glycerol, *myo*-inositol, D-mannitol, D-sorbitol, adonitol, D-lyxose, *meso*-erythritol, D-saccharate, mucate, *meso*-tartrate, D-malate, L-malate, *cis*-aconitate, *trans*-aconitate, citrate, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, *N*-acetyl-D-glucosamine, D-glucuronate, protocatechuate, *p*-hydroxybenzoate, quinate, benzoate, betain, putrescine, DL- α -amino-n-butyrate, histamine, DL-lactate, caprate, caprylate, succinate,

fumarate, glutarate, DL-glycerate, DL- α -amino-n-valerate, ethanolamine, D-glucosamine, itaconate, DL- β -hydroxybutyrate, L-aspartate, L-glutamate, L-proline, D-alanine, L-alanine, L-serine, malonate, propionate, L-tyrosine and 2-oxoglutarate. Produces acid from DL-lactate, D-tartrate, erythritol, mannitol and sorbitol. Does not assimilate L-sorbose, α -D-melibiose, D-raffinose, maltotriose, maltose, α -lactose, lactulose, methyl 1-*O*- β -galactopyranoside, methyl 1-*O*- α -galactopyranoside, D-cellobiose, β -gentiobiose, methyl 1-*O*- β -D-glucopyranoside, palatinose, α -L-rhamnose, α -L-fucose, D-melezitose, dulcitol, D-tagatose, maltitol, hydroxyquinoline- β -glucuronide, methyl 1-*O*- α -D-glycopyranoside, methyl 3-*O*-D-glucopyranose, L-tartrate, tricarballylate, L-tryptophan, phenylacetate, gentisate, *m*-hydroxybenzoate, 3-phenylpropionate, *m*-coumarate, trigonelline, L-histidine or tryptamine. All tested strains contain trace amounts of the cyclopropane fatty acid C_{19:0 cyc}. The DNA G + C content of strain PS 3a^T is 57.5 mol %. The type strain is PS 3a^T (= CFBP 5705^T = HAMBI 2444^T) (HAMBI, Culture Collection of the Division of Microbiology, University of Helsinki, Finland). Isolated from brown blotched mushroom sporophores originating from the Turku region of Southern Finland (Lat 60° N, Long 22-15° E).

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